Hydrological and Vegetative Controls on the Concentration and Composition of Dissolved Organic Nitrogen (DON).

Submitted by

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Statement of Authorship

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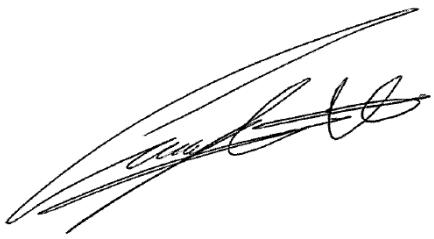

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Abstract

This thesis explores sources, potentially bioavailable forms, and fate of dissolved organic nitrogen (DON) within freshwater aquatic systems. With DON known to constitute up to 94 % of the dissolved nitrogen pool in aquatic systems and limited knowledge of forms, sources and bioavailability this research investigates a potential source (*Eucalyptus camaldulensis*) of DON to riverine systems.

This thesis examines the dissolved combined amino acid (DCAA) composition of DON leached from terrestrially aged *E.camaldulensis* leaves, the adsorption potential of DCAAs to different substrates at different pH and influences such as adjacent land use practices, floodplain connections and major townships and confluences on the DON concentration and composition along a longitudinal gradient.

This work demonstrates that DON released from terrestrially aged *E.camaldulensis* leaves occurs predominantly in the form of DCAAs and prior to leaching, this material is strongly partitioned to the mesophyll section of the leaf. DCAA nitrogen accounted for 57 % of total leaf nitrogen while dissolved inorganic nitrogen (DIN) only accounted for 2 %. This thesis also shows numerous sources along the length of a largely unregulated river to influence changes in DON composition and DCAA proportion. In particular, major townships, major confluences, floodplain connection and adjacent land use. Substrate and pH are also shown to effect DCAA adsorption and could potentially act as a DON sink in stream.

This work shows that DCAAs can be at least as important as DIN in providing potentially bioavailable nitrogen for in-stream processes and should be considered when constructing nitrogen budgets, monitoring aquatic nitrogen levels or studying nutrient dynamics in river systems where land use in an important consideration. Through

studying sources of organic nitrogen, influences on concentration, composition and adsorption of DCAAs to stream substrate this doctorate contributes to a greater understanding of DON within Australian freshwater systems and highlights the importance of this often abundant, yet overlooked nutrient source to in stream food webs.

Chapter 1 General Introduction

1.1 A general introduction to this study

Worldwide, understanding the sources and forms of dissolved nutrients and the processes that regulate them is of critical importance to the management of aquatic systems. From headwaters to estuaries, anthropogenic impacts and inorganic and organic inputs can lead to a change in the form of dissolved nutrients, threatening both freshwater biodiversity and water resources. Knowledge of the major nutrients entering waterways is a crucial factor when implementing effective management decisions. For this reason, it is imperative that we develop a stronger understanding of sources, bioavailability (the extent to which these sources re metabolised by aquatic organisms) and potential effects of each nutrient fraction when in excess or short supply.

Dissolved nutrients can encompass a large range of materials that fall into two broad categories; organic and inorganic. Due to the rise of anthropogenic inputs from agriculture and fossil fuels, the role of inorganic nutrients (NH_4^+, NO_3^-, NO_2^- and PO_4^-) is well established (Camargo & Alonso, 2006; Stevens *et al.*, 1993; Tromboni & Dodds, 2017) however, the organic portion is not as well understood (Pellerin *et al.*, 2004; Vitousek *et al.*, 1979). Historically, dissolved organic matter (DOM) was considered refractory due to its high C: N ratio, therefore, sources, forms and bioavailability have received much less attention in relation to aquatic ecosystem management and function (Wiegner *et al.*, 2006). However, it has come to light in recent years that DOM is metabolically important in rivers as it supplies both energy and nutrients in the form of dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) (Wiegner *et al.*, 2006). Due to the occurrence of blackwater events (the washing of large amounts of organic plant material into waterways) the DOC portion of the organic pool has been

identified as a critical component of river management (Kerr, Baldwin, & Whitworth, 2013; Whitworth, Baldwin, & Kerr, 2014; Whitworth, Baldwin, & Kerr, 2012) however, much less work has focused on DON.

1.2 Dissolved organic nitrogen

The chemical composition of the DON pool is dictated by the sources of DOM to the river or wetland and can originate from a variety of anthropogenic and natural sources such as agricultural activities, floodplain material, riparian vegetation, atmospheric deposition, groundwater recharge and autochthonous production (Wiegner *et al.*, 2006). Due to the large array of materials that contribute to DON, sources and concentrations have been difficult to quantify in the past (Saunders III *et al.*, 2017). It has been suggested that around 20 % of organic nitrogen species are low molecular weight bioavailable compounds such as dissolved free amino acids (DFAAs), dissolved combined amino acids (DCAAs) (proteins) and urea that are readily used as a nutrient source by plants, microbes and bacteria (Fan *et al.*, 2018; Stepanauskas, Laudon, & Jørgensen, 2000). The other 80 % is made up of recalcitrant compounds such as chitin and melanin that are often unavailable and need to be broken down further for the nitrogen to be accessible as a nutrient source. Due to the wide variety of sources, characterising the DON pool is complex; therefore, it is often calculated by difference from the total dissolved nitrogen (TDN) less the dissolved inorganic nitrogen (DIN) (Saunders III *et al.*, 2017). Analysing molecules such as peptides and amino acids is not without difficulty as some methods can be compromised by interfering molecules (Vakondios, Koukouraki, & Diamadopoulos, 2014; Whiffen, Midgley, & McGee, 2007). This is particularly evident when analysing proteins; many colorimetric-based protein assays can be compromised by humics and phenolics within the sample (Vakondios, Koukouraki, & Diamadopoulos, 2014; Whiffen, Midgley, & McGee, 2007).

Consequently, there are still major knowledge gaps in understanding sources of DON and the bioavailable portion within river and floodplain environments (Brookshire *et al.*, 2005; Campbell *et al.*, 2000).

1.3 Land use and anthropogenic sources

Over the last decade, there has been a large amount of experimental work examining the effects of inorganic nitrogen species in aquatic systems (Bernhardt *et al.*, 2003; Buda & DeWalle, 2009; Montreuil, Merot, & Marmonier, 2010; Seitzinger, Kroeze*, et al.*, 2002). However, DON can be a major component of the dissolved nitrogen pool and has largely been ignored when constructing nitrogen budgets for the management of human impact (Campbell *et al.*, 2000). A study by Seitzinger *et al.* (2010) reported global increases in terrestrial nitrogen export for DIN by 5 Tg yr^{-1} and DON by 0.5 Tg yr^{-1} . While DIN increases seem high, other work (Campbell *et al.*, 2000; Stanley & Maxted, 2008; Wiegner *et al.*, 2006) has found DON to contribute between 8 and 94 % of TDN within freshwater environments. This variation in DON contribution can be partly explained by association with human activity. For example, Stanley and Maxted (2008) examined changes in the dissolved nitrogen pool across 324 streams in Wisconsin, USA. The major trends observed were that TDN in largely undisturbed systems was dominated by organic species (68.4 %) while greater human presence was associated with a higher DIN proportion (74.9 %).

1.4 Natural sources

DON contribution is partly attributed to natural sources such as plant material and natural precipitation and can change due to variation in season, type of organic input, soil substrates and floodplain connection (Forshay & Stanley, 2005; Seitzinger, Styles*, et al.*, 2002; Timperley *et al.*, 1985). Fertility of rivers and wetlands alike are often reliant upon lateral links between the floodplain and river system for the transfer of nutrients

(Glazebrook & Robertson, 1999; Wolfenden *et al.*, 2018). Transfer of organic material such as leaf litter from the floodplain to the river channel has become an important part of energy cycling in freshwater ecosystems, especially in Australian river and floodplain systems. (Baldwin, 1999; Kerr, Baldwin, & Whitworth, 2013; Whitworth, Baldwin, & Kerr, 2014; Whitworth, Baldwin, & Kerr, 2012).

Floodplains of south-east Australia are characterised by low-lying areas of ground adjacent to rivers with a high degree of variation in both frequency and time of inundation (Whitworth, Baldwin, & Kerr, 2012). Before river regulation, Australian floodplains were usually inundated during winter and spring due to rainfall and snowmelt of the upper catchments (Whitworth, Baldwin, & Kerr, 2012). During this time, hydrological connections from the floodplain and the river channel facilitate the transport of carbon and nutrients, influencing the productivity of the entire system (Baldwin, 1999; Whitworth, Baldwin, & Kerr, 2012). *Eucalyptus camaldulensis* is a common floodplain and lowland riparian species in mainland Australia, particularly in south-east Australia where it has formed extensive stands of up to 60,000 ha (Reid & Quinn, 2004). Maximum leaf fall for *Eucalyptus* species occurs in summer and collects upon the floodplain until it is inundated or washed into the river channel during winter floods (Briggs & Maher, 1983).

1.5 Leaf leaching and decomposition

Over the dry summer period, fallen leaves are aged and decomposed by bacteria and fungi which kills the leaf tissue, and weakens the structural integrity of the leaf. Upon wetting, a rapid release of soluble compounds occurs, which can constitute between 20 and 40 % of total leaf mass (Gessner, Chauvet, & Dobson, 1999). However, leaching only constitutes the first phase of leaf litter breakdown in streams and further nutrients are lost from leaf tissue throughout subsequent stages (Gessner, Chauvet, & Dobson, 1999). The

second and longest phase of leaf breakdown is termed ‘conditioning’ in which the palatability of leaf litter for invertebrate shredders is enhanced through the colonisation of microbes and fungi of high nutritional value (Gessner, Chauvet, & Dobson, 1999). A large proportion of dissolved organic material (DOM) in rivers worldwide originates from plant material deposited directly into the river or indirectly through floodplain interactions (Baldwin, 1999; Gessner, 1991; Junk, Bayley, & Sparks, 1989; Meyer, Wallace, & Eggert, 1998).

1.6 Fungal sources and interactions

Throughout periods of desiccation and ageing, leaves are further broken down by processes involving three types of organisms: detritivores referred to as shredders, fungi and bacteria (Baldy, Gessner, & Chauvet, 1995). By comparison with the large amount of work carried out on invertebrate shredders and bacteria, there has been far less work carried out on fungal associations with leaf litter breakdown (Baldy, Gessner, & Chauvet, 1995). Aquatic hyphomycetes are the major fungal decomposers found in freshwater flowing streams and their decomposition of leaf material is important in making food available for macroinvertebrates that feed upon detritus (Bärlocher, 1992; Suberkropp & Chauvet, 1995). *Eucalyptus* leaves are historically thought of as poor substrates due to the release of inhibitory polyphenols upon initial immersion (Baldwin, 1999). However, recent studies have shown that aquatic hyphomycetes do in fact colonise and breakdown aged *Eucalyptus* leaves once these substances have been leached (Harris *et al.*, 2016; Kerr *et al.*, 2013; Nikolcheva & Bärlocher, 2005; Suter *et al.*, 2011).

Suter *et al.* (2011) demonstrated extensive fungal colonisation within terrestrially aged *Eucalyptus pauciflora* leaves by using Focal Plane Array (FPA) infrared mapping and staining leaf sections with lacto-phenol cotton blue (LPBC), a staining technique specific to fungal chitin. Fungal colonisation was found to occur in both the vascular

tissue and the mesophyll cells of the leaf sections, with the same areas of the leaf found to have high protein content, as revealed by the Amide I absorbance band of proteins in the mid-infrared spectrum (representative of protein). Fungi that colonise leaf material and are transported to streams may themselves be a source of DON, as fungal cell walls are a polymer of N-acetylglucosamine (Suter et al. 2011). Potentially, as the breakdown of leaves and fungal cells occur this material may also be released from the leaf upon wetting (Kerr *et al.*, 2013).

1.7 Floodplain interactions and longitudinal trends

Field studies of both floodplain and longitudinal concentration and composition worldwide, have proven to be important in supporting river management (Hadwen *et al.*, 2010; Kaushal *et al.*, 2014; Stanley & Maxted, 2008; Stepanauskas, Laudon, & Jørgensen, 2000) such studies are however, limited in Australia. These studies are often undertaken in an effort to understand anthropogenic impact upon nutrient cycling and to examine the effect of variables such as land use, point source nutrients, effluent release, floodplain interactions and hydrological influence (Stanley & Maxted, 2008). One major problem with longitudinal field studies is the high variability between flow and season. One way to account for these changes is to sample at different points on the hydrograph, over different seasons (Hadwen *et al.*, 2010; Moran *et al.*, 2014).

As longitudinal experiments are of a large scale and effort, it has been more convenient to address longitudinal nutrient relationships conceptually. Conceptual models such as the River Continuum Concept (RCC) of Vannote *et al.* (1980) and the Flood Pulse Concept (FPC) of Junk, Bayley, and Sparks (1989) have been used in an attempt to explain the spatial and temporal functioning of rivers. The RCC states that ecosystem dynamics downstream are linked with ecosystem processes upstream and it is generally assumed that nutrient concentrations will increase longitudinally (from upstream to

downstream sites) (Thorp & Delong, 1994). Another aspect that the RCC addresses is the ratio of riparian overhang to river channel width; with a decrease in riparian overhang downstream, it is thought that there will be less direct deposition from riparian species. However, overbanking and floodplain interactions that are a major feature in Australian lowland river systems don't necessarily conform to the general models for the RCC. The Flood Pulse Concept (FPC) of Junk, Bayley, and Sparks (1989) attempts to explain these interactions by stating that the principle driving force responsible for food web processes and nutrient delivery to rivers is the flood pulse, having a more substantial effect upon localised nutrient concentrations than nutrient spiralling (conversion of nutrients between organic and inorganic forms spatially along a river) discussed in the RCC. The flood pulse acts as a two-way transfer system between the river channel and adjacent floodplains with the majority of organic matter moving towards the river channel (Baldwin, 1999).

Concentrations and bioavailability of both DON and DOC in rivers can be influenced by numerous natural and anthropogenic sources that can make trends both complicated and difficult to conceptualise (Petrone, Richards, & Grierson, 2009; Seitzinger, Styles*, et al.*, 2002). Many events along a river continuum influence the form, concentration and bioavailability of DON; these include biogeochemical processes such as mineralisation to inorganic nitrogen species by heterotrophic bacteria and river regulation (Berman & Bronk, 2003). Humans have greatly altered many riverine systems within Australia with the introduction of dams, weirs and extraction of water for irrigation, which have changed the extent and duration of flooding events (Baldwin *et al.*, 2013). In turn, this also changes the natural flux of nutrients through a river system.

Brookshire *et al.* (2005) conducted a longitudinal study of DON at summer base flow in Hugh White Creek, USA in which DON was found to decrease in the lower

reaches of the stream due to rapid ammonification and nitrification. It was also found that dissolved organic nitrogen (DON) uptake was highly responsive to the amount of inorganic nitrogen within the stream, showing pronounced uptake of DON and DOC when DIN concentrations were low (Brookeshire *et al*. 2005). Other studies have found factors such as regulation (Hadwen *et al.*, 2010), land use (Seitzinger, Styles*, et al.*, 2002) and the input of organics through floodplain connection (Whitworth, Baldwin, & Kerr, 2014) to be major factors in controlling nutrient concentration and form.

Longitudinal trends in dissolved nutrients likely depend on not one or two but several contributing factors along the length of the waterway. While it is important to understand sources and influences on DON as it is transported to the stream channel, it is also important to understand DON sinks such as biogeochemical and adsorption processes that are occurring in stream.

1.8 Adsorption of dissolved organic nitrogen

The four main sinks of DON are microbial uptake by river autotrophs and heterotrophic organisms, photochemical decomposition and abiotic adsorption (Berman & Bronk, 2003). Due to the variety of molecules that constitute DON, many persist in the natural environment in different ways. Proteins (dissolved combined amino acids) (DCAAs) and dissolved free amino acids (DFAAs) in aquatic systems are thought to be a biologically available form of DON and therefore, an important aspect of riverine food web dynamics (Lundeen *et al.*, 2014; Lusk & Toor, 2016). Both DCAAs and DFAAs interact with organic and inorganic molecules onto which they may adsorb such as mineral and biofilm surfaces (Aufdenkampe *et al.*, 2001; Marmstrong & Bärlocher, 1989). This process may lead to a preferential removal of certain amino acids/proteins and preservation or degradation of the bound molecules (Hedges & Hare, 1987). Adsorption of proteins can occur within minutes and reach a steady state within 1.5 hours of exposure to a suitable

substrate, especially in turbulent water (Ding & Henrichs, 2002; Keil & Kirchman, 1994). Once adsorbed, microbial degradation of dissolved proteins occurs much slower and the desorption processes may take a long time if the DCAA/DFAA concentration within the river remains constant (Keil & Kirchman, 1994). Understanding adsorption properties of DCAAs both in stream and within sediment is integral for understanding stream function and nutrient availability (Yu *et al.*, 2013).

In general, basic amino acids such as lysine, arginine and histidine are thought to be more strongly adsorbed than neutral or acidic amino acids (Benetoli *et al.*, 2007; Hedges & Hare, 1987; Zaia, 2012). However, the ability for amino acids to adsorb changes at different pH levels. Altering the pH can change the surface charge on the substrates (particularly for clay minerals) and the protein molecule. Depending upon the pH of the stream the protein molecule can be negatively, neutrally or positively charged influencing its susceptibility to adsorption (Yu *et al.*, 2013).

Naturally occurring clay minerals such as illite, kaolinite and montmorillonite are layered aluminosilicates and capable of strongly adsorbing various biomolecules including proteins, amino acids, DNA and RNA within the aquatic environment (Ding & Henrichs, 2002; Yu *et al.*, 2013). Clays are found globally and exhibit several properties that encourage adsorption of amino acids and proteins, such as large surface area per unit weight, cationic exchange, and catalytic properties (Ding & Henrichs, 2002; Zaia, 2012). In most clay minerals there are two adsorption sites; the interlamellar channels and the surface of the clay mineral (Yu *et al.*, 2013). Dominant adsorption sites depend on both the clay mineral as well as structure and properties of the biomolecule (Yu *et al.*, 2013).The adsorption sites on illite (the most abundant clay mineral in the Murray-Darling Basin) are only found on the external surface due to its non-expanding layers (Yu *et al.*, 2013). While smectics such as montmorillonite are expanding layered alumina-

silicates and have numerous internal and external surfaces available for adsorption (Yu *et al.*, 2013). Generally, they also have higher adsorption capacity than non-expanding minerals such as kaolinite and illite (Yu *et al.*, 2013). In general, smaller protein molecules can be adsorbed to the interlayers of clay minerals through cationic exchange while larger molecules can be adsorbed via electrostatic forces (depending on surface charge of clay mineral, the protein and the pH of the environment) (Yu *et al.*, 2013). Clay minerals also have both hydrophilic and hydrophobic regions which attract proteins due to their amino acid chains which exhibit different polarities (Yu *et al.*, 2013).

1.9 This study

This thesis explores the sources, forms, potential bioavailability and fate of dissolved organic nitrogen (DON) within aquatic systems, with a particular focus on dissolved combined amino acids (DCAAs). Studies conducted outside of Australia indicate that DON can constitute up to 94 % of the dissolved nitrogen pool in aquatic systems. Given the limited knowledge of DON forms, sources and bioavailability, this research investigated a potential source (*Eucalyptus camaldulensis*) of DON to riverine systems, influences such as adjacent land use practices and floodplain connections, changes in DON composition along a longitudinal gradient and the effect of adsorption processes on DON concentrations. Through studying the complete cycle of organic nitrogen from source to sink, the aim of this thesis was to develop a greater understanding of DON within Australian freshwater systems and highlight the importance of this often abundant, yet overlooked nutrient source to in-stream food webs.

These concepts will be examined in greater detail in the following four experimental chapters.

Chapter Two: *The effect of microbial inhibitors on the measurement of N and P-containing nutrients in aquatic samples.*

This chapter trials a series of microbial inhibitors to identify one that is most appropriate for environmental experiments and the determination of dissolved organic nitrogen (DON). Before carrying out detailed studies on DON, an appropriate set of inhibitors needed to be examined. A target inhibitor should inhibit growth of microbial assemblages, be inert to the range of target molecules in the experiment and not interfere with analytical techniques. A suite of five microbial inhibitors were trialled and DON concentrations were measured over a 24 hour leaching period.

Chapter Three: *The potential of River Red gum (Eucalyptus camaldulensis) to act as an important source of dissolved organic nitrogen (DON) to aquatic systems through direct deposition and floodplain interactions.*

Leaf leaching experiments were carried out to determine DON concentrations and labile forms (proteins) released from leaves during the initial phase of leaf breakdown (inundation and leaching). Time course leaching experiments were carried out over a 24 hour period and a portion of the DON was characterised as DCAAs (protein). The cellular content of *Eucalyptus camaldulensis* leaves before and after leaching was also examined using Fourier-transform infrared (FTIR) microspectroscopy at the Australian Synchrotron.

Chapter Four: *Longitudinal trends in concentration and composition of dissolved organic nitrogen (DON) in a largely unregulated river system.*

This chapter explores longitudinal trends in DON along a largely unregulated river, the Ovens River, Victoria. No studies of this type have been carried out in Australian rivers. Furthermore, less is known of the composition of the organic fraction, particularly that which is readily biodegradable. To address these unknowns, changes in DON concentrations, composition and dissolved combined amino acid (DCAA) proportions were investigated along a longitudinal gradient in relation to major confluences,

townships, floodplain connectivity and adjacent land use activities. The Ovens river is approximately 203km long and represented an ideal system to investigate changes in nitrogen along its length.

Chapter Five: *Determining the extent of adsorption of amino acids onto naturally occurring substrates: a potential sink of dissolved organic nitrogen (DON).*
Since abiotic interactions are rarely considered with riverine DON studies, this chapter explored the in-stream fate of DON through adsorption onto stream substrates (kaolinite clay mineral and a natural river sediment). Batch experiments were run at different pH conditions and the DCAA concentrations and composition before and after adsorption analysed to determine whether different proteins/peptides are preferentially adsorbed by different substrates at different pH levels.

Each of the experimental chapters are written as discrete papers. Chapters three and four have been published, with the two unpublished chapters (two and five) in preparation for submission to peer-reviewed journals. Therefore, between each chapter is a short linking narrative to cohesively guide the reader. Finally, the results of each of the experimental chapters are discussed in chapter six, before outlining prospective directions for further study.

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Chapter 2 The effect of microbial inhibitors on the measurement of N and P-containing nutrients in aquatic samples.

Authorship statement

The following chapter is in preparation for submission to *(yet to be decided)*.

Author contribution: I collected the data and I analysed the data with advice from ES, GR and JP. I prepared the manuscript for publication with editing advice from both ES and GR (as per the normal supervisor role).

The effect of microbial inhibitors on the measurement of N and P-containing nutrients in aquatic samples.

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2.1 Abstract

Dissolved organic nitrogen (DON) can comprise up to 80 % of the dissolved nitrogen pool in riverine ecosystems and often encompasses a broad range of organic nitrogen containing materials. Some forms of DON such as dissolved combined amino acids (DCAAs) are highly available to microbes therefore, sample preservation within time course experiments is important for accurate analyses. While many microbial inhibitors are available, it is not always possible to cease microbial activity without producing some undesired analytical effects. We conducted 24 hour leaching experiments using *Eucalyptus camaldulensis*, a common leaf litter species found along the low-land rivers and wetlands of south-east Victoria, Australia. Leaching kinetics of dissolved organic nitrogen (DON), dissolved organic carbon (DOC), total dissolved phosphorus (TDP) and inorganic nutrients; oxides of nitrogen (NO_x) and ammonia (NH_4^+) were conducted with a range of microbial inhibitors to determine a suitable inhibitor for time-course leaching experiments. This was coupled with analysis of DCAAs to determine whether the type of inhibitor had an effect upon the composition of protein released from the leaf. Overall, no single inhibitor was entirely suitable for the measurement of all N-containing nutrients, even though DCAAs could be measured in the presence of both mercuric chloride ($HgCl_2$) and sodium azide (NaN_3) the DCAA composition was different between $HgCl_2$ and NaN_3. When compared to the control experiment total recovery of DCAAs were in a similar range to NaN_3 while recovery with $HgCl_2$ was lower but DCAAs were of a similar proportion to control. This indicates that neither treatment is entirely suited to the measurement of DCAAs as a source of DON. However, when compared with the control experiment NaN_3 was better able to recover DOC than $HgCl_2$ therefore, from the suite of microbial inhibitors NaN_3 was determined to be the best compromise as a microbial inhibitor for experiments of this nature.

2.2 Introduction

Organic molecules targeted for analysis in biological and environmental samples can be lost through adsorption, abiotic chemical reactions and microbial consumption (Winslow *et al.*, 2001). Microbial consumption is potentially a major sink of target organic molecules, with the rate of consumption relying on factors such as microbial population density, concentration of target organics, other molecules that may be a preferential target and the biodegradability of the target molecule (Winslow *et al.*, 2001). Microbial inhibitors cease microbial activity allowing preservation of the sample. However, with diverse microbial communities and an array of organic molecules in environmental samples, it is rarely possible to inhibit all microbial organisms without some negative analytical side effects. To conduct robust experiments on targeted biodegradable molecules it is important to understand the limitations that microbial inhibitors have on the range of target molecules (Oremland & Capone, 1988).

Dissolved organic nitrogen (DON) is a potentially bioavailable and important component of the riverine nitrogen pool however, it has received much less attention than inorganic nitrogen species (Harris *et al.*, 2018; Martin & Harrison, 2011). This is in part due to the large assortment of biomolecules that comprise DON and the uncertainty associated with accurate measurements, as DON is determined by the difference between total dissolved nitrogen (TDN) and dissolved inorganic nitrogen (DIN) (Campbell *et al.*, 2000; Frankovich & Jones, 1998). DON can constitute up to 80 % of the entire TDN pool so it is vital that there is an understanding around DON measurements and their limitations if its role in ecological systems is to be understood (Harris *et al.*, 2018; Martin & Harrison, 2011). Leaching experiments are one type of analysis carried out in DON studies and these experiments require a good understanding of the analytical limitations for measurement of relevant species in the presence of various inhibitors.

To understand the amount of bioavailable DON that is generated by inundation of leaf litter, an analytical approach was developed whereby measurement of dissolved organic carbon (DOC), TDN, oxides of nitrogen (NO_x), ammonia (NH_4^+) and one component of DON, namely total amino acid (proteins, peptides and free amino acids) were carried out on samples, in the presence of a microbial inhibitor. Our target in this work was to trial a series of microbial inhibitors and identify one that is most appropriate for work of this nature. For our purposes, an ideal microbial inhibitor should have several key characteristics; (i) it should inhibit growth of microbial assemblages in the presence of the target molecule (ii) it must be unreactive to the range of target molecules within the water sample (iii) it should not interfere with any of the analytical techniques that will be used on the water sample (iv) it must be soluble in water and (vi) it should be a reasonably safe substance to handle and store in the laboratory.

2.3 Methods

2.3.1 Leaf collection

Eucalyptus camaldulensis (River red gum) leaves were collected during summer leaf fall of 2012 from Wonga Wetlands, Albury, NSW, Australia (36.0686ºS, 146.8543ºE). Leaf nets were placed under the tree canopy to ensure fallen leaves were captured before contact with the ground to minimise exposure to soil microorganisms. Leaves chosen for inhibitor experiments had all fallen within one day, dried naturally in the field and were not exposed to precipitation between senescence and collection.

2.3.2 Experimental methods

Six replicate leaf-leaching experiments (5 inhibitors and a control) were carried out with 5 replicates of each inhibitor and 5 replicates with no inhibitor (control). The different microbial inhibitors used are as follows; 2.5 mM sodium azide (NaN_3) (Baldwin, 1999), 180 μM mercuric chloride ($HgCl_2$) (Lee *et al.*, 1992) 500 mg/L copper sulfate ($CuSO_4$)

(Winslow *et al.*, 2001), autoclaving leaves at 120°C for 120 minutes (Tuominen, Kairesalo, & Hartikainen, 1994) 2g/L phenol (C_6H_6O) (Stoilova *et al.*, 2006) and a control experiment with no inhibitor. The individual inhibitors and their mode of anti-microbial action is shown below in Table 2.1.

Table. 2.1 Tested microbial inhibitors and their mode of anti-microbial action.

Treatment	**Anti-microbial action**
Control	None.
Copper sulfate	Interference with microbial respiration, combines with thiol groups of enzymes (Winslow *et al.*, 2001)
Sodium azide	Inhibits microbial respiration (Wilson & Chance, 1967).
Mercuric chloride	Combines with the thiol groups of enzymes (Winslow *et al.*, 2001).
Autoclaving at 120°C for 20 minutes	Moist heat and high pressure causes inactivation of enzymes.
Phenol (carbolic acid)	At high concentrations precipitates cellular proteins, at lower concentrations inactivates enzymatic systems (Winslow *et al.*, 2001)

Leaves to be autoclaved were placed in a beaker, capped with foil to avoid exposure to steam and placed in the autoclave for 120 minutes at 120 °C. All other treatments were prepared as 5 L with the appropriate concentration and decanted into 5 separate 1 L Schott bottles. For each leaching experiment ~0.25 g of *E. camaldulensis* leaves were placed in each of the replicate inhibitor solutions; zero time was taken to be the point at which the water was added to the Schott bottle. Schott bottles were capped and gently stirred once every hour for the first 12 hours and before the final measurement to

minimise concentration gradients, with samples taken at 0, 10 and 24 hours. Water samples were filtered through 0.45 μM pore-size cellulose-acetate membrane filters (Cameo) and stored frozen until analysed. Samples were analysed for concentrations of dissolved organic carbon (DOC), dissolved inorganic nitrogen species (DIN; NH_4^+, NO_x), TDN and dissolved combined amino acids (DCAAs). All analytes were converted to μg-N (or μg-C) per gram of leaf. The pH of the leaching solution was not buffered and measured to be approximately 6 at the beginning and end of the 24 hour leaching period.

2.3.3 Chemical analyses

2.3.3.1 Nutrient analysis

Total dissolved nitrogen (TDN), oxides of nitrogen (NO_x; NO_2-N and NO_3-N) and ammonium (NH_4^+) were measured by flow injection analysis (FIA) (Lachat Quikchem 8000 Flow injection analyser; TDN range: 0.02 – 5.0 mg-N/L; oxides of nitrogen and ammonium ranges: 0.005 – 2.5 mg-N/L). TDN samples were digested using a NaOH-$K_2S_2O_8$ persulfate oxidation procedure at 120 ºC for one hour (Hosomi & Sudo, 1986). NO_x produced was analysed by FIA as described above. DOC was measured by combustion using an Akta Jenika 3100 N/C Analyser (DOC range: 0.3 – 250 mg/L). All procedures employed method blanks and quality controls to check against potential sample contamination or calibration drift. All results are reported with two standard error (2 SE).

2.3.3.2 Total dissolved amino acid analysis

All protein digestions and total amino acid analyses (DCAAs and dissolved free amino acids (DFAAs)) were conducted using new (pyrolysed) 5 mL glass sample vials. For DCAA analysis, 1 mL of filtered leachate was freeze dried, 200 μL of 6N HCl (containing 0.02 % phenol) added and the samples hydrolysed under an argon atmosphere at 110 °C for 24 hours. Hydrolysed samples were cooled and transferred to 1.5 mL

Eppendorf tube using an additional 0.3 mL of Milli-Q water to rinse the hydrolysis vial and assist with sample recovery. Samples were then freeze-dried and reconstituted with 80 µL (3:1 borate buffer: Milli-Q water mixture) and tagged with 20 µL of 6-aminoquinolyl-N-hydroxysuccinimyidyl carbamate (AQC) reagent, with vortex mixing after each addition step. Samples were heated at 55 °C for 10 minutes before adding 100 µL of 0.1 % formic acid and mixing. Derivatized samples were transferred to 200 µL centrifuge tubes and spun at 10,000 rpm for 5 minutes to remove any solid material and then transferred to 250 µL low volume inserts (Agilent) for analysis.

Tagged amino acid samples were analysed by liquid chromatography – mass spectrometry (LC-MS/MS). The LC-MS/MS system consisted of a Shimadzu Nexera X2 UPLC coupled to a Shimadzu 8030 triple quadrupole mass spectrometer, operated in positive ion electrospray ionisation (ESI) mode (Shimadzu corporation Kyoto, Japan). Individual tagged amino acids were detected in multiple reaction monitoring (MRM) mode, with collision parameters optimized individually. Source conditions were: sheath gas temperature 315 °C, gas flow 10 $L.min^{-1}$, nebulizer pressure 45 psi and capillary voltage 3.8 kV. Separation was achieved using a gradient eluent with a Waters Aquity UPLC BEH C18 column (2.1 X 150 mm; pore size 1.7 µM) maintained at 50 °C. The mobile phase consisted of 0.1 % formic acid (eluent A) (Sigma-Aldrich, St Louis, USA) and 100 % OPTIMA LCMS grade acetonitrile (eluent B) (Fischer Scientific, Pittsburgh, USA) with flow of 0.55 mL/min.

Calibration standards were made using amino acid standard H (Waters Corporation) spiked with glutamine (GLN), asparagine (ASN) and tryptophan (TRP) allowing measurement of all proteinogenic amino acids over the range 0.01 – 2 pmol/uL (equivalent to 0.01 – 2 µM). A quality control sample of selected amino acids was prepared at 1 pmol/uL containing HIS, ARG, GLN, LYS and ILE (amino acid three letter

abbreviations are detailed in Table S2.2). Blanks were included in each sample run, including: (i) a reagent blank which consisted of 60 µL borate buffer, 20 µL Milli-Q water and 20 µL AQC, (ii) a sample blank which was 40 µL of Milli-Q water, hydrolysed and derivatized as detailed above and (iii) a 0.1 % formic acid blank. An insulin standard (bovine pancreas; Sigma Aldrich, St Louis, USA) was included in every sample set to test for amino acid recovery (recoveries ranged from 32 to 75 %; see Table S2.1). For comparison with concentrations measured by non-specific methods, amino acid concentrations were converted to µg-N/L.

Some amino acids are not recoverable using the HCl hydrolysis method; tryptophan is destroyed and cystine is often difficult to determine from the hydrolysed samples (Fountoulakis & Lahm, 1998). During acid hydrolysis asparagine and glutamine are deamidated to aspartic acid and glutamic acid, respectively, making it impossible to determine the contributions of each amino acid individually; as is common practice, these amino acid pairs will be referred to as ASX and GLX (Table S2.2) (Anders *et al.*, 2003).

2.4 Results and discussion

2.4.1 Dissolved organic carbon

Dissolved organic carbon (DOC) could be measured in all experiments however there was large variation between treatments (Figure 2.1). Within the control experiment DOC concentrations increased from 9580 ± 3180 (T0) to 188,000 ± 14,700 (T24) µg-C/g of leaf over the 24 hour leaching period, with similar concentrations measured (6590 ± 762 - 167,000 ± 19,900 µg-C/g of leaf) measured in the presence of NaN_3. For all other treatments DOC after 24 hours was significantly less than in the controls; 112,800 ± 29,300 µg-C/g of leaf in the presence of $HgCl_2$, 11,000 ± 894 µg-C/g of leaf in the presence of C_6H_6O, 65,600 ± 11,300 µg-C/g of leaf with the autoclave and 79,100 ± 30,400 µg-C/g of leaf in the presence of $CuSO_4$.

NaN_3 and control experiments followed the same pattern over the 0, 10 and 24 hour measurements, without significant difference in DOC concentrations between NaN_3 and the control. These experiments had the highest DOC concentrations and given NaN_3 is likely inhibiting microbial activity, these experiments suggest that no detectable DOC consumption is occurring in either treatment over the 24 hour period. DOC concentrations in the C_6H_6O experiment were low throughout the leaf leaching experiment compared to the other treatments, which is surprising given that C_6H_6O is a source of DOC. No change in DOC was detected throughout the 24 hour period in the presence of phenol. DOC in the autoclave treatment did increase over the 24 hour incubation period, but was markedly reduced when compared to the control and azide treatment. While autoclaving samples sterilized the leaves, preventing any decomposition, these results suggest that the interaction of steam with the leaf surface did lead to pre-leaching of material and loss of DOC during the autoclaving process. Both $HgCl_2$ and $CuSO_4$ show intermediate levels of leaching compared to the other treatments which is likely due to the formation of metal-organic complexes through the binding of DOC with heavy metals (Hg and Cu) in solution (Guggenberger, Glaser, & Zech, 1994).

2.4.2 Dissolved inorganic nitrogen

DIN species concentrations varied greatly between treatments. For the control experiment NH_4^+ concentrations increased from 19 ± 5 to 2430 ± 572 µg-N/g of leaf over the 24 hour leaching period. This compared to an increase from 68 ± 17 to 290 ± 67 µg-N/g of leaf in the $HgCl_2$ experiment, 1090 ± 281 to 1510 ± 862 µg-N/g of leaf in the presence of $CuSO_4$, 46 ± 18 to 3330 ± 1340 µg-N/g of leaf in the presence of NaN_3, 57 ± 33 to 84 ± 55 µg-N/g of leaf with the autoclave treatment and 28 ± 21 – 501 ± 54 µg-N/g of leaf when C_6H_6O was used as a microbial inhibitor. All treatments show increasing NH_4^+ concentrations over the 24 hour leaching period however, it is clear that the extremely

low NH_4^+ concentrations in the presence of $HgCl_2$ demonstrate mercury interference with NH_4^+ analysis, previously shown to occur (Kérouel & Aminot, 1997).

For all treatments excluding NaN_3 the majority of NO_x was released immediately after immersion, with little or no change over the 24 hour period; treatments within the margin of error. Oxides of nitrogen (NO_x) concentrations for the control experiment increased from 29.5 ± 20 to 46 ± 16 µg-N/g of leaf over the 24 hour leaching period. This compared to changes over the same time period of: 55 ± 24 to 73 ± 16 µg-N/g of leaf with the addition of $HgCl_2$, 91 ± 34 to 176 ± 74 µg-N/g of leaf with the addition of $CuSO_4$, 100 ± 56 to 660 ± 213 µg-N/g of leaf with the addition of NaN_3, 49 ± 21 to 60 ± 25 µg-N/g of leaf with the autoclave treatment and 34 ± 17 to 52 ± 17 µg-N/g of leaf when C_6H_6O was used as a microbial inhibitor. While concentrations of NO_x varied between treatments, there was no major observable difference between treatments apart from the NaN_3 treatment. Oxides of nitrogen were higher in the NaN_3 experiment with an average of 660 µg-N/g of leaf at T24. This is possibly due to the partial oxidation of NaN_3; the much lower T0 value for the NaN_3 treatment suggests formation of NO_x species over the time period of the leaching experiment.

2.4.3 Total dissolved nitrogen

TDN concentrations increased from 265 ± 309 to 3496 ± 1180 µg-N/g of leaf in the control experiment and from 806 ± 523 to 4526 ± 2463 µg-N/g of leaf with the addition of $HgCl_2$, displaying a similar leaching profile over the 24 hour period. Similar to the control and $HgCl_2$ treatments TDN concentrations in the presence of $CuSO_4$ increased over the 24 hour leaching period, but over a lower range of values, from 474 ± 174 to 1225 ± 627 µg-N/g of leaf. TDN concentrations in the presence of NaN_3 were much higher than all other treatments ranging between 88,000 ± 7230 – 88,800 ± 8100 µg-N/g of leaf (data not presented). The autoclave experiment exhibited highest TDN

concentrations at T0 (2822 ± 3190 µg-N/g of leaf) however, concentrations did not change over the 24 hour period (T24; 1893 ± 907 µg-N/g of leaf) indicating an immediate release of TDN upon immersion.

TDN was unable to be determined for the C_6H_6O treatment set as a precipitate was formed during the digestion phase of sample preparation, which is likely due to the precipitation of organic material in the sample (Winslow et al., 2001) including the C_6H_6O itself. The lower levels of TDN in the presence of $CuSO_4$ suggest some form of precipitation of nitrogenous compounds occurred. Conversely, the blue colour generated by the addition of $CuSO_4$ would potentially lead to erroneously elevated TDN adding further weight to its unreliability as an inhibitor if TDN is required (Liu, Feng, & Jiang, 2001). As NaN_3 is itself a form of nitrogen, it is clearly responsible for the anomalously high TDN concentrations, making it impossible to determine TDN in the presence of this inhibitor (Bräse *et al.*, 2005). TDN in autoclaved samples was greatest at the outset of the experiment, and remained similar for the duration of the experiment. We suggest that cell lysis may be occurring during the autoclave process, leading to a pulse of TDN upon immersion with very little further change.

2.4.4 Total dissolved phosphorus

Total dissolved phosphorus (TDP) could be measured in all treatments apart from the C_6H_6O inhibited samples that precipitated during the digestion phase of analysis. In the control experiment TDP concentrations increased from 97 ± 36 to 1258 ± 107 µg-P/g of leaf over the 24 hour time course, compared to: 75 ± 8 to 1120 ± 234 µg-P/g of leaf in the $HgCl_2$ experiment, 1477 ± 97 to 2268 ± 186 µg-P/g of leaf with $CuSO_4$, 86 ± 19 to 1447 ± 90 µg-P/g of leaf with the addition of NaN_3 and 107± 12 to 802 ± 187 µg-P/g of leaf with the autoclave treatment. TDP concentrations were greatly elevated at T0 and remained consistently high in the presence of $CuSO_4$ over the 24 hour leaching period. This

suggests a potential interference in the analytical method such that the measurement of TDP in the presence of $CuSO_4$ would be unreliable. The TDP in the autoclaved samples were able to be analysed accurately, and lend support to the suggestion that as with TDN, TDP was lost from the sample during sterilizing.

2.4.5 Dissolved organic nitrogen

The treatments that permitted DON detection (one of our target group of molecules) were: autoclaving which decreased from 2716 ± 3172 to 1749 ± 841 µg-N/g of leaf over the time course and the control experiment which increased from 2 ± 10 to 490 ± 39 µg-N/g of leaf over the time course. By way of example, if all data were considered valid, then at the 24 hour measurement DON constituted 14 % of TDN in the control experiment and 92 % of TDN with the autoclave treatment. However, for the measurement of DON and TDN none of the microbial inhibitors in this experiment are entirely suitable (Table 2.5); NaN_3 is itself a source of organic-N and caused extremely elevated TDN and DON values, autoclaving causes a large pulse of nutrients to be released at T0, likely due to cell lysis and leaching, $CuSO_4$ alters the colour of samples and therefore, interferes with DIN analysis, C_6H_6O formed a precipitate during digestion and $HgCl_2$ causes depressed NH_4^+ values and therefore inflated DON values.

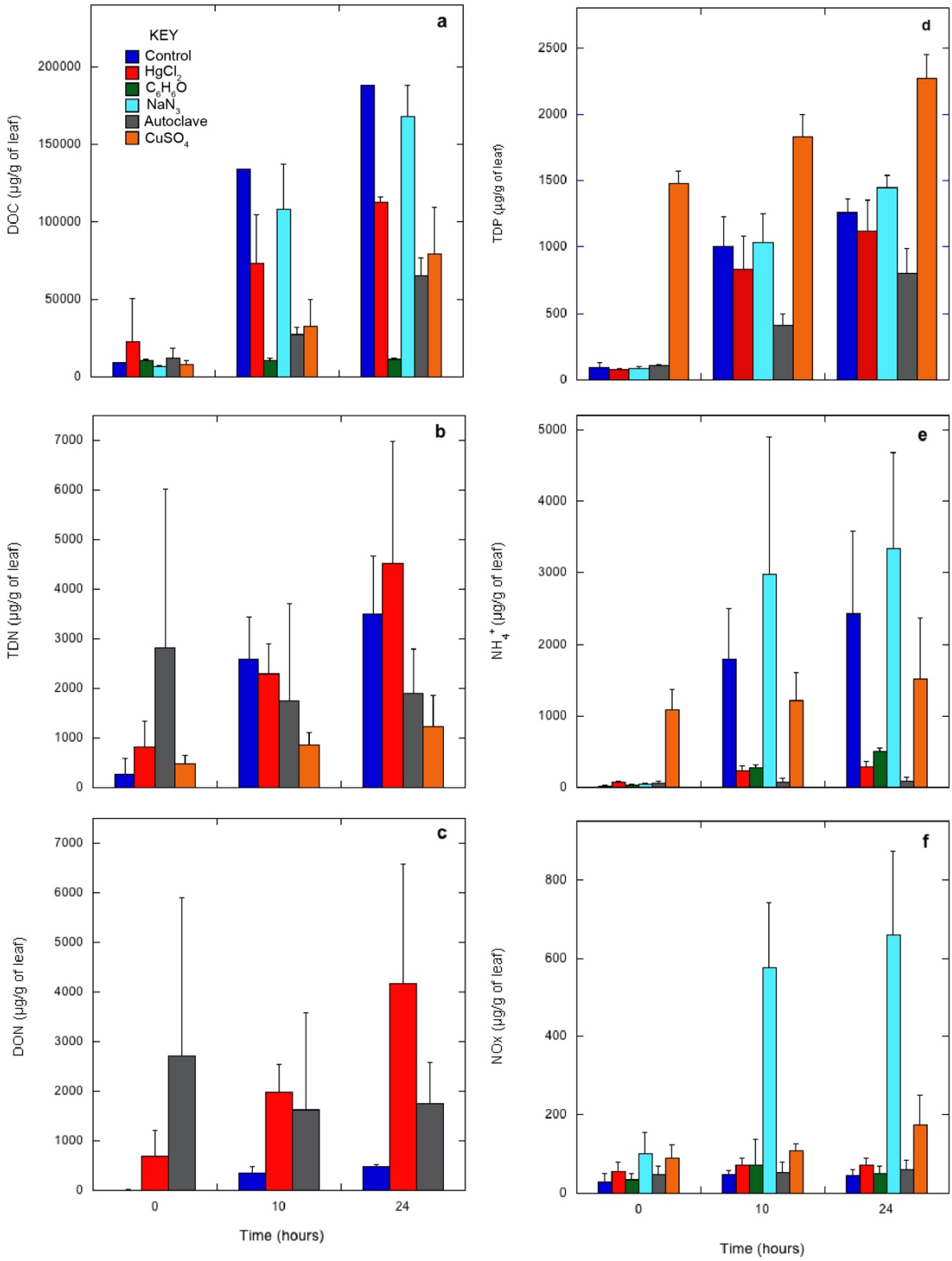


Figure. 2.1 Summary of dissolved organic carbon (DOC) (a), total dissolved nitrogen (TDN) (b), dissolved organic nitrogen (DON) (c), total dissolved phosphorus (TDP) (d) (ammonia) $NH4^{+}$ (e) and oxides of nitrogen (NO_x) (f) concentrations (normalised to leaf mass) at T0, T10 and T24 for 24 hour leaching experiments, values shown for all inhibitor treatments and the control experiment are the average of 5 replicate samples ± 2 SE.

2.4.6 Dissolved combined amino acid concentration

DCAA (and therefore DCAA-N) concentrations increased over time in all treatments with the exception of the treatment containing $CuSO_4$ which for unknown reasons interfered with DCAA analysis (Table 2.5, Figure 2.2). Within the control experiment DCAA-N concentrations increased from 14 ± 2 to 150 ± 15 µg-N/g of leaf during the 24 hour leaching period. NaN_3 concentrations increased from 12 ± 2 to 216 ± 22 µg/g of leaf, $HgCl_2$ concentrations increased from 10 ± 2 to 67 ± 6 µg-N/g of leaf and the autoclave treatment increased from 23 ± 2 to 74 ± 5 µg-N/g of leaf over the 24 hour leaching period (Table 2.2). Across all treatments and sampling times the most abundant amino acids at T24 were GLX (8 – 20 % total aa-N) and GLY (12 – 36 % total aa-N). While the amino acids that were leached at lowest concentrations after 24 hours were CYS (0 – 1 % total aa-N) and HIS (1 – 3 % total aa-N).

DCAA-N concentrations are similar between control and NaN_3 treatments (Table 2.2) indicating that there is no bacterial decomposition of DCAAs over the 24 hour period. Interestingly, there is some reduction of DCAA-N in the $HgCl_2$ treatment. Autoclave DCAA-N concentrations are low at T10 and T24 throughout the experiment when compared to other treatments, but similar at T0. This is not surprising due to the pulse of TDN released from autoclaved leaves upon immersion (T0). For two of the treatments where both DON and DCAA-N could be reliably measured (control and $HgCl_2$) the two groups of compounds followed similar leaching profiles, both DON and DCAA-N increased over time for control and $HgCl_2$. However, for the autoclave treatment DON concentrations were highest at T0 and lowest at T10, while DCAA-N concentrations increased over the time course but were substantially lower than other treatments at T24. As previously mentioned, a portion of TDN is lost from leaves on autoclaving therefore, it is reasonable to assume that a portion of DCAAs is also lost through the autoclaving process resulting in a lower of amount DCAA-N leached.

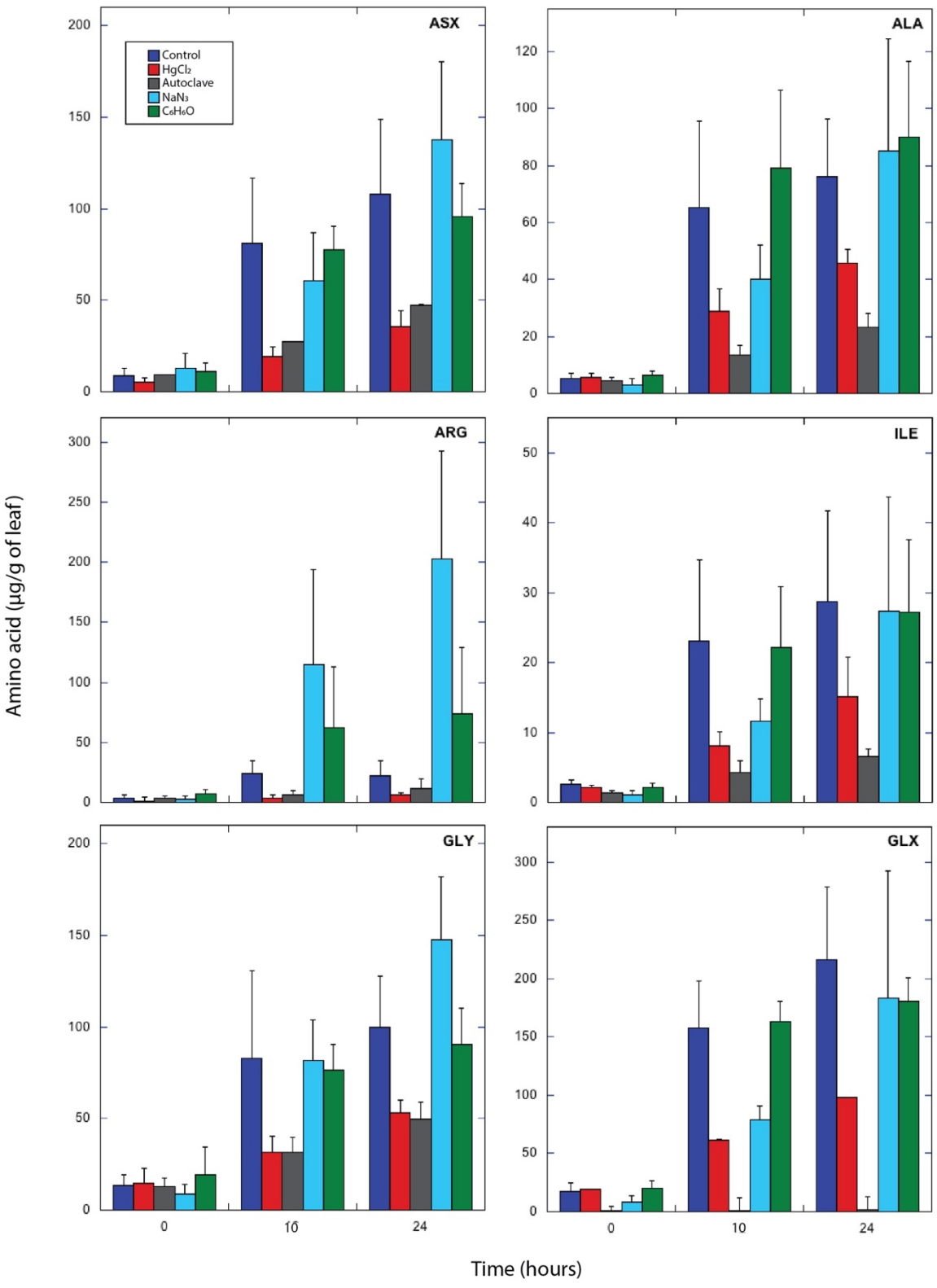
2.4.7 Dissolved combined amino acid composition

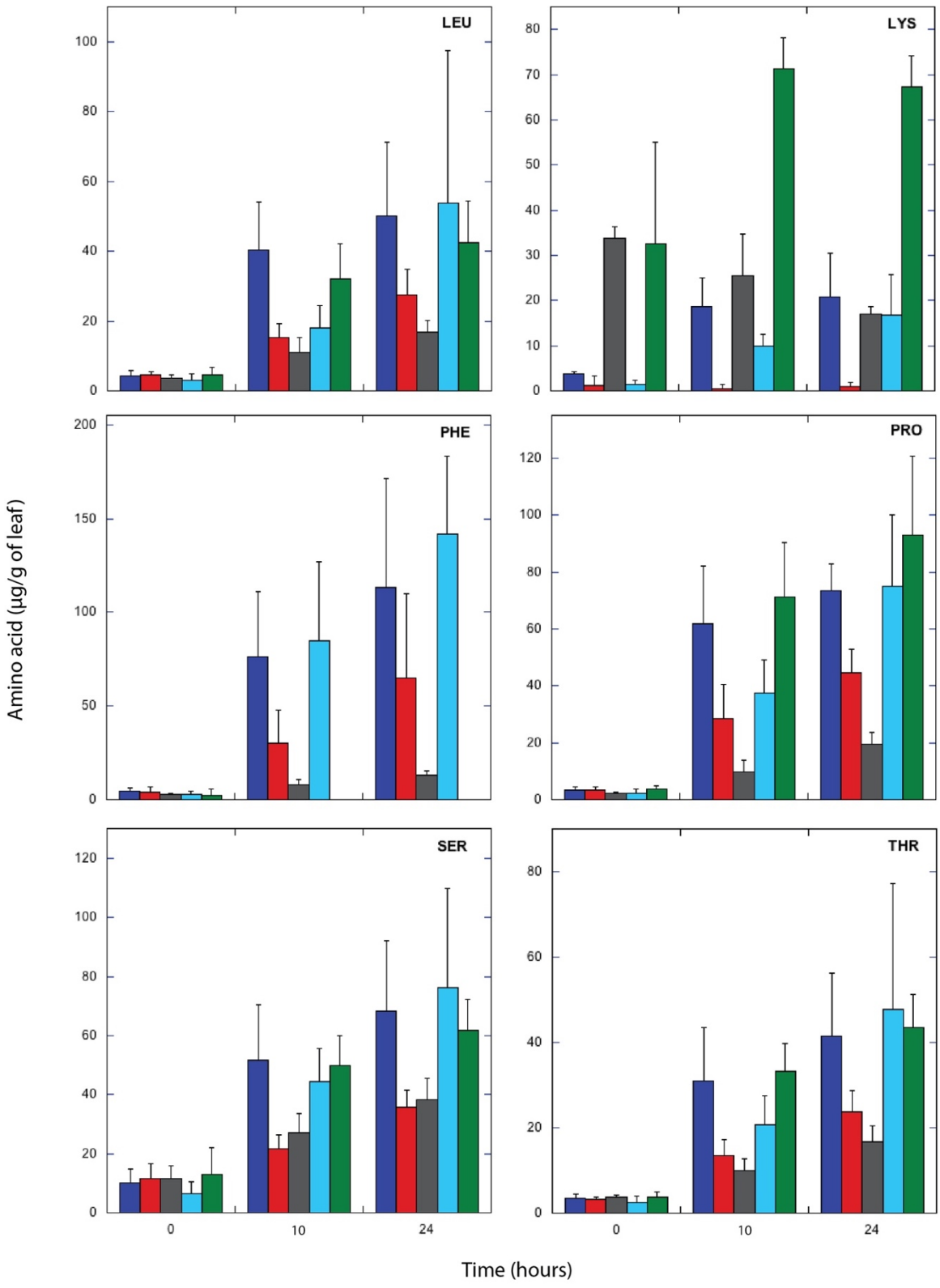
When considering time as a factor within treatments, the composition of the leached DCAA changed most between the T0 and T10 measurements ($p<0.05$) (Table 2.3) with no significant change occurring between T10 and T24 in any of the treatments apart from autoclaving. The difference observed between the T0 and T10 sampling times can be largely attributed to the change in the proportion of LYS, averaging 25 % of leached DCAAs at T0, 9 % at T10 and only 3 % at T24.

Within the T10 and T24 sampling times all treatments were significantly different in the composition of amino acids leached apart from control and $HgCl_2$ ($p>0.05$) which did not differ significantly throughout the entire time course experiment.

Overall, inhibitor type had an effect upon leached DCAA composition, suggesting that there was a differential leaching response depending on inhibitor type. When comparing between treatments (Table 2.4) those that significantly differ ($p < 0.05$) from each other at the T0 sampling time are; control and NaN_3, control and autoclave, NaN_3 and $HgCl_2$, NaN_3 and autoclave, $HgCl_2$ and C_6H_6O and $HgCl_2$ and autoclave. Autoclave samples were significantly different to all inhibitors at T0 except C_6H_6O ($p = 0.5331$) however, this was not the case at the T10 ($p = 0.0061$) or T24 ($p = 0.0075$) measurements. This difference is again driven by the large proportion of LYS at T0 where DON concentration is highest, and supports the idea that the autoclave treatment leads to a release of a particular type of protein upon immersion. It is clear from both the principal coordinate analysis and permutational analysis of variance (PERMANOVA) of amino acid proportion (Figure 2.2) that control and $HgCl_2$ experiments were not significantly different from each other at any of the sampling times ($p > 0.05$). The largest DCAA contributors across these two treatments are ASX, GLX and GLY at T0 and PRO, ALA, VAL, PHE, GLX, THR, and LEU at 10 and T24 measurements. Figure 2.3 shows NaN_3 to be somewhat similar to control and $HgCl_2$ treatments, however pairwise comparisons show NaN_3 to be

significantly different ($P<0.05$) from HgCl2 and control treatments across all sampling times. This is more pronounced in Figure 2.4 where a clear difference in DCAA proportion is observed in the NaN_3 treatment at T10. This is likely driven by a higher concentration of both SER, ASX and ARG with the addition of azide and a high concentration of PRO across the $HgCl_2$ time course.


ASX
ALA
ARG
ILE
GLY
GLX
Control
HgCl₂
Autoclave
NaN₃
C₆H₆O
Amino acid (µg/g of leaf)
Time (hours)
0
10
24


LEU
LYS
PHE
PRO
SER
THR
Amino acid (µg/g of leaf)
Time (hours)
0
10
24

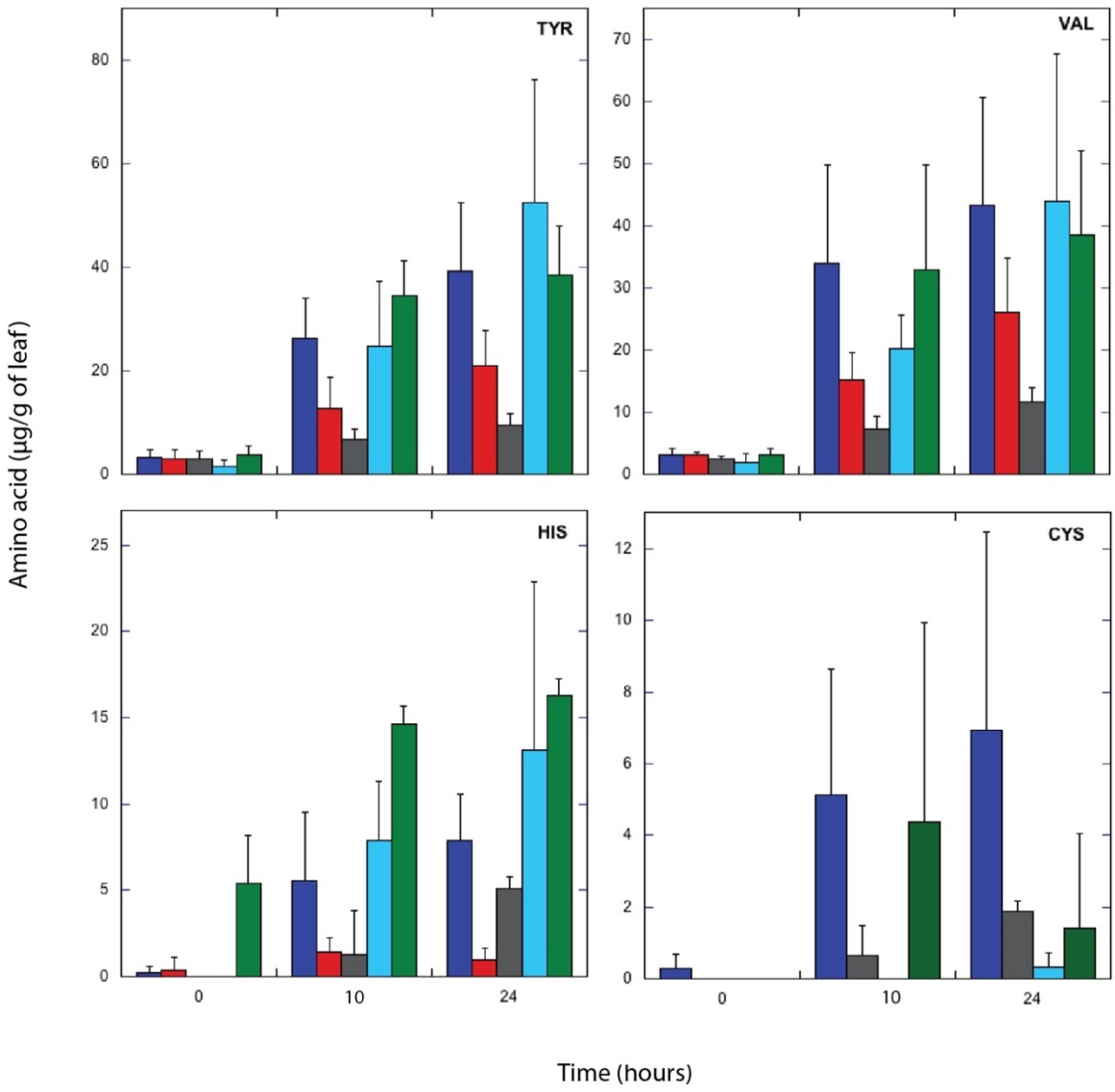


Figure 2.2 Individual amino acid leaching profiles across all inhibitor treatments and control at 0, 10 and 24 hour sampling times.

Table. 2.2 Combined amino acid nitrogen (DCAA-N) and dissolved organic nitrogen (DON) concentration across all treatments at 0, 10 and 24 hours. Values are average of 5 replicates ± 2 SE.

Treatment	Time	DON (µg-N/g of leaf)	DCAA-N (µg-N/g of leaf)
Control	0	2.16 ± 10.21	14.03 ± 2.21
	10	358.42 ± 121.0	119.04 ± 16.39
	24	489.81 ± 38.95	149.52 ± 15.03
Mercuric chloride	0	683.38 ± 518.11	13.13 ± 2.48
	10	1988.24 ± 547.95	44.68 ± 5.50
	24	4164.30 ± 2415.74	74.80 ± 5.96
Sodium azide	0	-	9.73 ± 2.05
	10	-	91.27 ± 11.09
	24	-	179.95 ± 22.15
Autoclaving at 120°C for 20 minutes	0	2716.24 ± 3171.84	13.58 ± 1.76
	10	1630.37 ± 1944.70	36.88 ± 4.00
	24	1749.08 ± 840.51	57.60 ± 4.56
Copper sulfate Autoclaving at 120°C for 20 minutes	00	-2716.24 ± 3171.84	-13.58 ± 1.76
	1010	-1630.37 ± 1944.70	-36.88 ± 4.00
	2424	-1749.08 ± 840.51	-57.60 ± 4.56

Table. 2.3 Pair-wise tests of proportion of amino acids leached between sampling times using treatment as a factor.

Treatment	Sampling times compared (hours leached)	P- value
Control	0, 10	**0.036**
	0, 24	**0.008**
	10, 24	0.993
Mercuric Chloride	0, 10	**0.0082**
	0, 24	**0.0068**
	10, 24	0.9615
Phenol	0, 10	0.0931
	0, 24	0.0546
	10, 24	0.8207
Sodium azide	0, 10	**0.0171**
	0, 24	**0.0158**
	10, 24	0.8904
Autoclave	0, 10	**0.0085**
	0, 24	**0.0059**
	10, 24	**0.0164**

Table. 2.4 Pair-wise tests of amino acid proportions leached between different treatments using sampling time as a factor, significant values ($p < 0.05$) are in bold.

Sampling time (hours)	Treatment 1	Treatment 2	p-value
0	Control	Sodium azide	**0.0422**
	Control	Mercuric chloride	0.0909
	Control	Phenol	0.1474
	Control	Autoclave	**0.0082**
	Sodium azide	Mercuric chloride	**0.0155**
	Sodium azide	Phenol	0.0329
	Sodium azide	Autoclave	**0.0084**
	Mecuric chloride	Phenol	**0.0301**
	Mecuric chloride	Autoclave	**0.0079**
	Phenol	Autoclave	0.5331
10	Control	Sodium azide	**0.0392**
	Control	Mercuric chloride	0.6016
	Control	Phenol	**0.0432**
	Control	Autoclave	**0.0160**
	Sodium azide	Mercuric chloride	**0.0152**
	Sodium azide	Phenol	**0.0166**
	Sodium azide	Autoclave	**0.0070**
	Mecuric chloride	Phenol	**0.0059**
	Mecuric chloride	Autoclave	**0.0081**
	Phenol	Autoclave	**0.0061**
24	Control	Sodium azide	**0.0181**
	Control	Mercuric chloride	0.5385
	Control	Phenol	**0.0097**
	Control	Autoclave	**0.0076**
	Sodium azide	Mercuric chloride	**0.0079**
	Sodium azide	Phenol	**0.0149**
	Sodium azide	Autoclave	**0.0165**
	Mecuric chloride	Phenol	**0.0075**
	Mecuric chloride	Autoclave	**0.0066**
	Phenol	Autoclave	**0.0075**

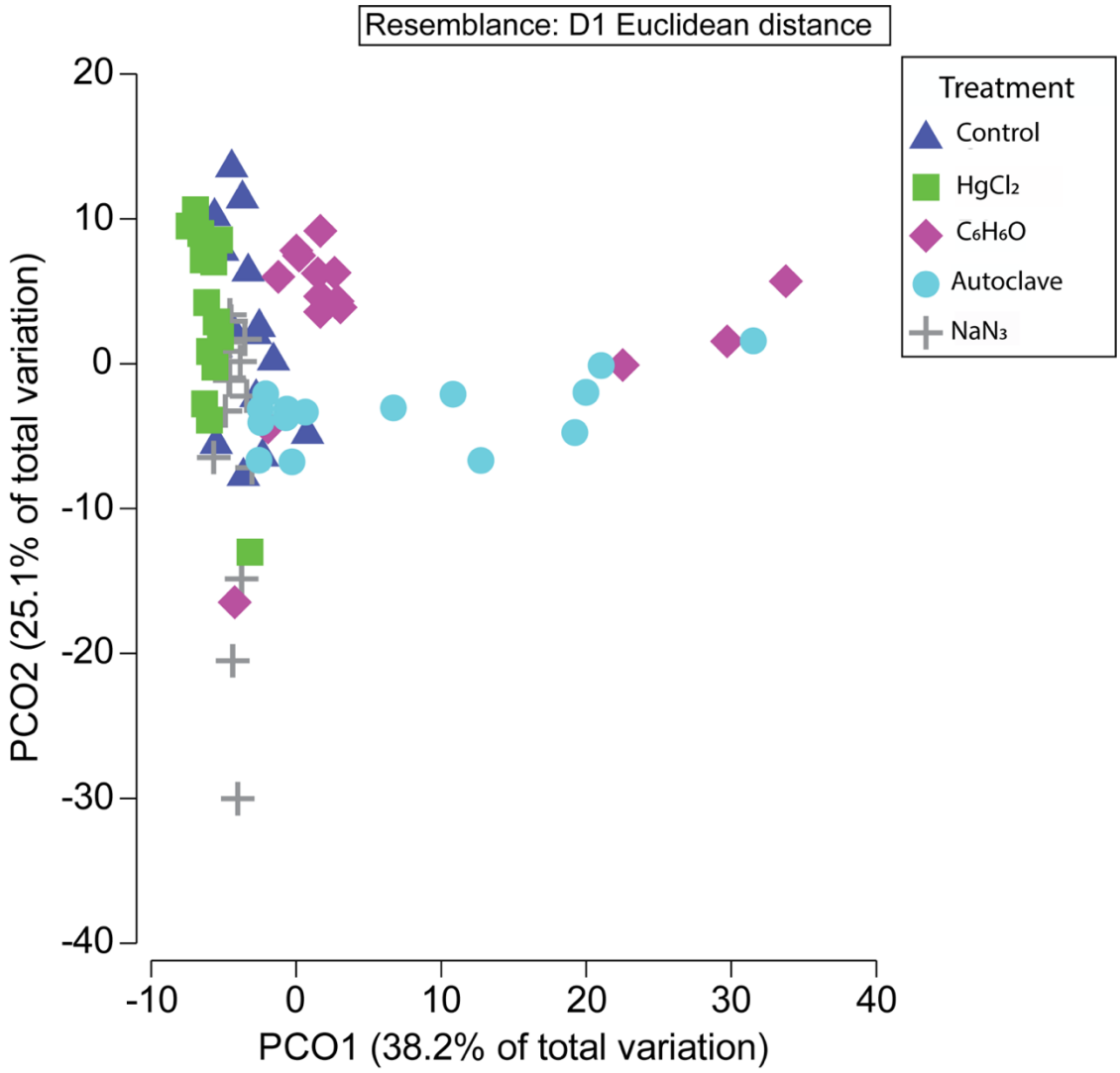


Figure 2.3 Principal coordinate analysis (PCoA) of amino acid proportion showing all experimental treatments aggregated by time.

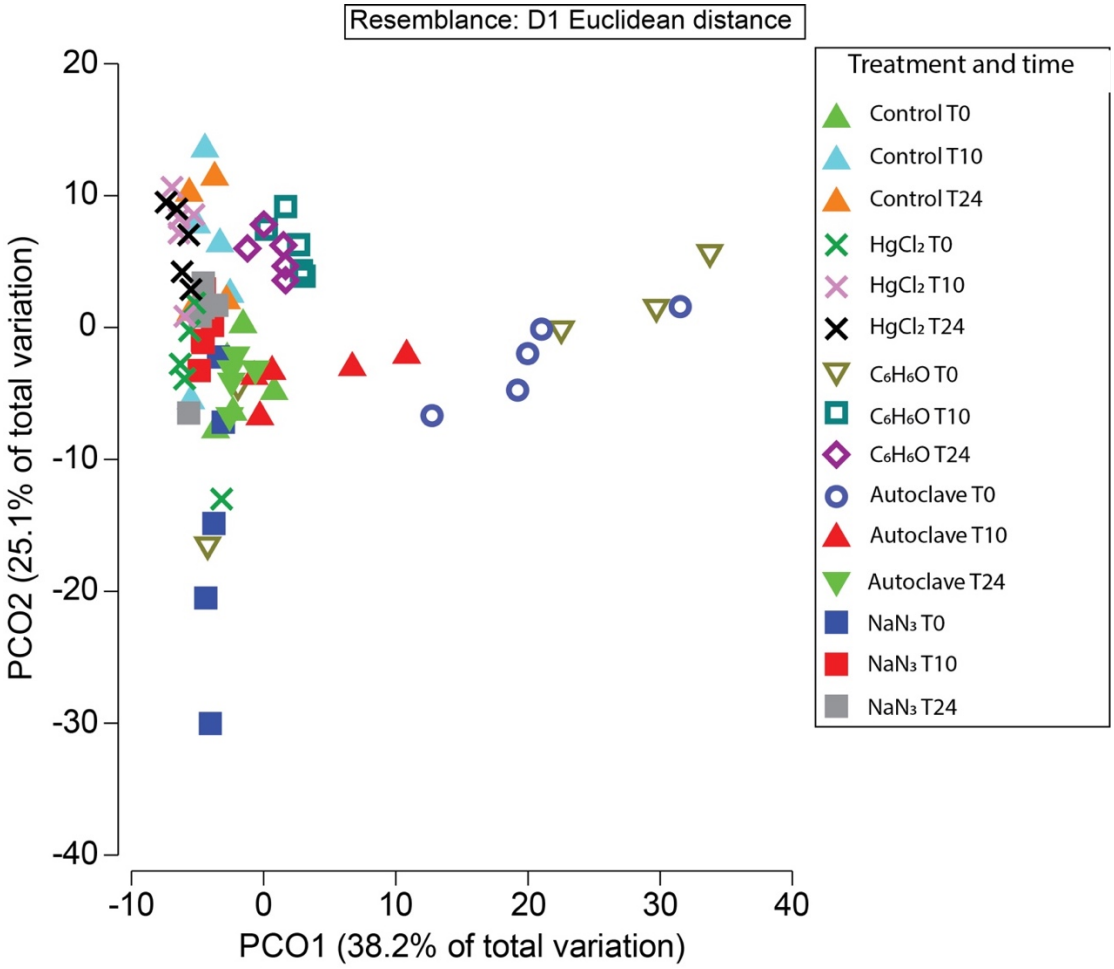


Figure. 2.4 Principal coordinate analysis (PCoA) of amino acid proportion showing all experimental treatments across all sampling times.

Table. 2.5 Microbial inhibitors and their ability (✓) or inability (×) to measure target molecules.

Microbial inhibitor	DON	TDN	TDP	DOC	NH_4^+	NO_x	DCAAs
$HgCl_2$	×	×	✓	✓	×	✓	✓
C_6H_6O	×	×	×	×	✓	✓	×
NaN_3	×	×	✓	✓	×	×	✓
Autoclave	×	×	×	×	×	×	×
$CuSO_4$	×	×	✓	✓	✓	✓	×

2.4.8 Selection of inhibitors for time course studies

An ideal inhibitor for time course studies should allow for analysis of all the same chemical components as a sample with no inhibitor (control), but should also not interfere in any way with the analytical procedure. Depending on the length that leaching periods are carried out, analytes can be expected to be at similar concentration, or greater than inhibitor-free experiments due to the absence of microbial activity. As mentioned above, for the measurement of DON and TDN none of the microbial inhibitors in this experiment are entirely suitable (Table 2.5); NaN_3 is itself a source of organic-N and caused extremely elevated TDN and DON values, autoclaving causes a large pulse of nutrients to be released at T0, likely due to cell lysis and leaching, $CuSO_4$ alters the colour of samples and therefore, interferes with DIN analysis, C_6H_6O formed a precipitate during digestion and $HgCl_2$ causes depressed NH_4^+ values and therefore inflated DON values. TDP and DOC were able to be measured in the presence of NaN_3, $HgCl_2$ and $CuSO_4$ however, recovery of both was highest with NaN_3. As for inorganic-N containing nutrients NO_x was able to be measured by $HgCl_2$, C_6H_6O and $CuSO_4$ however, NH_4^+ could only be reliably measured with C_6H_6O and $CuSO_4$. DCAAs and DCAA-N were unable to be measured with $CuSO_4$ and both the C_6H_6O and autoclave treatments appear to have influenced the release rate of DCAAs. Most appropriate for the analysis of DCAAs were $HgCl_2$ and NaN_3 however, most amino acids were measured at higher concentrations with the addition of NaN_3.

In conclusion, no single inhibitor fulfilled all the criteria and could allow detection of all target nutrients from single samples. It was possible to use a combination of inhibitors to allow inferences to be made about diverse compounds. NaN_3 is the best compromise for experiments that are aiming to measure DCAAs and DCAA-N as a component of DON (the target molecule). While DCAA proportions in the NaN_3 experiment were found to be significantly different to those in the control and $HgCl_2$

experiments, recovery of DCAAs was almost always higher. This difference may be attributed to NaN_3 effectively inhibiting microbial consumption. While NaN_3 interfered with some analytical techniques TDP and DOC could be measured in the presence of NaN_3 at higher recovery rates than the other microbial inhibitors tested.

2.5 Acknowledgements

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2.7 Supporting information

The effect of microbial inhibitors on the measurement of N and P-containing nutrients in aquatic samples.

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Summary of the supplementary information

- Number of pages:5 (including this page)
- Number of figures: 1 (multi-part)
- Number of tables: 2

Table. S2.1 Individual amino acid recoveries for bovine insulin standard.

Amino acid	Theoretical concentration (pmol/µL)	Average measured concentration (pmol/µL)	Recovery (%)
HIS	0.57	0.18	32.21
ARG	0.29	0.19	64.30
SER	0.86	0.58	67.75
GLY	1.14	0.86	75.41
ASX	0.86	0.59	68.48
GLX	2.00	1.37	68.44
THR	0.29	0.15	53.24
CYS	0.86	0.68	78.82
ALA	0.86	0.55	63.90
PRO	0.29	0.14	48.39
LYS	0.29	0.49	48.39
TYR	1.14	0.53	46.42
VAL	1.43	0.51	35.92
ILE	0.29	0.05	16.69
LEU	1.72	0.94	54.69
PHE	0.86	0.30	35.26

Table. S2.2 Three letter abbreviations for amino acids described in our study. Note that glutamine and asparagine are de-aminated during acid hydrolysis, thus are detected as glutamic and aspartic acid respectively. Hence our use of the abbreviation ASX and GLX.

Amino acid	**Three letter abbreviation**	**Amino acid**	**Three letter abbreviation**
Aspartic acid and asparagine	ASX	Cystine	CYS
Serine	SER	Tyrosine	TYR
Glutamic acid and Glutamine	GLX	Valine	VAL
Glycine	GLY	Methionine	MET
Histidine	HIS	Lysine	LYS
Arginine	ARG	Isoleucine	ILE
Threonine	THR	Leucine	LEU
Alanine	ALA	Phenylalanine	PHE
Proline	PRO	Tryptophan	TRP

Linking Narrative: Chapter 2 to 3

The previous chapter provided evidence that sodium azide (NaN_3) is the best compromise as a microbial inhibitor for time course leaf leaching experiments and the analysis of dissolved combined amino acid (DCAA) concentration and composition. In the following chapter, sodium azide is used as a microbial inhibitor in time course leaf leaching experiments to determine whether *Eucalyptus camaldulensis* leaves are an important source of dissolved organic nitrogen (DON) and the contribution that DCAAs and dissolved free amino acids (DFAAs) make to DON leached from the leaves over 24 hours.

Chapter 3 Proteins are a major component of dissolved organic nitrogen (DON) leached from terrestrially aged *Eucalyptus camaldulensis* leaves.

Authorship statement

The following chapter appears as published in *Environmental Chemistry* with the exception of formatting changes such as font type and chapter numbering on headings and figures.

Harris, C., Silvester, E., Rees, G., Pengelly, J., & Puskar, L. (2016). Proteins are a major component of dissolved organic nitrogen (DON) leached from terrestrially aged *Eucalyptus camaldulensis* leaves. *Environmental Chemistry*, *13*, 877–887.

Author contribution: I completed data collection with advice from ES, LP and JP regarding data analysis, especially in regards to leaching kinetics. I prepared the manuscript for publication with editing advice from both ES and GR (as per the normal supervisor role). A statement from co-author LP immediately follows.

Dear Board of Graduate Research, La Trobe University,

Re: Proteins are a major component of dissolved organic nitrogen (DON) leached from terrestrially aged *Eucalyptus camaldulensis* leaves

This is to confirm that my contribution to the above named work was in the alignment and optimisation of the infrared microscope at the Australian Synchrotron Microspectroscopy beamline and assistance in the collection of data.

Best regards,

Ljiljana

Dr. Ljiljana Puskar

Infrared Beamline IRIS

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Proteins are a major component of dissolved organic nitrogen (DON) leached from terrestrially aged *Eucalyptus camaldulensis* leaves.

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3.1 Abstract

Understanding sources and forms of dissolved nitrogen is of critical importance to the management of aquatic systems worldwide. Dissolved organic nitrogen (DON) often constitutes the largest portion of the dissolved nitrogen pool yet is commonly overlooked as a nutrient source to aquatic food webs, likely due to its bound nature within organic material and the non-specific methods by which it is measured. In this study, we have determined the protein and peptide (dissolved combined amino acid (DCAA)) contribution to DON leached from *Eucalyptus camaldulensis* leaves over 24 hours. The distribution of proteinaceous material in unleached and leached leaves has been characterised using Fourier transform infra-red (FTIR) microspectroscopy to determine the likely source of DCAA within the leaf tissue. DCAA were found to be a significant component (38.5 %) of the leached DON, however >90 % of the leaf protein remained in the leaves after 24 hours. FTIR microspectroscopy shows that proteinaceous material is strongly partitioned to fungal colonised palisade cells in the leaf mesophyll, with evidence for depletion of this material after leaching. Comparison of leaching kinetics in the presence and absence of a microbial inhibitor (sodium azide) suggests that microbial uptake/adsorption commences within the timescales of these leaching experiments. The work shows that DON in the form of peptides and proteins leached from leaf litter is a likely source of bioavailable nutrients to in-stream systems and eventual export to oceans.

3.2 Introduction

Dissolved materials in river systems worldwide, are an important source of carbon and nutrients for in-stream processes (Giller & Malmqvist, 1998). The role of inorganic nutrients in aquatic ecosystem function is well established (Vitousek *et al.*, 1979) however, the contribution of organic compounds as sources of bioavailable nutrients is not as well documented (Howarth, 1988). Dissolved organic nitrogen (DON) from surface waters can account for a large portion of total dissolved nitrogen (TDN) in surface waters (Campbell *et al.*, 2000; Seitzinger, 1988). DON comprises a range of compounds, from readily bioavailable compounds such as amino acids and nucleotides, through to complex and refractory N-containing humic materials (Campbell *et al.*, 2000; Stanley & Maxted, 2008). As a consequence, DON is typically determined by the difference between TDN and N-containing inorganic nutrients (Howarth, 1988; Martin & Harrison, 2011). Consequently, there are still major knowledge gaps in understanding the sources of DON and the proportion that is bioavailable within river and floodplain environments (Frankovich & Jones, 1998; Howarth, 1988; Kaushal & Lewis, 2003; Stepanauskas, 1999).

River floodplain systems of south eastern Australia are characterised by areas of low lying ground adjacent to rivers, with a high degree of variation in both frequency and time of inundation (Seitzinger & Sanders, 1997). Hydrological connections from the floodplain and the river channel facilitate transportation of carbon and nutrients influencing the productivity of the entire system. *Eucalyptus camaldulensis* dominates these floodplains and when leaves on the floodplain are inundated, they leach between 20 – 40 % of their total leaf mass within a few days of submersion (Boulton & Boon, 1991; Briggs & Maher, 1983). Thus, a large proportion of dissolved organic material (DOM) in these rivers (and similarly elsewhere in the world) originates from plant material

deposited directly into the river or indirectly through floodplain interactions (Baldwin & Mitchell, 2000; Boulton & Boon, 1991; Brookshire *et al.*, 2005; Seitzinger *et al.*, 2002). The chemical speciation of DON in 'terrestrially aged' leaves on a floodplain will likely depend upon the period of time they have been on the floodplain floor; when leaves fall on a floodplain, they quickly become colonised by fungi and bacteria and undergo terrestrial breakdown processes, with the rate of breakdown controlled (in part) by the availability of soil moisture (Briggs & Maher, 1983; Meyer, Wallace, & Eggert, 1998).

This study investigates DON release from terrestrially aged *E. camaldulensis* leaves over an initial 24 hour leaching period to answer three main questions: (i) are these leaves an important source of DON, (ii) what contribution do free amino acids and proteins make to DON leached from these leaves, and (iii) which region of the leaf is the primary source of labile DON? To do this we have measured leachate concentrations of dissolved free amino acids (DFAA) and dissolved combined amino acids (DCAA i.e. proteins and peptides). Fourier transform infra-red (FTIR) microspectroscopy of unleached and leached leaf sections has been used to determine the likely origin of DON from the decomposed leaf tissue. As part of this work we have also investigated the kinetics of DON leaching, and possible role of microbial uptake of released DON over the initial 24 hour period.

3.3 Methods

3.3.1 Source and characterisation of leaves

E. camaldulensis leaves were collected from forest litter at 12 Mile reserve, a floodplain adjacent to the Murray River, near the town of Howlong (S 37º38'47.2", E 146º22'86.1"; January of 2011). These 'terrestrially aged' leaves were stored in paper bags in the laboratory until required. The composition of the leaves was analysed for total carbon and

nitrogen content (LECO CNS2000 Analyser; three whole leaf replicates) (Table S3.1) as well as protein (amino acid) content (four whole leaf replicates; see below).

3.3.2 Leaching experiments

Two leaf leaching experiments were carried out, one in which a microbial inhibitor (2.5 mM sodium azide, NaN_3) (Baldwin, 1999) was used, and the other with no inhibitor. As will be discussed, azide interferes with the TDN assay whereas dissolved organic carbon (DOC) analysis is not affected by azide. In each leaching experiment approximately 0.5 g of *E. camaldulensis* leaves were placed in conical flasks with 2 L of Milli-Q water (18 MΩ cm; 5 replicates); zero time was taken to be the point at which the water was added. Flask openings were covered with aluminium foil to prevent airborne contamination and kept in a temperature controlled room at 20 °C. The flasks were gently stirred every hour to minimize concentration gradients, with solution samples taken every 2 hours for the first 12 hours and a final sample at 24 hours. Water samples were filtered through 0.45 µM pore-size cellulose-acetate membrane filters (Cameo) and stored frozen until analysed. These samples were analysed for concentrations of DOC, inorganic nitrogen species (NH_4^+, NO_x), TDN, DFAA, DCAA and total DNA. All analytes were converted to µg/g of leaf. The pH of the leaching solution was not buffered and measured to be approx. pH 8 (most likely controlled by leaf leachate); not affected by addition of NaN_3 (weak base).

3.3.3 Dissolved organic carbon, total dissolved nitrogen and nutrient analyses

Total dissolved nitrogen (TDN), oxides of nitrogen (NO_x; NO_2-N and NO_3-N) and ammonium (NH_4^+) were measured by flow injection analysis (FIA) (Lachat Quikchem 8000 Flow injection analyser; TDN range: 0.02 – 5.0 mg-N/L; oxides of nitrogen and ammonium ranges: 0.005 – 2.5 mg-N/L). TDN samples were digested using a (NaOH-$K_2S_2O_8$) persulfate oxidation procedure at 120 ºC for one hour (Hosomi & Sudo, 1986).

NO_x produced was analysed by FIA as described above. DOC was measured by either wet oxidation using an OI Analytical model 1010 Total Organic Carbon Analyser or by combustion using an Akta Jenika 3100 N/C Analyser (DOC range: 0.3 – 250 mg/L). Cross-comparison between these two instruments confirmed similar values for the same samples. All procedures employed method blanks and quality controls to check against potential sample contamination or calibration drift.

3.3.4 Total dissolved amino acid analysis

All amino acid digestions and analyses were conducted using new (pyrolyzed) sample vials. For whole leaf samples, replicate leaves were separately ground and homogenised to a fine powder using a mortar and pestle. Approximately 0.2 g of the powder was digested in 5 mL of 6 M HCl containing 0.02 % phenol at 150 °C for 1 hour under a nitrogen atmosphere. The solution was then diluted to a range that was appropriate for measurement (determined by serial dilution), freeze dried and then derivatized using the procedure described below. For DFAA analysis, one mL of filtrate was freeze-dried and the residue derivatized directly. For DCAA analysis 100 µL samples were freeze dried, 200 µL of 6 M HCl (containing 0.02 % phenol) added and the samples then hydrolysed under a nitrogen atmosphere at 150 °C for one hour (Fountoulakis & Lahm, 1998; Jørgensen & Jensen, 1997; Tsugita *et al.*, 1987). Hydrolysed samples were cooled, freeze dried and then derivatized. Reconstitution and derivatization of amino acids was performed using the Waters AccQ•Tag Chemistry Package (Millipore corporation 1993). Some amino acids are not recoverable using this hydrolysis method; tryptophan is destroyed and cysteine is often difficult to determine from the acid-hydrolysed samples, although we measured some in the whole leaf (Fountoulakis & Lahm, 1998). The addition of sodium azide may also affect determination of cysteine and methionine as it causes oxidation of methionine and cysteine producing cysteic acid, methionine sulfoxide and

methionine sulfone in addition to the amino acids(Manneberg, Lahm, & Fountoulakis, 1995).

The HPLC system consisted of a Waters 717 auto sampler, inline degasser, Waters 600 controller, and a Waters 2475 fluorescence detector (Waters Corporation, Milford, MA, USA). The separation system was a Waters C-18 (3.9mm x 150mm) column maintained at 37 °C with an eluent flow rate of 1 mL/min. The mobile phase was acetonitrile and AccQ•TAG gradient acetate-phosphate buffer, as per the AccQ•TAG method. The excitation wavelength of the fluorescence detector was 250 nm and the emission wavelength 395 nm. α-aminobutyric acid was included with all samples as an internal standard. Insulin from bovine pancreas (Sigma-Aldrich, St Louis, MO, USA) was used as a quality control sample. High and low calibration standard mixtures of all detectable amino acids (Amino Acid Standard H (Waters, Corporation, Milford, MA, USA) and blanks were run with each sample batch. The final concentration of amino acids was converted to µg/g of leaf. All amino acids measured in this work are represented by their three letter abbreviations; these are listed in Table S3.2. Differences in the amino acid abundances between whole leaf, non-azide leach and azide leach were analysed by a Principle Co-ordinate Analysis (PCoA) (Figure S3.1) and a Permutational Analysis of Variance (PERMANOVA).

3.3.5 DNA quantification

DNA quantification was conducted using the Quant-iT Pico Green procedure to detect double-stranded (ds) DNA (Life technologies, Invitrogen, Australia). Samples were prepared according to the Quant-iT Pico Green manual and analysed on a Fluoroskan Ascent (Thermo Scientific, Australia). ds DNA standards of 0, 10, 100 and 1000 ng/mL were provided by the manufacturer (Life Technologies, Invitrogen, Australia).

3.3.6 Fourier transform infrared microscopy

Leaf sections were analysed by Fourier transform infrared (FTIR) microscopy to determine the distributions of key biomolecule types. Two types of FTIR microscopy were used in this work: Focal Planar Array-FTIR with a conventional infrared source (FPA-FTIR; ~10 μM lateral resolution) and synchrotron sourced FTIR (S-FTIR; 5 μM resolution). Infrared (IR) maps were acquired in transmission mode, from both the mid-vein and the mesophyll regions of unleached and leached leaves.

The leaves used for FTIR microscopy were prepared in separate experiments to those described for leaching studies. These leaves (5 replicates) were cut in half (transversely) prior to leaching, with one half kept aside (unleached; T0) and the other half leached under the same conditions as the nutrient release experiment for 24 hours (leached; T24; no azide). The five replicates of T0 and T24 leaves were fixed with 4 % formalin and embedded in paraffin for histological sectioning (Heraud *et al.*, 2007). Transverse (axial) 8 μM sections of leaf tissue for IR microscopy were mounted onto poly-L-lysine coated CaF_2 slides (0.5 mm × 25 mm; Crystran, UK) and the paraffin removed by submersion into 3 separate xylene baths immediately prior to infrared analysis. Adjacent 6 μM transverse sections were stained with lacto-phenol cotton blue (LPCB) for fungal chitin (Bärlocher, 1980).

FPA-FTIR: Large area IR images of leaf sections (0.3 × 0.3 mm) were collected in transmission mode using a 15× objective and condenser (0.4 numerical aperture) of a Hyperion 3000 infrared microscope (Bruker Optik GmbH, Ettlingen, Germany). The microscope is equipped with a focal plane array (FPA) imaging detector and coupled to a Vertex 70 FTIR spectrometer (Bruker Optik GmbH, Ettlingen, Germany). FPA maps consisted of multiple 'blocks', each containing 4096 data points (64 × 64 element array), with 2 × 2 pixel binning to give a spatial resolution of ~10 microns. For each mapped area 64 scans were co-added and referenced against a background on the calcium fluoride free

of leaf material. The spectral resolution was 6 cm^{-1}, with Blackman-Harris 3-term apodization function. Opus 6.5 software (Bruker Optik GmbH, Ettlingen, Germany) was used to control the instrument.

S-FTIR: Higher lateral resolution infrared maps were acquired with a Hyperion 2000 infrared microscope (Bruker Optik GmbH, Ettilingen, Germany) connected to the infrared light source at the Australian Synchrotron (Clayton, Australia). The microscope (36× objective and condenser; numerical aperture 0.5) was equipped with Vertex 80V IR spectrometer with a photovolatic liquid nitrogen cooled mercury-cadmium telluride (MCT) detector (Bruker Optik GmbH, Ettilingen, Germany). Measurements were performed in transmission mode at 6 cm^{-1} spectral resolution with 32 co-added scans at each point, controlled with OPUS 6.5 software.

Data analyses were implemented with OPUS 7.0 software using the Chem Imaging module with band assignments from Heraud *et al.* (2007), Kerr *et al.* (2013) and Silverstein and Bassler (1962). A standard set of wavenumber ranges were integrated, including: (i) 3000 – 2800 cm^{-1} (ν(C-H) of most organic molecules) (ii) 1705 – 1570 cm^{-1} (including ν(C=O) of proteins (commonly known as amide I) and $\nu_{as}(COO^-)$ of carboxylate groups), (iii) 1565 – 1480 cm^{-1} (including ν(C-N) & ν(N-H) of proteins (commonly known as Amide II)), (iv) 1260 – 1210 cm^{-1} ((ν(C-C) & ν(C-O) typical of lignified material), and (v) 1180 – 950 cm^{-1} (ν(C-O-C) of carbohydrates). The absorbance at each wavenumber range was visualised using a heat map; for all maps the minimum absorbance was set to zero and the maximum to a consistent value for each leaf region (mid-vein or mesophyll) and wavenumber range, across all replicates.

Spectroscopic components contributing to the wavenumber region encompassing amide I and amide II were analysed using a Principal Component Analysis in Unscrambler 10.3 (CAMO Software AS, Oslo¸ Norway). For this analysis 100 random

single point spectra were extracted from the FPA-FTIR maps of the mesophyll region (50 from T0 and 50 from T24; 10 from each of the 5 replicates). In order to determine significance of differences between T0 and T24 (and therefore by inference a difference in protein content), these same spectral data were used to prepare a resemblance matrix, based on Euclidean distances between samples. A multivariate Permutational Analysis of Variance (PERMANOVA) was used to examine the relationship between spectra at T0 and T24, with time used as a single factor and running 9999 permutations (Andersson, 2003).

3.3.7 Kinetic fitting

Kinetic data was simulated using numerical integration program based on a Runge-Kutta algorithm. Rate constants were fitted to experimental data by a least squares minimization using a Simplex-based algorithm (Press *et al.*, 1990).

3.4 Results

3.4.1 Whole leaf analyses

The average total carbon and nitrogen contents of three replicate *E. camaldulensis* leaves were 49.83 ± 0.24 % (498.30 ± 0.24 mg-C/g) and 1.71 ± 0.06 % (17.07 ± 0.64 mg-N/g), corresponding to the C:N ratio of 29.2:1 (mass basis) (Table S3.1). The total amino acid content of the leaves (4 replicates) was 58.84 mg/g, with the relative abundance of the individual amino acids shown in Figure 3.1a. The contribution of these amino acids to leaf nitrogen was calculated to be 9.55 mg-N/g-leaf, taking into account the differing nitrogen content of each amino acid, equivalent to 57 % of total leaf nitrogen.

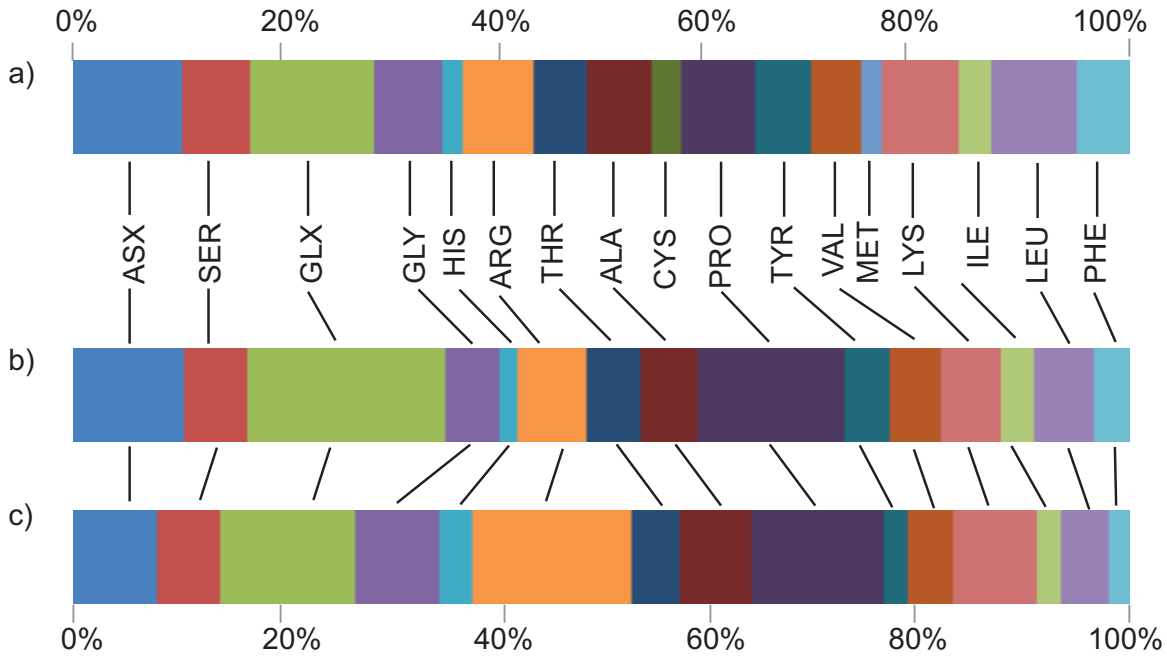

Figure. 3.1 (a) Average proportion of total amino acids within four replicates of whole Eucalyptus camaldulensis leaves; (b) Average amino acids leached after 24 hours (non-azide); and (c) relative nitrogen contribution of leached amino acids after 24 hours.

3.4.2 Amino acid leaching

Dissolved free amino acids (DFAA) were below detection limits in all leachate samples however dissolved combined amino acids (DCAA) were readily detected after acid hydrolysis. Individual DCAA leaching profiles for both the azide and non-azide leaching experiments are shown in Figure S3.2. The relative abundance of amino acids leached after 24 hours is shown in Figure 3.1b (non-azide data shown; azide data in Figure S3.3). Comparison of the amino acid distributions in leaf leachates (both azide and non-azide) with whole (unleached) leaf reveal that 88 % of variation is accounted for by the leaching process (pairwise comparison; whole leaf – azide, $p = 0.09$; whole leaf – non azide, $p = 0.05$). Differences in amino acid distributions between azide and non-azide leachates account for 7.6 % of the total variation ($p = 0.06$) (Figure S3.1). The whole leaf shows several amino acids which are more strongly represented including: PHE, LEU, GLY, CYS, MET and LYS, while others (PRO, GLU and ASP) are more strongly represented

in leachate (see Figure 3.1). This suggests some preferential leaching of proteins from the whole leaf.

Taking into account both the amino acid concentrations and nitrogen contents, the leading contributors of DCAA nitrogen in the non-azide leaching experiment were: ARG (14.9 %), GLU (12.8 %), PRO (12.5 %) and LYS (8.1 %) (Figure 3.1c). In the azide leaching experiment the dominant DCAA nitrogen contributors were: ARG (15.2 %), GLU (12.8 %), PRO (11.2 %) and ASP (8.6 %). The total DCAA nitrogen leached after 24 hours was 601 ± 17 µg-N/g-leaf, in the absence of azide, equivalent to ~6 % of leaf protein nitrogen. In the presence of azide the DCAA nitrogen released was 865 ± 111 µg-N/g-leaf, equivalent to ~9 % of leaf protein nitrogen. Despite of the differences in the amount of leaf nitrogen leached between the non-azide and azide experiments, it is clear that >90 % of the leaf protein remains associated with the leaf after 24 hours leaching. Sodium azide is known to cause oxidation of both methionine and cysteine resulting in partial measurement of these amino acids (Manneberg, Lahm, & Fountoulakis, 1995). However, as methionine and cysteine are absent in both the azide addition and non-azide leaching experiments this effect was not of concern.

3.4.3 Leaching kinetics

Concentrations of dissolved inorganic nitrogen (DIN; NH_4^+ and NO_x) were low in both azide and non-azide leach experiments (data shown in Figure S3.4). In the non-azide experiment ammonium concentrations reached 265 µg-N/g-leaf after 24 hours whereas 357 µg-N/g-leaf was released in the azide experiment. In both experiments ammonium leaching is closely following first order kinetics (non-azide: $k \approx 0.47$ hr^{-1}; $r^2 = 0.99$; azide: $k \approx 0.24$ hr^{-1}; $r^2 = 0.99$). Concentrations of NO_x species were very low in both the non-azide and azide leaching experiments, with maximum concentrations of 5 µg/g and 30 µg-

N/g-leaf released, respectively; the higher NO_x concentrations in the azide leachate may be due to partial oxidation of the inhibitor (Figure S3.4).

TDN concentrations were approximately 10× higher in the azide leaching experiment compared to the non-azide experiment, attributed to oxidation of the inhibitor during NaOH – persulfate digestion, and rending it impossible to reliably calculate the DON in this experiment (Figure S3.4). In the non-azide experiment DON showed a rapid release to a peak value of 1490 µg-N/g-leaf after eight hours, followed by an apparent decrease and then slow increase to 1562 µg-N/g-leaf after 24 hours (Figure 3.2a). Ninety-nine % of the TDN that initially leached from the leaves was DON, decreasing to ~85 % over the remaining 24 hours. DCAA nitrogen (aa-N) in the non-azide experiment showed a similar profile to DON, with a peak value of 524 µg N/g-leaf leached after 6 – 8 hrs, followed by a slight decrease between 8 – 10 hrs, and then an increase to 601 µg N/g-leaf at 24 hours (Figure 3.2a). This general leaching behaviour was observed for all amino acids in the non-azide experiment, as expected given that leaching entities are proteins or peptides and not individual amino acids. Overall, aa-N accounted for 38.5 % of the DON that leached from the leaves in 24 hours (Figure 3.2a). DCAA nitrogen leaching behaviour (aa-N) in the azide addition experiment was distinctly different to that observed in the non-azide experiment, with the data closely following first-order kinetics ($k= 0.148$ hr^{-1}; $r^2= 0.996$) and a significantly higher aa-N concentration in solution after 24 hrs (865 µg N/g leaf).

DOC release in the non-azide and azide leaching experiments closely approximated first order kinetics (non-azide: $k = 0.12$ hr^{-1}; $DOC_\infty = 32{,}750$ µg C/g leaf; $r^2= 0.991$; azide: $k = 0.105$ hr^{-1}; $DOC_\infty = 44{,}500$ µg C/g leaf; $r^2 = 0.997$; Figure 3.2b). The amount of DOC released from the leaves differed significantly between the two experiments, with less DOC leached in the non-azide experiment, particularly from 8 hrs

onwards. Part of the DOC leached is carbon associated with DCAA, the profiles for which are shown in Figure 3.2c. DCAA carbon (aa-C) shows the same behaviour as that observed for aa-N in the non-azide and azide leach experiments. After 24 hours, aa-C release in the non-azide experiment was 1975 μg C/g leaf, compared to 2851 μg C/g leaf in the azide experiment. aa-C represents 6.5 – 7.0 % of DOC, and the difference in aa-C leached after 24 hrs (871 μg-C/g) represents 8.3 % of the difference in DOC between the two experiments.

The DON:DOC ratios for the non-azide leaching experiment decreased from an initial value of 0.22:1 to a range of 0.07:1 – 0.09:1 for the first 8 hours, and finally to 0.05:1 after 24 hours (Figure 3.2d), indicating that the relatively nitrogen-rich compounds leach faster than nitrogen poor-compounds. The N:C ratio in the DCAA leached from the leaves is 0.3:1, similar to that observed for the initial leaching, however DCAA represents a very small proportion of DON at zero time (~11 %). DNA which is also relatively nitrogen rich (N: C in the range 0.5:1 – 0.2:1) was released during leaching, but in very small amounts relative to DON (12 – 20 μg-DNA/g after 24 hrs).

The leaching profiles observed for DON, DCAA and DOC in the absence of the microbial inhibitor suggest either an inhibited release or re-adsorption (uptake) of selected organic molecules. Given that the initial release rates are very similar in both the non-azide and azide addition experiments (see Figures 3.2a-c), a re-adsorption process appears more likely. The aa-N leaching (non-azide) data has been fitted to a surface induction and re-adsorption mechanism (Figure S3.5) where the DCAA release rate is the same as that observed in the azide leaching experiment, and the adsorption site concentration equal to the difference between aa-N concentrations in the non-azide and azide experiments at 24 hours. The aa-N leaching profiles have also been scaled to the aa-C leaching data (using the average C:N ratio for DCAA of 3.34:1; Figure 3.2c) and the DON leaching profile

(using the average DON: aa-N ratio of 2.91:1; Figure 3.2a). Comparison of the simulated kinetics with experimental data for DON suggests that non-DCAA DON leaches faster than DCAA. We do not attempt to apply this mechanism to DOC as aa-C represents a small proportion of DOC.

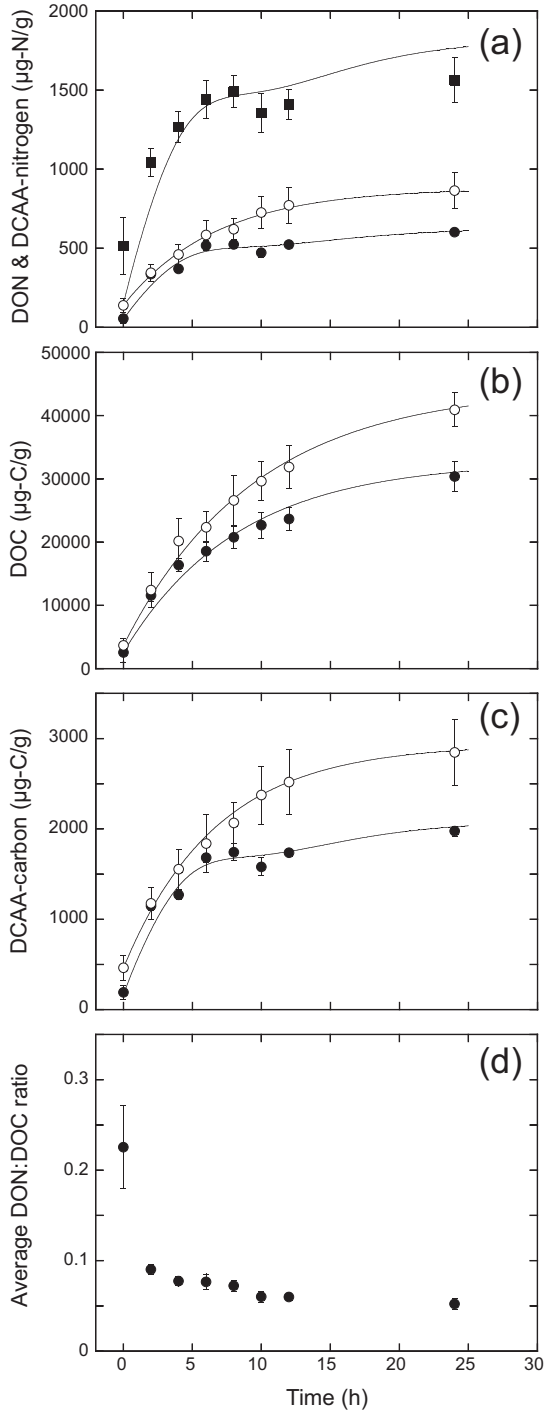

Figure. 3.2 (a) Time-course leaching experiments with (○) and without (●) azide addition showing: average concentration of dissolved organic nitrogen (DON) (■) and dissolved combined amino acid nitrogen (DCAA-N); (b) dissolved organic carbon (DOC); (c) dissolved combined amino acid carbon (DCAA-C); and (d) average dissolved organic nitrogen: dissolved organic carbon (DON:DOC) ratios over a 24 hour period for *Eucalyptus camaldulensis* leaves. Data points are the means of five replicate samples ± 2 SE.

3.4.4 Leaf section histology

Histological sections of *E. camaldulensis* leaves stained with lacto-phenol cotton blue (LPCB) are shown in Figure 3.3 for both the mid-vein and mesophyll regions, prior to leaching (T0) and after 24 hours leaching (T24). LPCB staining (which is specific for fungal chitin) (Sano *et al.*, 1993) indicates extensive fungal colonisation in both the mesophyll and mid vein regions of the leaf. In the mid-vein region fungal material is located immediately adjacent to the vascular bundle and between the xylem and phloem fibres. This distribution of fungal tissue has been shown previously to coincide with the regions of high protein content in the mid-vein of fresh *E. camaldulensis* leaves (Kerr *et al.*, 2013). Within the mesophyll fungal colonisation appears most prominent in the palisade cells (keeping in mind that eucalypt leaves are isobilateral and that palisade cells are situated on both sides of the mesophyll), which are also regions of high protein content in fresh leaves. While LPCB staining is not quantitative, fungal tissue clearly remains in T24 samples.

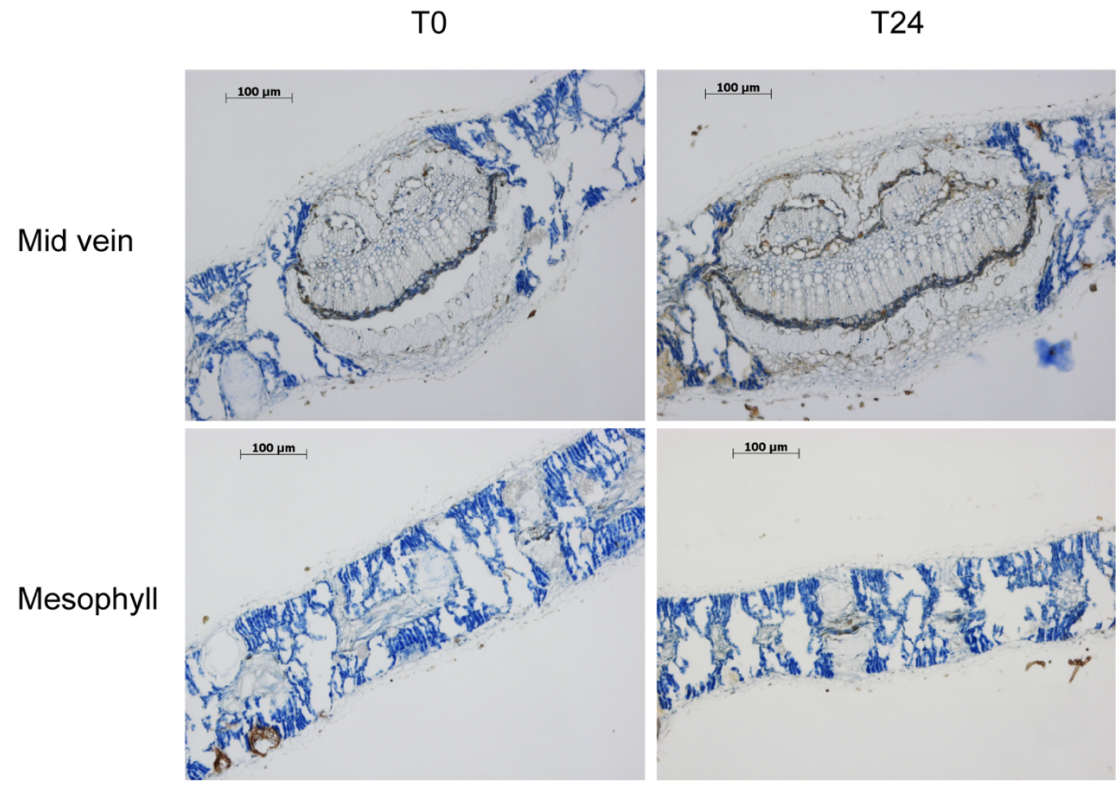

Figure. 3.3 Histological sections of the mid-vein (upper panels), and mesophyll (lower panels) stained with lacto-phenol cotton blue (LPCB) staining showing the presence of fungal material in terrestrially aged *Eucalyptus camaldulensis* leaf before (T0, left panels) and after leaching for 24 hours (T24, right panels).

3.4.5 Leaf section Fourier transform infrared mapping

Broad-scale FPA-FTIR maps of transverse sections of unleached (T0) and leached (T24) *E. camaldulensis* leaves were acquired from both mid-vein and mesophyll regions (5 replicates each). Representative maps (and associated microscopic images) for T0 and T24 samples are shown in Figure 3.4 for both regions; IR maps shown are for the wavenumber ranges: (i) 1705 – 1570 cm^{-1} (including ν(C=O) of proteins, commonly known as amide I), (ii) 1180 – 950 cm^{-1} (ν(C-O-C); carbohydrates) and (iii) 1260 – 1210 cm^{-1} ((ν(C-C) & ν(C-O); lignin). All FPA-FTIR replicates are shown in Figure S3.6 and Figure S3.7. The amide I map shows strong absorbance in the leaf mesophyll in T0, coinciding with the distribution of fungal colonised palisade cells (Figure 3.3). The absorbance of this band is reduced in T24, indicating that the concentrations of biomolecules containing this functional group have decreased after leaching. Amide I

absorbance is indicative of protein (Kerr *et al.*, 2013; Silverstein & Bassler, 1962) but can also be due to the presence of other biomolecules with amide linkages, including chitin (Kerr *et al.*, 2013). This wavenumber range will also capture the carboxylic band of pectins (unlikely to be present in decomposed leaves) as well as (weaker) aromatic absorption bands of polyphenolics (see later). While the decrease in Amide I absorbance between T0 and T24 in Figure 3.4 suggests strong leaching from the mesophyll, significant variation was observed between replicate samples (see Figures S3.6 and S3.7).

Similar to the results of Kerr *et al.* (2013) areas of high amide I absorbance in the mid-vein region are restricted to the region between the xylem and phloem fibres, and in the mesophyll immediately adjacent to the vascular bundle; distribution that is very similar to that observed for fungal tissue by LPCB staining (Figure 3.3). It is likely the biomolecules containing amide I do decrease in concentration in the vascular bundle between 0 and 24 hours, but the contribution to overall protein leached from the leaves is likely small compared to mesophyll.

The distributions of carbohydrates (as measured by the ν(C-O-C) band; 950 – 1180 cm^{-1}) in both the mesophyll and mid-vein regions are very similar to that for lignified tissue (as measured by the ν(C-C) & ν(C-O) bands; 1210 – 1260 cm^{-1}). This strong association is due to the presence of structural carbohydrates (cellulose and hemicellulose) in xylem; as expected, there is very little change in the amount of this tissue over the 24 hour leaching period as microbial access to this resource requires de-polymerisation of the lignin (Kerr *et al.*, 2013). Depletion of carbohydrates from the palisade cells in the mesophyll may occur over this time period but was not specifically analysed in this work.

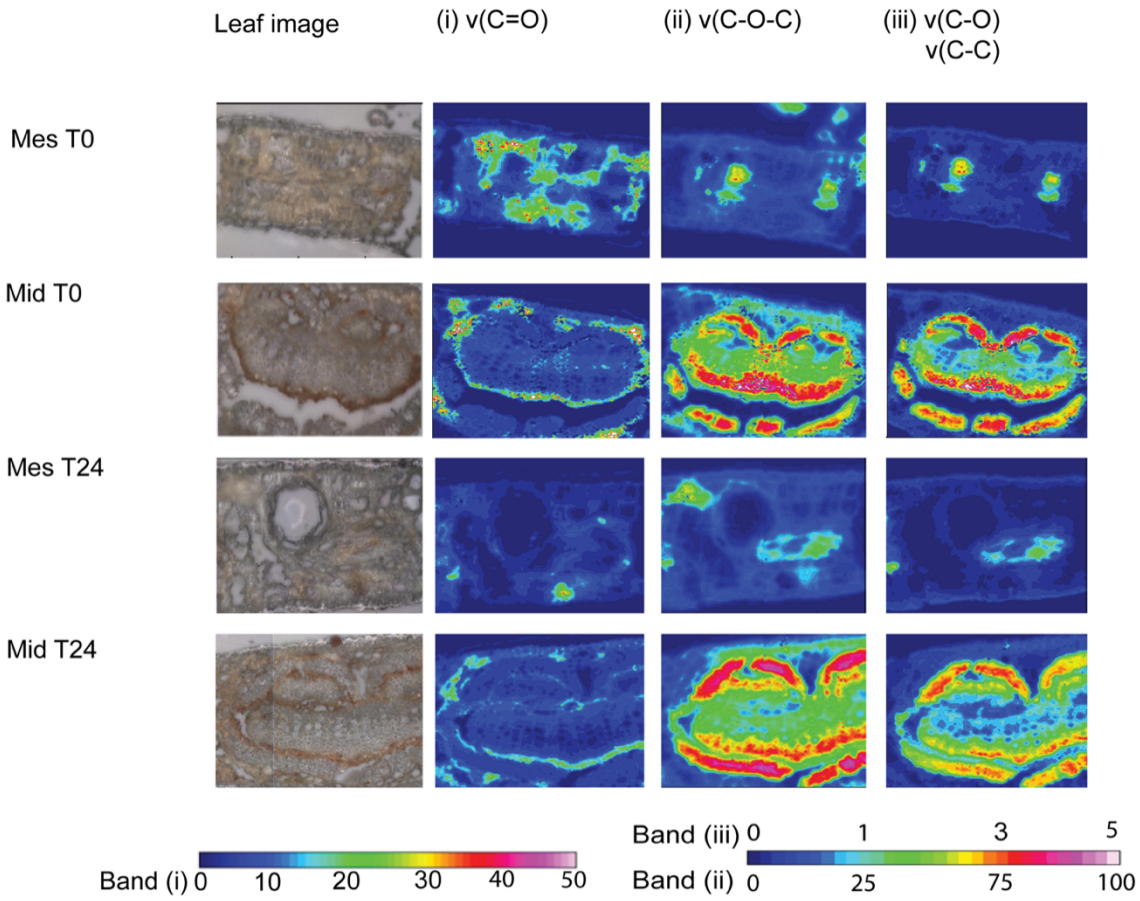

Figure. 3.4 Bright micrographs and focal planar array Fourier-transform infrared (FPA-FTIR) transmission images of transverse sections of *Eucalyptus camaldulensis* leaves, unleached (T0) and leached (T24) from mesophyll (Mes) and mid-vein (Mid) regions of leaf. IR maps are shown for wavenumber regions corresponding to: (i) C=O stretching modes of Amide I region (1705-1570 cm^{-1}); (ii) C-O-C stretching modes of carbohydrates (1180-950 cm^{-1}); and (iii) C-O and C-C stretching modes (characteristic of lignin) (1260-1210 cm^{-1}). The colour bars represent the intensity of the integrated band.

High resolution synchrotron-FTIR (S-FTIR) maps of individual mesophyll palisade cells are shown in Figure 3.5a for T0 and T24 samples. Also shown is the S-FTIR map of a fresh *E. camaldulensis* leaf (Fresh) for comparison. IR maps shown are for wavenumber ranges: (i) 1705 – 1570 cm^{-1} (ν(C=O); amide I) and (ii) 3000 – 2800 cm^{-1} (ν(C-H); organic molecules); the ν(C-H) map is included to assist in locating the palisade cells. Single pixel (5 × 5 μm) spectra from the palisade cells in each of the samples, over the wavenumber range 1800 – 1400 cm^{-1}, are shown as examples in Figure 3.5b. The spectrum for Fresh is similar for that observed previously for high protein regions of fresh

E. camaldulensis leaves, and with a peak at 1656 cm^{-1} representative of proteins with an α-helix structure (Susi & Byler, 1983), and similar to that observed elsewhere for photosynthetic cells (Heraud *et al.*, 2007). A distinct broadening of this band at lower wavenumbers is likely due to a combination of β-sheet structured proteins, auxiliary pectins and aromatic materials (Heraud *et al.*, 2007; Kerr *et al.*, 2013). Terrestrial aging (Fresh to T0) appears to result in physical separation (detachment) of the palisade cells and increase in the concentration of biomolecules containing the amide I (and amide II) bands. This increase is either due to shrinkage in cell volume, or a real increase in the amount of this material in the cells. The T24 sample shows a decreased intensity of the amide I (and amide II) bands, suggesting that the associated biomolecules are being lost from the palisade cells, consistent with that observed for amide I band in FPA-FTIR maps. The amide I band for T0 and T24 is distinctly different to that for Fresh, with a shift in the (protein) amide I peak to 1645 cm^{-1} and a prominent peak at 1628 cm^{-1}. Protein amide I bands in the range 1645 – 1656 cm^{-1} can be assigned to random mixtures of protein secondary structures (Kong & Yu, 2007); the band at 1626 cm^{-1} may be due to β-sheet proteins, but is also consistent with the amide I band of β-chitin; the reported amide II band of β-chitin at ~1560 cm^{-1} is however not evident in the spectra of either T0 or T24 (Noishiki *et al.*, 2003). Aromatic bands of polyphenolic materials also occur in this range and the presence of at least some aromatic material in the palisade cells is supported by the phenolic band (shoulder) at 1517 cm^{-1}, more prominent in the spectrum shown for T24 than T0.

In order to test whether the mesophyll spectra change significantly with leaching time we extracted 50 spectra (in the wavenumber range 1800 – 1400 cm^{-1}) from palisade cells (FPA-FTIR maps) for T0 and T24 (10 pixel points from each of the 5 replicates). Principal Components Analysis shows that 2 components explain 96 % of

variability, with component 1 (91 %) describing the intensity of the absorption bands (absorbance lower for T24 compared to T0) and PC2 (5 %) describing variations in the relative intensities of the two amide I peak at 1645 cm^{-1} and 1628 cm^{-1} as well an additional (likely phenolic) band at ~1510 cm^{-1} (see Figure S3.8). In order to test significance of the separation of T0 and T24, Principle Co-ordinate Analysis (PCoA) and a Permutational Analysis of Variance (PERMANOVA), based on the resemblance matrix were run for the 100 spectra (Figure 3.5c). A strong difference between samples was observed (pseudo-F = 29.755, p = 0.0001) but more importantly 86.1 % of variation is explained by the difference in leaching time (p = 0.001). We infer from this that the concentration of biomolecules containing amide groups in the mesophyll decreases between T0 and T24, most likely due to leaching. The amide I band peak shape does not differ significantly between T0 and T24 indicating that there is no preferential leaching of biomolecules (e.g. protein and chitin) from the palisade cells between.

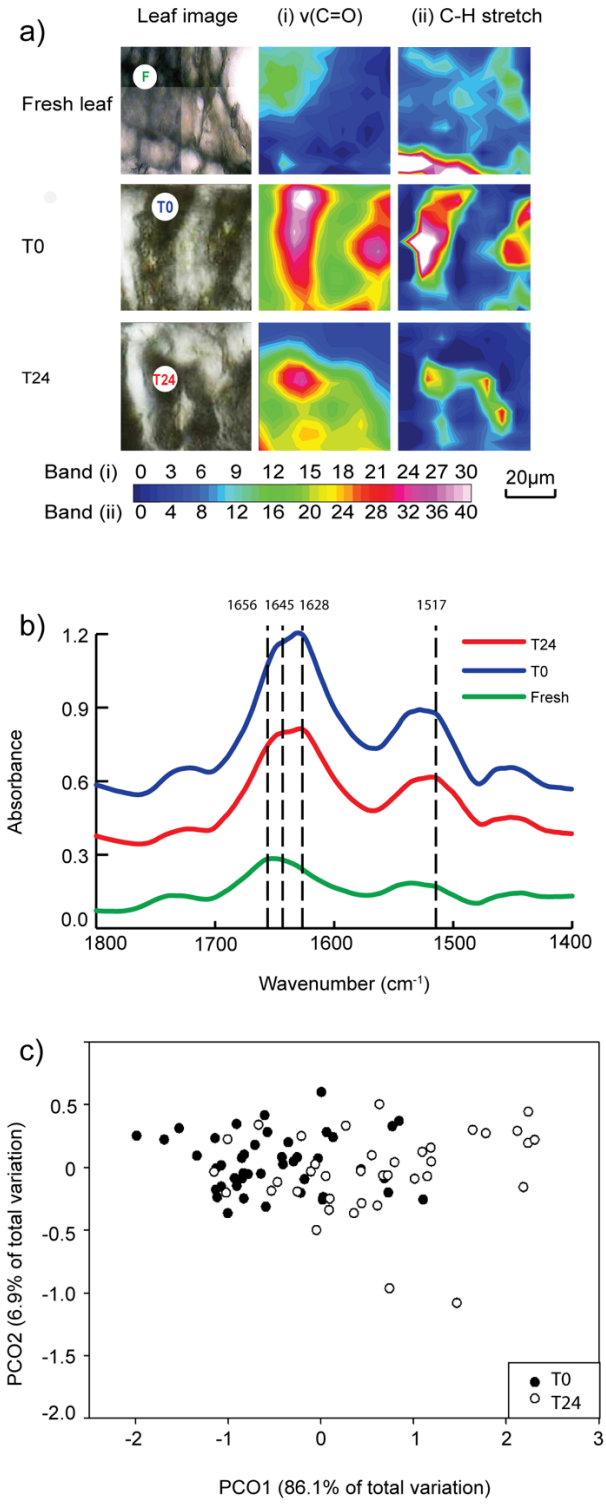

Figure. 3.5 (a) Bright field micrographs and synchrotron-sourced Fourier-transform infrared (S-FTIR) maps of fresh, unleached (T0) and leached (T24) *Eucalyptus camaldulensis* mesophyll cells. IR maps shown are (i) Amide I region (1705-1570 cm^{-1}); and (ii) C-H stretching mode region (3000-2800 cm^{-1}). (b) Single-pixel extracted spectra from palisade cells, from points indicated on the brightfield micrographs in (a). (c) Principal coordinate analysis (PCoA) analysis of FTIR spectra (1800-1400 cm^{-1}) for single pixel spectra (50 unleached (T0) and 50 leached (T24)) extracted from focal planar array (FPA)-FTIR maps of leaf mesophyll. The colour bars in (a) represent the intensity of the integrated band.

3.5 Discussion

Previous studies on leaf decomposition in aquatic systems have focused on the rapid release of dissolved organic carbon (DOC) and the longer term physical breakdown processes (Wallace, Ganf, & Brookes, 2008; Wetzel & Manny, 1972). These studies have been instrumental in understanding leaching mechanisms and the potential of the leached nutrients to satisfy demand in aquatic systems (Briggs & Maher, 1983; Corrigan & Oelbermann, 2013; Francis & Sheldon, 2002; Wallace, Ganf, & Brookes, 2008; Wetzel & Manny, 1972). A lesser amount of work has examined dissolved organic nitrogen (DON) as a potential nutrient source from leaf leachate, and only in terms of non-specific DON (Corrigan & Oelbermann, 2013; Wetzel & Manny, 1972). In this work we have determined the protein and peptide (DCAA) contribution to DON leached from *E. camaldulensis* leaves over 24 hours and examined leaf sections using FTIR microspectroscopy to determine the likely source of DCAA in the leaf tissue. In doing this we provide strong context for studying the bioavailability of DCAA in freshwater ecosystems, and an enhanced understanding of the leaf decomposition process.

3.5.1 Eucalyptus camaldulensis leaves as a source of amino acids

In the terrestrially aged leaves used in this work aa-N accounted for 57 % of leaf N and DIN accounted for 2 % (based on the amount of DIN leached). The remainder (41 %) is presumably associated with other organic N-containing molecules. There was no detectable DFAA in leachate samples, DFAAs are presumably lost during periodic rain flushing processes or rapidly assimilated by leaf microbes. DCAAs were found to account for 40 % of the total DON released (a smaller proportion than that of whole leaf aa-N), indicating that there may be smaller amounts of more labile DON species being released simultaneously. Conversely, DCAAs are a small contributor to DOC, so a large

proportion of the carbon containing molecules that are released have a low nitrogen content.

Dissolved organic nitrogen to carbon ratios were initially high (0.22:1) indicating a pulse of labile nitrogenous material upon submersion of the leaves; this material was not DCAA or DNA and was not identified in this work. After leaching for 24 hours 89 % of the total nitrogen content remained within the leaves along with 91 % total aa-N. Amino acid distributions were similar between azide and non azide experiments but differed from the whole leaf. While more work is required to quantify differences in amino acid distribution between whole leaves and leachate, this study shows a larger proportion of glutamic acid and proline within the leaf leachate than in the whole leaf. Conversely, while present in the whole leaf, methionine and cysteine (two amino acids involved in the development of secondary structures in proteins) were absent in both leaching experiments (Janson & Tischler, 2012).

Protein in the leaves was strongly partitioned to the mesophyll where fungal colonisation had taken place inside the palisade cells. While we cannot unequivocally confirm the presence of fungal chitin from the FTIR analysis, there is evidence for protein occurring in combination with fungal chitin from LPCB histological staining. S-FTIR shows that it is not green leaf protein in the mesophyll cells, but either de-natured or fungal protein. The fungal colonised palisade cells appear to the dominant source of aa-N in the terrestrially aged leaves. This is consistent with previous work that shows that woody vascular plant material is a poor source of amino acid nitrogen compared to other plant tissues where amino acid nitrogen accounts for 38 – 84 % of total nitrogen concentrations (Cowie & Hedges, 1992). Leaching from the mesophyll is also less likely to be impeded by locking within vascular tissue. The FTIR analysis suggests that the composition of the palisade cells remains unchanged after leaching, indicating that this

tissue leaches congruently. It appears most likely that the DCAA which appears in solution is that which has been released from degraded mesophyll tissue through aging and terrestrial breakdown processes (Gessner, Chauvet, & Dobson, 1999). Further terrestrial breakdown may lead to further pulses of DCAA during subsequent re-wetting events.

It is likely that some of the unidentified DON is fungal chitin (or the N-acetyl glucosamine monomer), as well as other high molecular weight organic-N compounds such as: lignin-bound peptides, fungal melanins, and humic acid precursors (Berman & Bronk, 2003; Kerr *et al.*, 2013). Fioretto *et al.* (2005) examined nitrogen dynamics during the period of lignin breakdown in three Mediterranean leaf species. After 3 years of exposure to microbial breakdown processes eight percent of the remaining leaf nitrogen was associated with lignin or lignin-like substances in *Quercus ilex* an evergreen oak species. Incorporation of nitrogen into lignin is likely mediated by fungi accessing lignin bound carbohydrates while simultaneously decomposing vascular material (Kerr *et al.*, 2013). The amount of nitrogen incorporated into lignin is therefore likely dependent upon the terrestrial ageing history.

3.5.2 Leaching kinetics

Leaching rates observed in this study (Figure 3.2) are generally similar to that observed elsewhere, with the majority of labile compounds leached within the first 24 hours (Briggs & Maher, 1983; Francis & Sheldon, 2002; Wallace, Ganf, & Brookes, 2008). 55 % of leachable DOC was released within the first 6 hours of immersion, a similar result to Wallace, Ganf, and Brookes (2008) who found 66 % of leachable DOC released from *E. camaldulensis* in the same time frame. DIN leaching rates were faster than both DOC and DON, likely due to the higher mobilities of NH_4^+ and NO_x.

Our experimental design included sodium azide as a microbial inhibitor to confirm that the first 24 hours of leaching is an abiotic process. Instead, a significant effect of the microbial inhibitor was observed for both DCAAs, DON and DOC. DCAA release in the presence of sodium azide was first order, consistent with that expected for direct leaching. However, in the absence of sodium azide there is evidence for either microbial activity or a re-adsorption of material back onto the leaf substrate. The non-azide data can be simulated according to a surface-induction and adsorption process (Figure S3.5) with adsorption commencing after approximately 8 hrs of leaching, and amounting to 250 μg-N/g-leaf after 24 hours. Armstrong and Bärlocher (1989a) reported a rapid (but small) adsorption of amino acids from leaf leachate onto the surface of stream-sediment biofilms. While the leaves in the present study had been dried and biofilm assumed to be dead, an additional study by Armstrong and Bärlocher (1989b) found no significant difference between active and dead biofilm surface adsorption and concluded that it is not governed by living bacteria but by physiochemical factors such as the surface structure of biofilm. Our work suggests that live biofilm does actively remove DCAA from solution, at least over the timescales investigated in this work.

It has been reported that *Eucalyptus* leaves leach large amounts of polyphenols and tannins during the initial leaching period which act as a defence to microbial colonisation (Hillis, 1966); such compounds could act in the same way as the sodium azide added in this study. Common phenolic compounds released from *Eucalyptus* vegetation include p-coumaric and ferulic acids, inhibiting microbial breakdown of leaves and activity of hydrolytic enzymes which aid in turnover of various cellular materials (DNA, RNA, proteins and other organic nutrients) (Canhoto & Graça, 1999; Canhoto & Graça, 2006; Chapuis-Lardy, Contour-Ansel, & Bernhard-Reversat, 2002). Factors such as oxidation combined wetting-drying cycles prior to sample collection likely influence

the amount and activity of polyphenolic material remaining within the leaf tissue. The terrestrially aged leaves used in this study had extensive fungal colonisation suggesting that microbial inhibitors in the leaf were no longer active.

3.5.3 Implications for aquatic ecosystems

This study has implications not only in wetland environments but also river systems that may receive a nutrient pulse during flood. In addition to in-stream processes, production within a riverine system is influenced by floodplain interactions, vegetation falling directly into the river and effluent sources along its length (Hadwen *et al.*, 2010; Seitzinger *et al.*, 2002). Conceptual work such as the flood pulse concept of Junk, Bayley, and Sparks (1989) attempts to explain floodplain interactions by proposing that a key driving force of food webs in stream environments is the flood pulse which has a more substantial effect upon nutrient concentrations than nutrient spiralling (conversion of organic and inorganic forms of nutrients along the length of a river).

Previously, Wetzel and Manny (1972) and Corrigan and Oelbermann (2013) have studied DON as nutrient source from leaf litter. Cowie and Hedges (1992) and Lee (1993) have considered marine sediments, bacteria and plankton as sources of dissolved free amino acids (DFAA) and dissolved combined amino acids (DCAA) within marine environments. This study provides evidence of DON in the form of protein from *E. camaldulensis* leaves contributing to the organic nitrogen pool in a freshwater environment. It also addresses the region of leaf that the material is located and where most labile and bound forms of nitrogen can be sourced. With approximately 0.17 % of leaf weight leached in the form of DON within 24 hours it is clear that leaf litter could play an important role in the delivery of DON to floodplain and riverine environments. Potentially, terrestrial leaf litter acts as a large and bioavailable source of organic

nutrients, particularly protein, to in-stream processes as well as an eventual source of nutrients exported to oceans.

3.6 References

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3.7 Acknowledgements

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3.8 Supporting information

Proteins are a major component of dissolved organic nitrogen (DON) leached from terrestrially aged *Eucalyptus camaldulensis* leaves.

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Summary of the supplementary information

- Number of pages: 11 (including this page)
- Number of figures: 8 (multi-part)
- Number of tables: 2

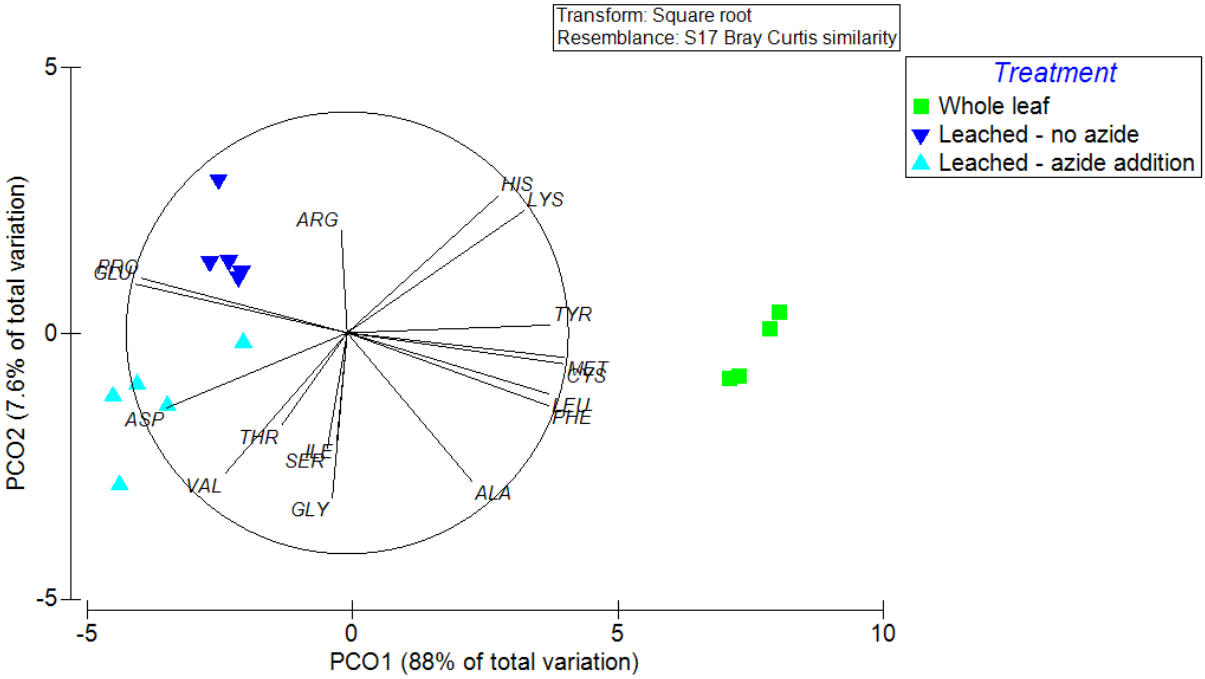
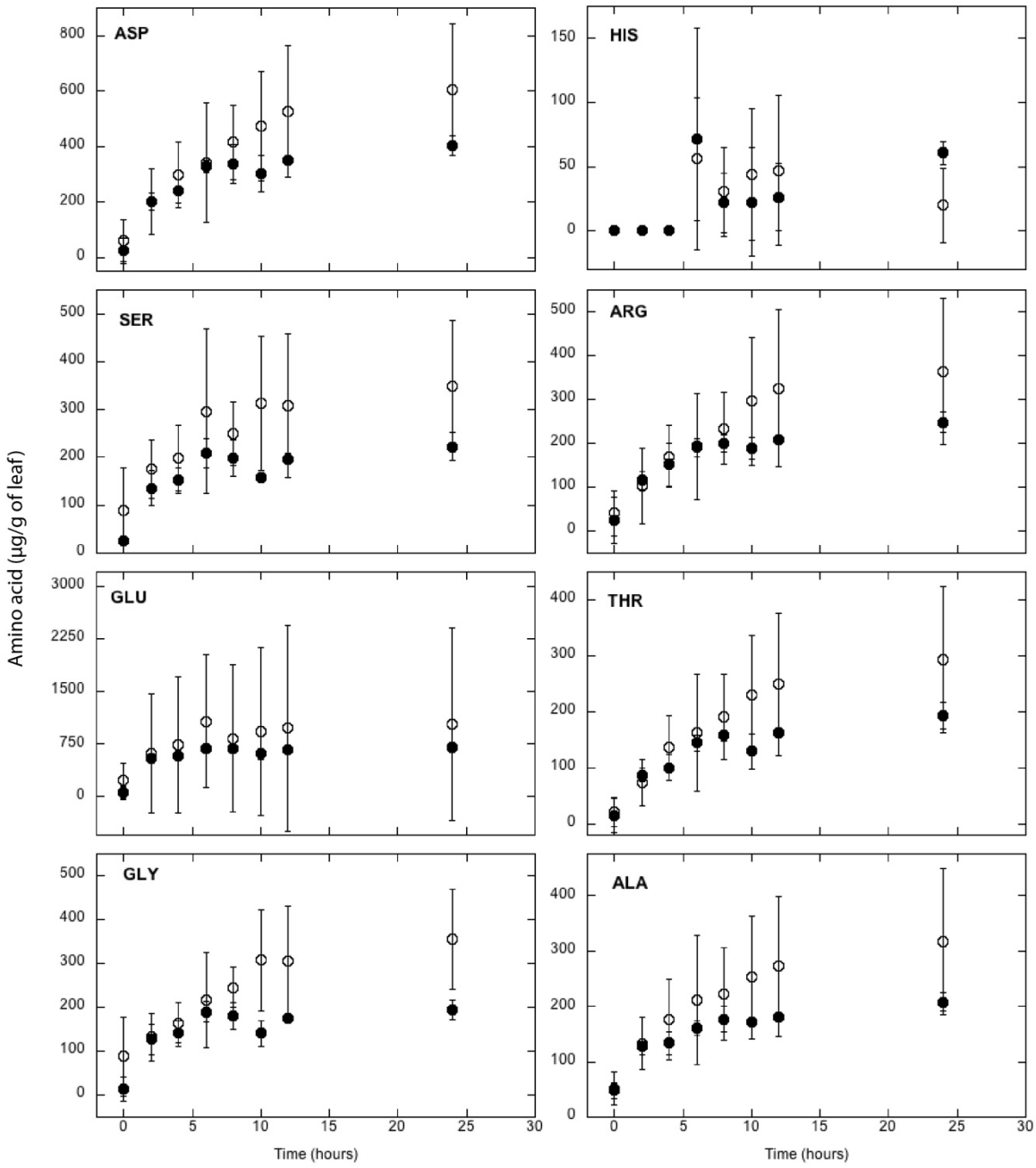


Figure. S3.1 Principal coordinate analysis (PCoA) of amino acid distribution in whole leaf and leachate samples (azide and no azide) after 24 hours.


ASP
HIS
SER
ARG
GLU
THR
GLY
ALA
Amino acid (µg/g of leaf)
Time (hours)
Time (hours)

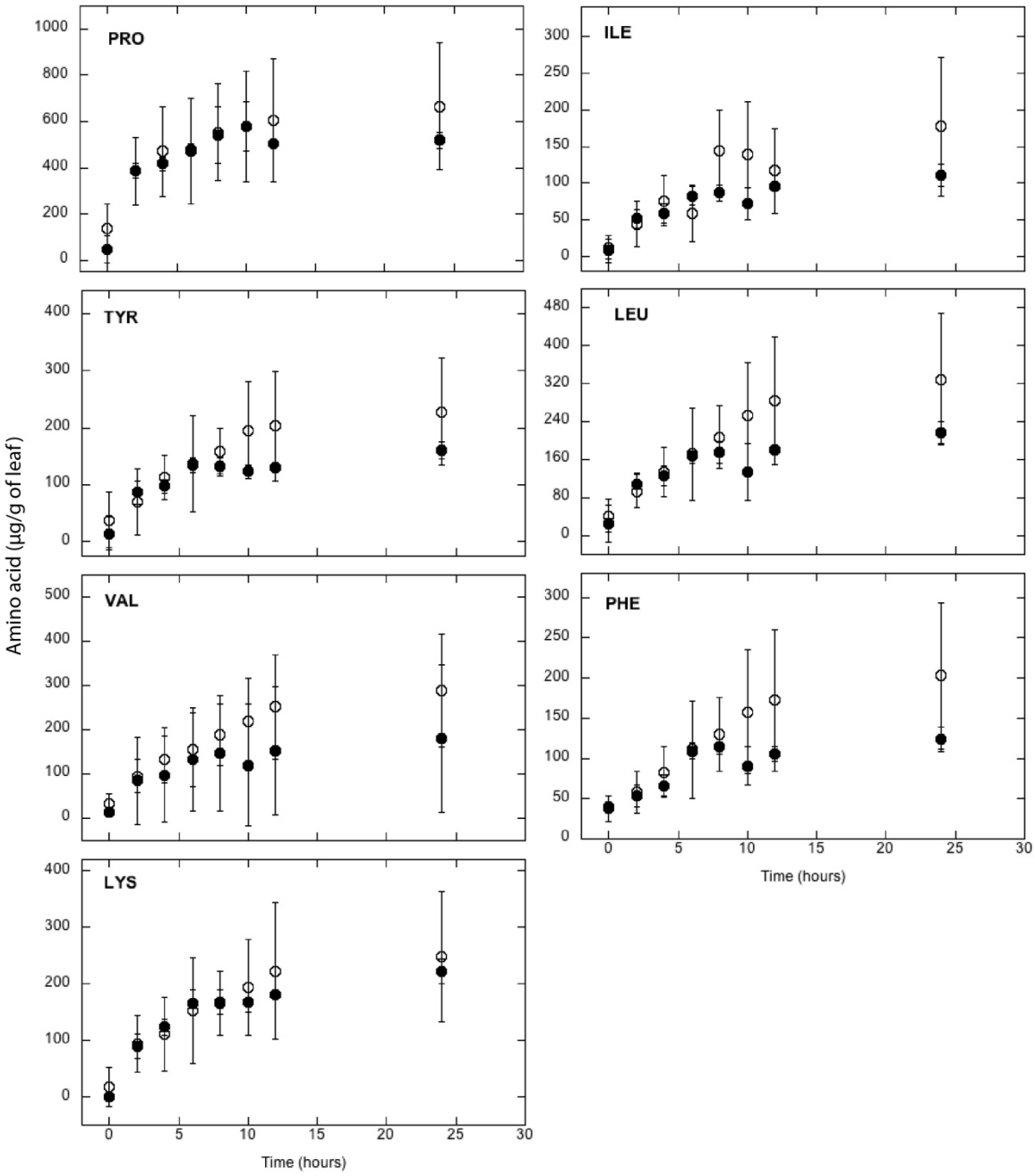


Figure. S3.2 Time course leaching experiments with (○) and without (●) azide addition showing average concentration of individual amino acids over a 24 hour leaching period. Data points are the means of five replicate samples ± 2 SE.

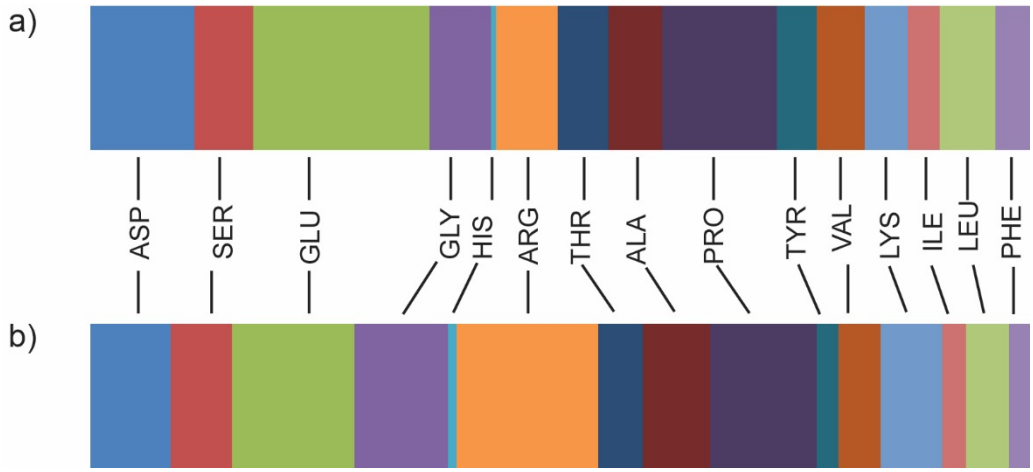


Figure. S3.3 Average proportion of total amino acids leached after 24 hours (a) and relative DON contribution of leached amino acids after 24 hours (b) in the azide addition experiment.

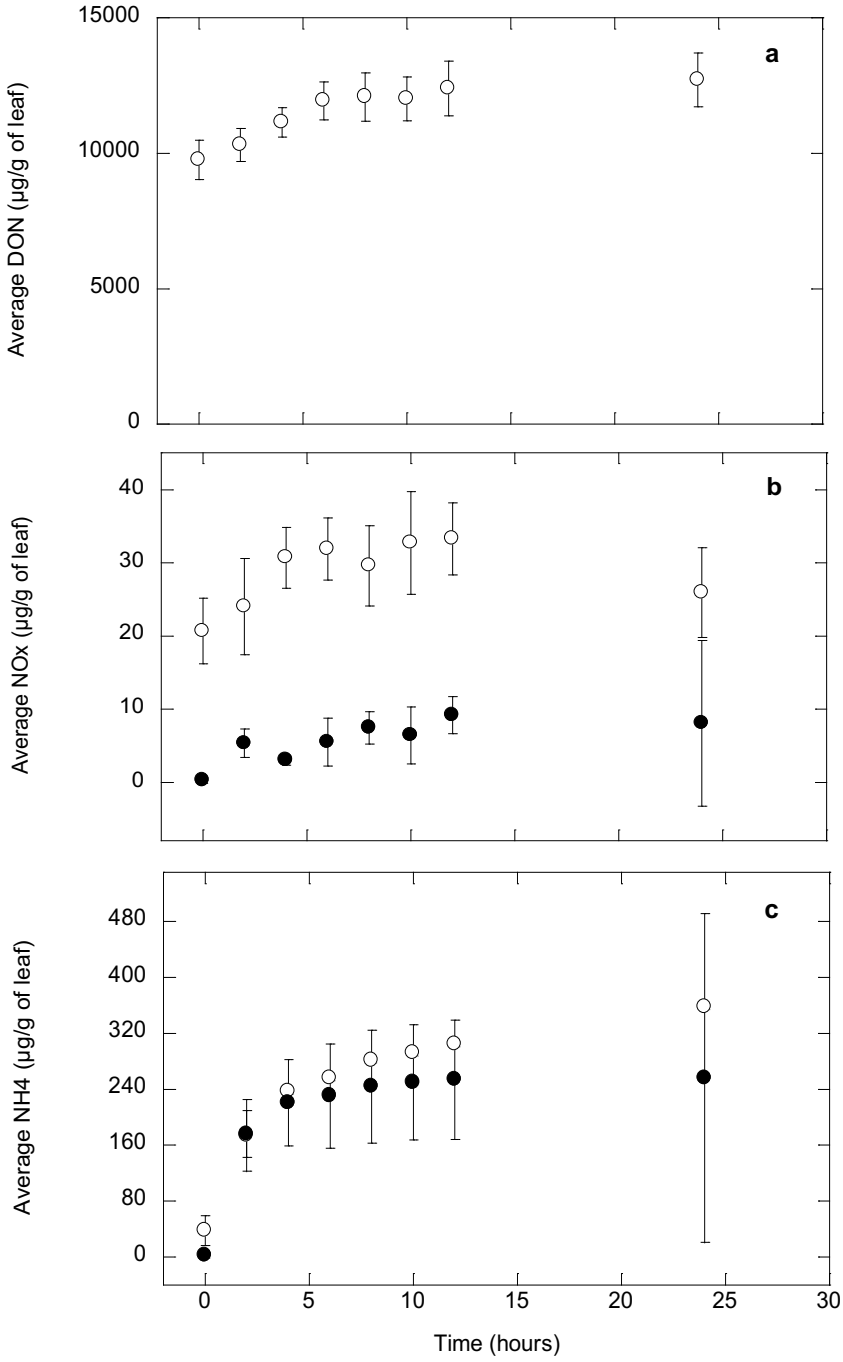


Figure. S3.4 Time course leaching experiments with (○) and without (●) azide addition showing average concentration of dissolved organic nitrogen (DON) (a) and inorganic nutrients (NO_x) (b) and ammonium (NH_4^+) (c) over a 24 hour period for *Eucalyptus camaldulensis* leaves. Data points are the means of five replicate samples ± 2 SE.

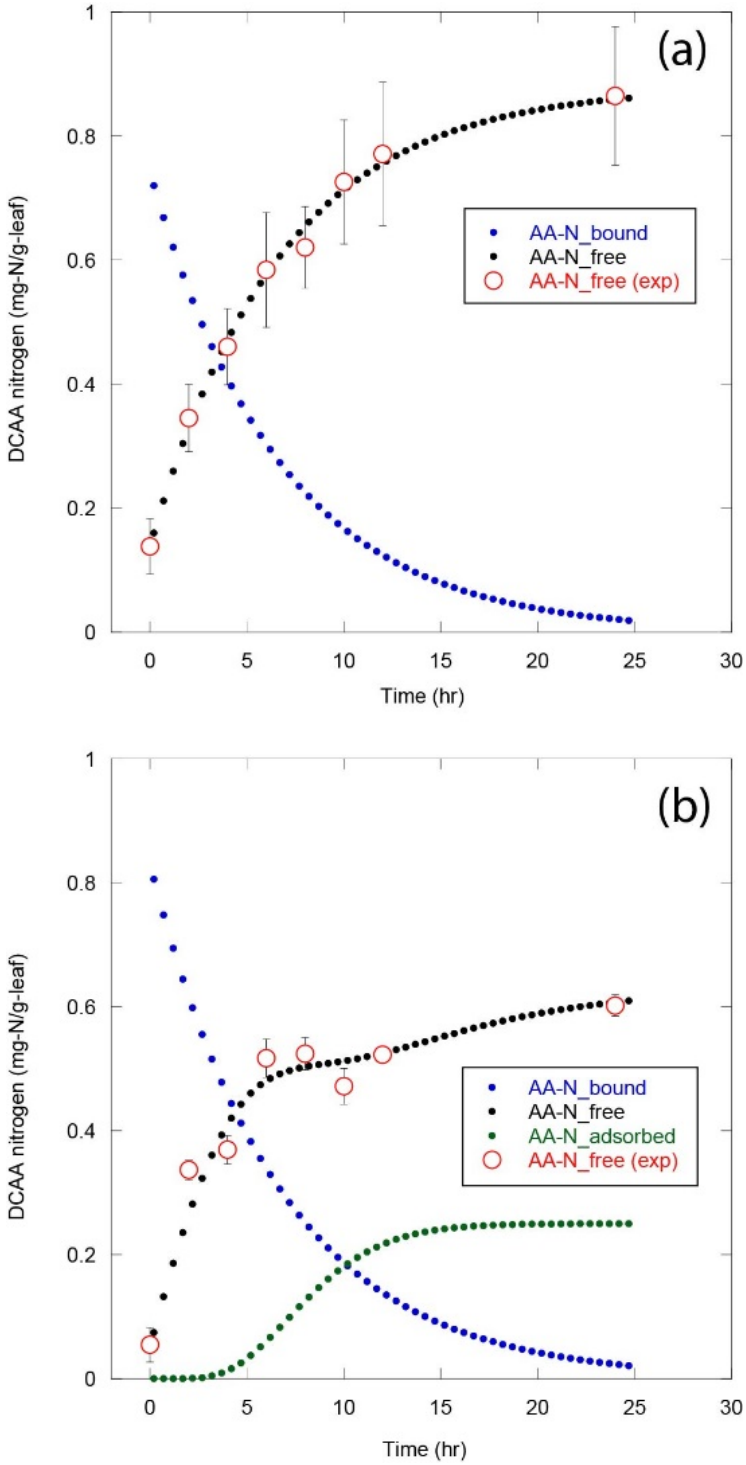


Figure. S3.5 (a) Simulation of DCAA-nitrogen leaching kinetics in the presence of azide according to first-order process, and (b) Simulation of DCAA-nitrogen leaching kinetics in the absence of azide, according to a surface site-induction and re-adsorption process. Mechanisms and fit parameters shown below.

Azide

AA-N_bound $\xrightarrow{k=0.149\ hr^{-1}}$ AA-N_free

AA-N$_{total}$ (mg-N/g)	**AA-N_bound$_{Initial}$ (mg-N/g)**	**χ^2**
0.880	0.742	0.827

Non-azide

AA-N_bound $\xrightarrow{k=0.149\ hr^{-1}}$ AA-N_free

Surf $\xrightarrow{k=0.757\ hr^{-1}}$ Surf* (×7)

AA-N_free + Surf* $\xrightarrow{k=3.91\ hr^{-1}}$ AA-N_adsorbed

AA-N $_{Total}$ (mg-N/g)	**AA-N_bound $_{Initial}$ (mg-N/g)**	**Surf $_{Initial}$ (mg-N/g)**	**χ^2**
0.880	0.830	0.250	4.18

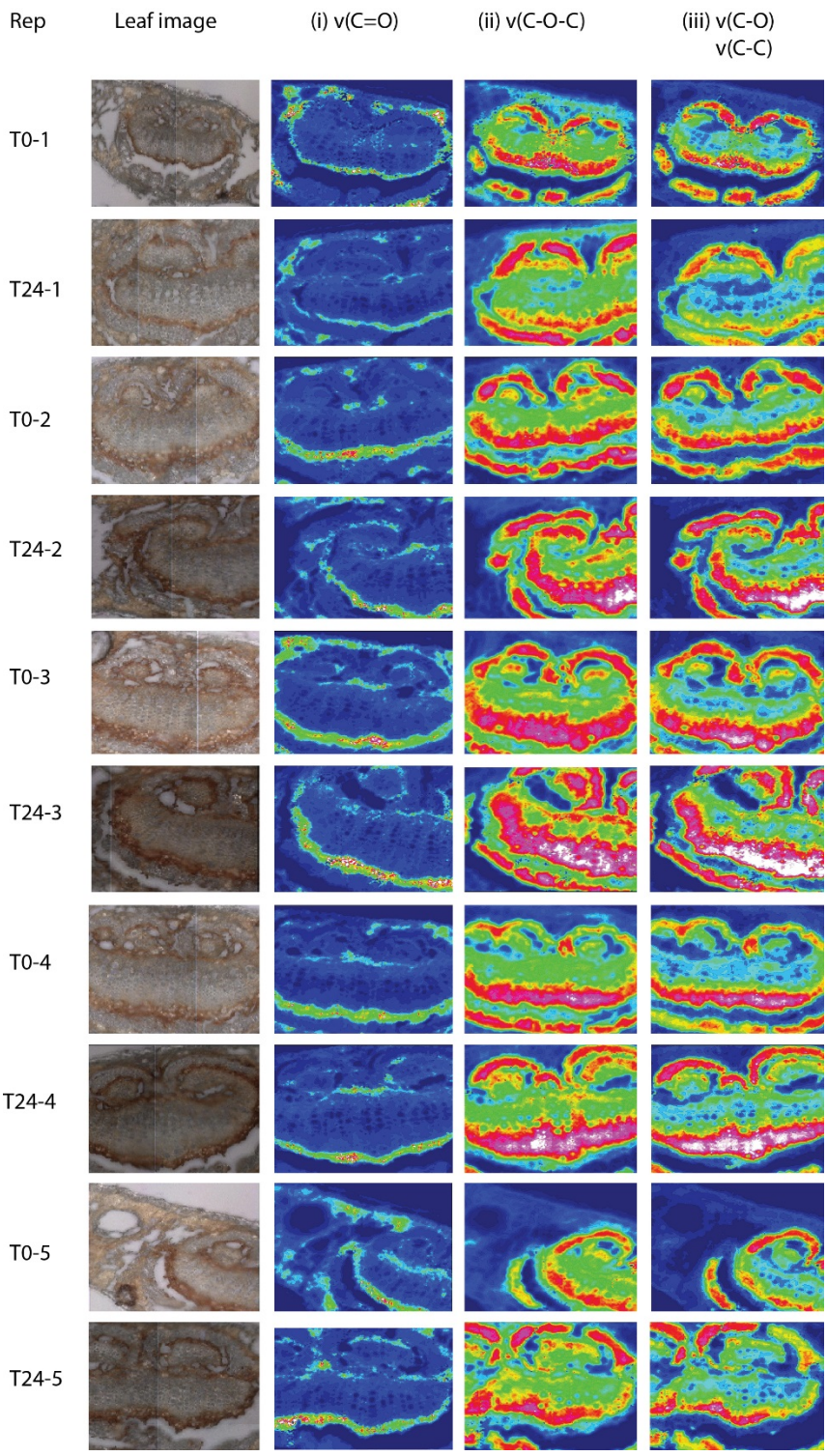


Figure. S3.6 Bright field micrographs and FPA-FTIR transmission images of transverse sections of *Eucalyptus camaldulensis* leaves, leached for 0 hours (T0) and 24 hours (T24) from mid-vein regions of leaf. IR maps are shown for wavenumber regions corresponding to: (i) stretching modes of Amide I region (1705-1570 cm^{-1}), (ii) C-O-C stretching modes of carbohydrates (1180 – 950 cm^{-1}), and (iii) C-O and C-C stretching modes (characteristic of lignin) (1260 – 1210 cm^{-1}).

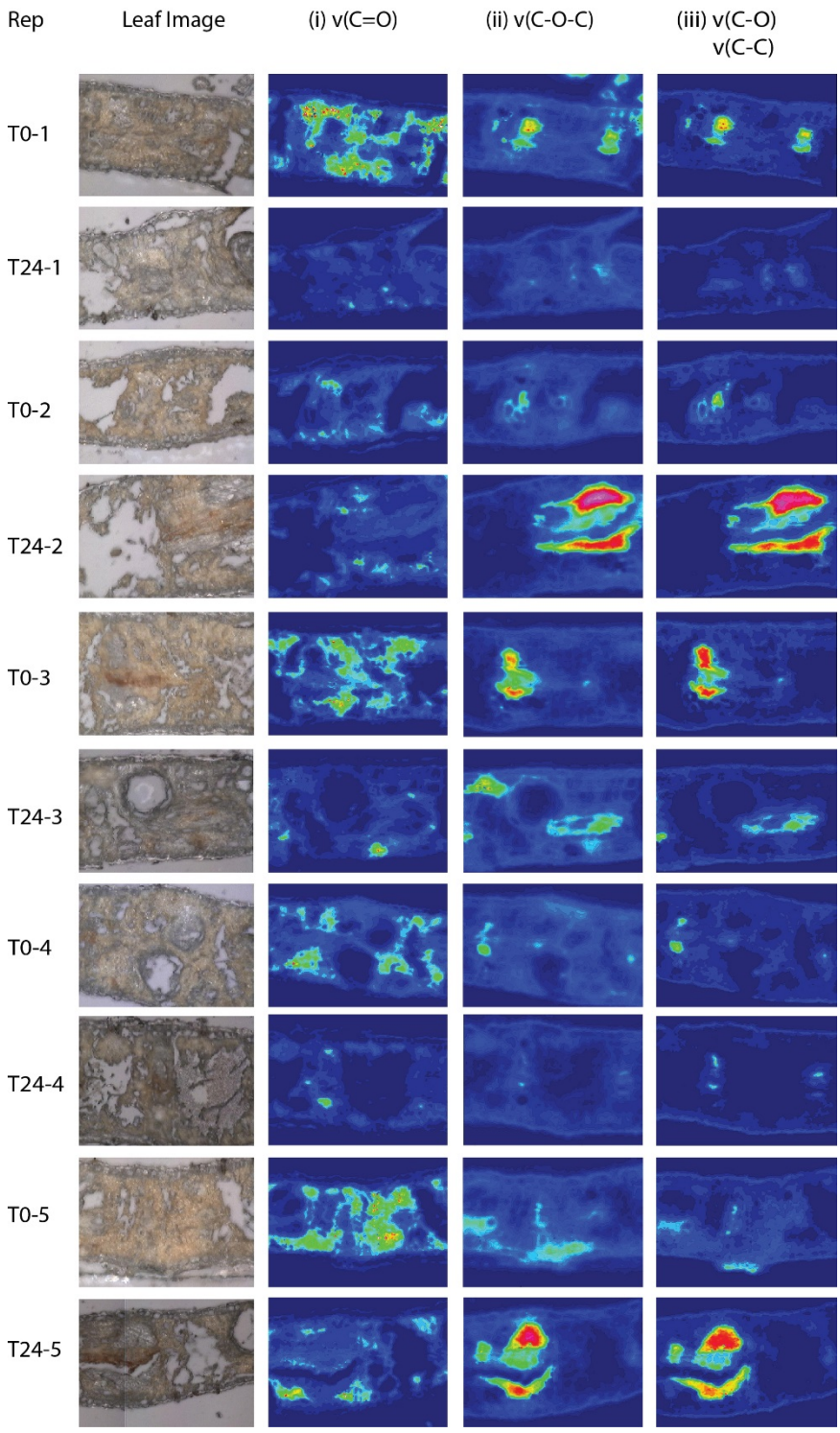


Figure. S3.7 Bright field micrographs and FPA-FTIR transmission images of transverse sections of *Eucalyptus camaldulensis* leaves, leached for 0 hours (T0) and 24 hours (T24) from mesophyll regions of leaf. IR maps are shown for wavenumber regions corresponding to: (i) stretching modes of Amide I region (1705-1570 cm^{-1}), (ii) C-O-C stretching modes of carbohydrates (1180 – 950 cm^{-1}), and (iii) C-O and C-C stretching modes (characteristic of lignin) (1260 – 1210 cm^{-1}).

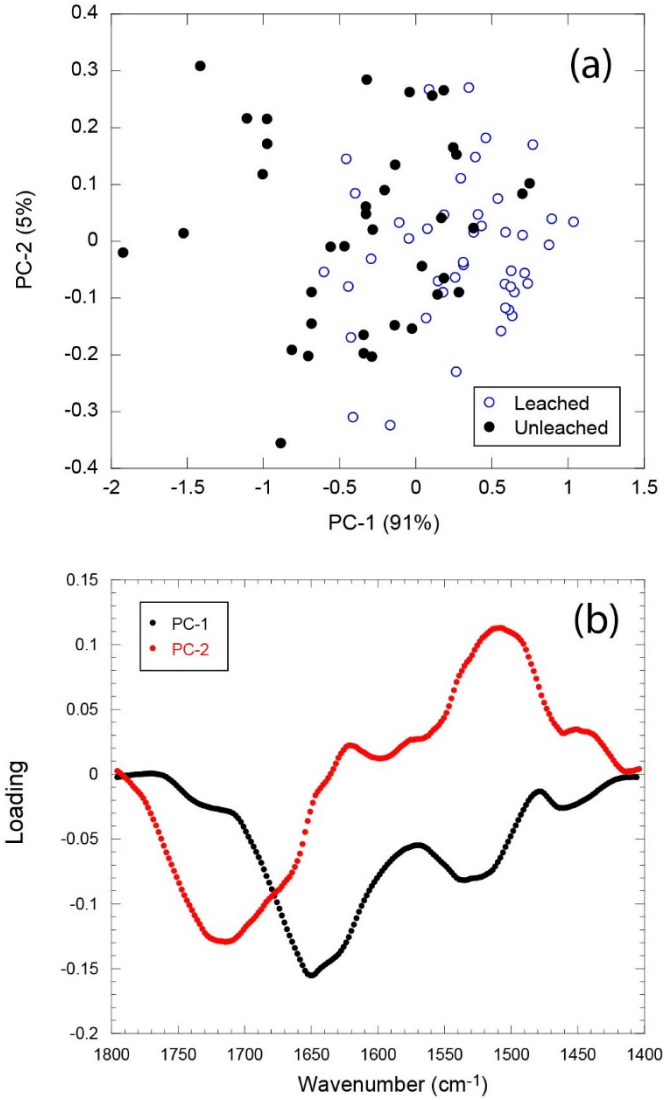


Figure. S3.8 (a) PCA scores plot for FTIR spectra (1800 – 1400 cm^{-1}) extracted from transverse sections of unleached and leached *Eucalyptus camaldulensis* leaves. (b) Loading plots for PC-1 and PC-2 which combined explain 97 % of the observed variance.

Table. S3.1 Total nitrogen analysis of three terrestrially aged *Eucalyptus camaldulensis* leaves.

Rep	%N	N (mg/g leaf)	%C	C (mg/g leaf)
1	1.65	16.5	50	500
2	1.76	17.6	49.5	495
3	1.71	17.1	50	500

Table. S3.2 Amino acid three letter abbreviations.

Amino acid	Three letter abbreviation	Amino acid	Three letter abbreviation
Aspartic acid	ASP	Cysteine	CYS
Serine	SER	Tyrosine	TYR
Glutamic acid	GLU	Valine	VAL
Glycine	GLY	Methionine	MET
Histidine	HIS	Lysine	LYS
Arginine	ARG	Isoleucine	ILE
Threonine	THR	Leucine	LEU
Alanine	ALA	Phenylalanine	PHE
Proline	PRO		

Linking Narrative: Chapter 3 to 4

In the previous chapter *Eucalyptus camaldulensis* was studied as a source of dissolved organic nitrogen (DON). It was found that ~40 % of DON occurred in the form of dissolved combined amino acids (DCAAs), a potentially bioavailable food source to in stream food webs. The following chapter investigates DON and DCAAs along a longitudinal gradient to determine whether the previously characterised portion of DON is influenced by factors such as major confluences, townships, floodplain connection and adjacent land use.

Chapter 4 Longitudinal trends in concentration and composition of dissolved organic nitrogen (DON) in a largely unregulated river system

Authorship statement

The following chapter appears as published in *Biogeochemistry* with the exception of formatting changes such as font type and chapter numbering on headings and figures.

Harris, C. W., Rees, G. N., Stoffels, R. J., Pengelly, J., Barlow, K., & Silvester, E. (2018). Longitudinal trends in concentration and composition of dissolved organic nitrogen (DON) in a largely unregulated river system. *Biogeochemistry, 139*(2), 139-153.

Author contribution: GR, ES and I completed data collection. RS, JP, KB and I completed analysis. I prepared the manuscript for publication with editing advice from both ES and GR (as per the normal supervisor role). A statement from co-authors KB and RS immediately follows.

To the Board of Graduate Research (Latrobe University),

I write to you to provide a quick summary of my involvement in the “Longitudinal trends in concentration and composition of dissolved organic nitrogen (DON) in a largely unregulated river system” paper which was produced by Clayton Harris as part of his Ph.D. studies.

For this project I used a Digital Elevation Model (DEM) of the Ovens River catchment to create a stream and sub-catchment area raster representation of the Ovens River Catchment. This was overlaid with sampling locations and the sub-catchments were grouped to define the upstream area of each sampling location. The land use classifications were then overlaid on the sub-catchment raster in ArcInfo to determine percentage cover for each of the major land-uses.

Please do not hesitate to contact me if you have any further queries.

Have a great day

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RE.: Statement from co-author, Dr Rick Stoffels

I'm writing to clarify my role as a co-author on the paper Harris, C. W., Rees, G. N., Stoffels, R. J., Pengelly, J., Barlow, K., Silvester, E. (2018) Longitudinal trends in concentration and composition of dissolved organic nitrogen (DON) in a largely unregulated river system. *Biogeochemistry*. 139(2): 139-153. I had no contribution to the study objectives or design. I collaborated with Clayton after the data was collected, to assist with advanced analysis of a difficult data set; additive modelling to determine discontinuities in a spatial series. Following instruction from Clayton concerning the questions he wanted answered, I tidied the data, wrote the code to complete the analysis and prepared a figure. Clayton wrote the paper.

Regards,
Rick Stoffels

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Longitudinal trends in concentration and composition of dissolved organic nitrogen (DON) in a largely unregulated river system

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4.1 Abstract

Dissolved organic nitrogen (DON) can comprise up to 80 % of the dissolved nitrogen pool in riverine ecosystems, but concentration and compositional responses to catchment conditions has received limited attention. We examined the suite of nitrogenous nutrients along the length of the Ovens River, Victoria, Australia, a river with identifiable regions of native vegetation, agricultural activity and floodplain forest connection, carrying out longitudinal surveys in winter during a period of high flow and in summer during a period of stable base flow. We examined: the concentrations of DON, the proportion of DON that occurs as dissolved combined amino acids (DCAAs), whether concentration and DCAA composition varied between flow and whether land use and tributaries have an impact upon nutrient concentration and DON composition. DON concentrations were greater than dissolved inorganic nitrogen (DIN) under both base flow and high flow conditions. Under base flow DON exhibited a continuous increase in concentration downstream (ranging from 50 to 300 µg/L), compared to a much larger increase under high flow (150 – 600 µg/L) coupled with a major discrete increase of ~350 µg/L at a tributary input (King River). Concentrations of NO_x (oxides of nitrogen) species were much higher under high flow conditions (range: 50 – 250 µg/L) compared to 0 – 50 µg/L at base flow, and showed a significant increase in concentration with distance downstream. A discrete change in NO_x concentrations was also observed at the King River confluence under high flow, although in this case causing a decrease in concentration of ~100 µg/L. DCAA concentrations varied little along the length of the river at base flow but increased with distance downstream at high flow. The DCAA concentrations were of the same order of magnitude as ammonium at both base and high flows and nitrate concentrations at base flow. The proportion of DON that was in the form of DCAA was reasonably uniform during high flow (3 – 6 %), but highly variable at

base flow (5 – 44 %). The amino acid composition of the DCAA varied along the river and differed between flow regimes (except below the confluence with the King River where amino acid composition under the two flow conditions converged) suggesting a strong influence of land use. We show that DON is potentially a large component (4 – 81 %) of the total nitrogen budget and given that 5 – 23 % is in the form of peptide/protein, represents an important source of nitrogen. DON and more specifically DCAAs should therefore be considered both when constructing nitrogen budgets and monitoring levels of in-stream nitrogen.

4.2 Introduction

Field studies of longitudinal nutrient concentration and composition have had a strong influence on river management by enhancing our understanding of the effects of river regulation, adjacent land use practices, and major tributaries, as well as nutrient availability (Baldwin, 1999; Hadwen *et al.*, 2010; Kaushal *et al.*, 2014; Mattsson *et al.*, 2015; Petrone, Richards, & Grierson, 2009; Wiegner *et al.*, 2006). These studies are often undertaken in an effort to understand anthropogenic impacts upon nutrient cycling and to examine the effect of factors such as land use, point source nutrients, effluent release, floodplain interactions and hydrological influences (Stanley & Maxted, 2008).

While studies on nitrogen processes have generally focused on inorganic species, studies have shown that the contribution of dissolved organic nitrogen (DON) can vary widely across streams. In forested catchments DON has been reported to constitute on average approximately 80 % and up to 90 % of the total nitrogen load (Kortelainen *et al.*, 2006; Kortelainen, Saukkonen, & Mattsson, 1997). A wider study of European streams showed the proportion of DON is variable across streams, comprising between 11 and 100 % of the total dissolved nitrogen load (Mattsson *et al.*, 2009).

Part of the reason why DON has received less attention than inorganic nitrogen species may be due to the large array of compounds that comprise DON (Kaushal *et al.*, 2014; Stanley & Maxted, 2008; Stepanauskas, 1999; Wiegner *et al.*, 2006), thus leading to complexity in understanding its potential biological function. DON is often determined as the difference between total dissolved nitrogen (TDN) and dissolved inorganic nitrogen (DIN) and as a consequence there are still major knowledge gaps in understanding the bioavailable portion within river and floodplain environments (Brookshire *et al.*, 2005; Campbell *et al.*, 2000; Seitzinger & Sanders, 1997; Seitzinger *et al.*, 2002). Presently, it is believed that of the DON in freshwater streams between 1 and 3 μM nitrogen is dissolved

combined amino acids (DCAAs) (proteins and peptides) that can be readily used as a nutrient source (Bronk *et al.*, 2007; Jørgensen, 1987; Stepanauskas, Laudon, & Jørgensen, 2000). The remaining DON is made up of larger and/or more recalcitrant compounds such as chitin, melanin, or bound up within lignin-like complexes that are less bioavailable, requiring further photochemical or biological decomposition processes prior to being used as a nutrient source (Berman & Bronk, 2003).

A limited number of longitudinal studies have provided some insight into how DON can vary along the length of a river. For example, at summer base flow in Hugh White Creek, USA, DON was found to decrease in the lower reaches of the stream due to rapid ammonification and nitrification. DON uptake was highly responsive to the amount of inorganic nitrogen within the stream, showing pronounced uptake of DON and dissolved organic carbon (DOC) when the inorganic concentrations were low (Brookshire *et al.*, 2005). Less information is available on role of catchments, concentrations of DON and its composition.

Our study focuses on the Ovens River of south-eastern Australia (Figure S4.1). The Ovens River is one of few largely unregulated rivers in south-east Australia, having a natural seasonal flow regime that leads to episodic flooding events (Baldwin *et al.*, 2006; Cottingham *et al.*, 2001; Hadwen *et al.*, 2010; Rees, Bowen, & Watson, 2005). The Ovens River has well recognized tributaries, one major urban settlement and floodplain connection in the lower reaches (Cottingham *et al*. 2001), which allows us to explore flow, land use and water chemistry relationships.

To investigate the longitudinal relationships of dissolved nitrogen species and dissolved organic carbon in this river system, we conducted two longitudinal field surveys; a summer survey at base flow and a winter survey (during the recession of a flood event) along the length of the Ovens River, Victoria. For both surveys samples were

taken for analyses of N-containing nutrients (including DCAAs) to determine the effects of major tributaries, changes in vegetation and land use. Data analyses were designed to test three hypotheses about the spatial and temporal variation in dissolved nutrients: First, concentrations increase with discharge and distance downstream, with this trend being unimpacted by the three main tributaries of the Ovens River. Second, concentrations increase with discharge and distance downstream, but with all the three main tributaries of the Ovens causing discontinuities in this trend. Third, concentrations increase with discharge and distance downstream with only one tributary (the King River) causing a discontinuity in this trend.

4.3 Methods

4.3.1 Site description

Our study focused on the Ovens River of south-eastern Australia. The Ovens River catchment has a total area of 7780 km^2 and drains the Victorian highlands (Hadwen et al. 2010) (Figure S4.1). It is one of the few unregulated rivers in south-east Australia, having a natural flow regime and episodic flooding events (Cottingham *et al.*, 2001; Hadwen *et al.*, 2010). Between 1st January 2012 and 31st December 2014, the mean daily discharge measured at Peechelba, the site lowest in the catchment, ranged from 133.5 ML/d in February 2013 to 40862.3 ML/d at peak flood flow in March 2012.

For both longitudinal surveys, the 203 km length of the Ovens River between Harrietville and Bundalong was divided into four reaches containing 29 sampling sites, delineated by three major tributaries (Buckland River, Buffalo River, King River). The number of sites within each reach ranged from 4 to 11 depending on the location of major tributaries, with an average of 7 km between adjacent sampling sites. The site uppermost in the catchment was immediately below the confluence of the East and West branches of the Ovens River (Harrietville), while the lower site was upstream of the confluence of the

Ovens River with the Murray River (Peechelba). For logistic purpose, two teams carried out the sampling campaign, each team commencing sampling at extremities of the river and meeting close to the middle site. A complete list of sites, their geographical location and distance downstream can be found in Table S4.1. Sample sites are designated as X-Y (X being the reach number from 1 (headwaters) to 4 (downstream) and Y being the specific site number within the reach).

We used a Digital Elevation Model (DEM) of the Ovens River catchment to create a stream and sub-catchment area raster representation of the Ovens River Catchment using Arc Info (ESRI Redlands, CA, USA). This was overlaid with sampling locations and the sub-catchments were grouped to define the upstream area of each sampling location. Land use was classified using the Australia Land Use Mapping (ALUM) classification Version 5 (BRS 2001). The land use classifications were then overlaid on the sub-catchment raster in ArcInfo and used to calculate the percentage of each land use upstream of a sampling location.

Land cover within the Ovens catchment was dominated by forest (Figure 4.1), ranging between 63 and 99 % of total land use across the catchment. This was followed by agricultural activities, which included both medium and low annual input pastures and land irrigated for lucerne and wheat and ranged from ~ 5 % of total land use in reach 1 to 34 % in reach 4. A substantial increase in agricultural land use occurred between reaches 3 and 4, increasing from 17 % to 23 % land cover between sites 3-6 and 4-1, a distance of 7.7 Km, and due to the higher agricultural land use in the King River catchment. Other land cover classes (urban and surface water) were less than 2 % of the total. The upper Ovens River catchment is dominated by diverse native vegetation, while River Red Gum (*Eucalyptus camaldulensis*) forest dominates in the lower reaches (80 % canopy cover),

particularly the floodplain forest bounding major sections of the lowest reach (Cottingham et al. 2001; Hadwen et al. 2010).

4.3.2 Diurnal pilot study

Prior to the longitudinal sampling campaign, a pilot study of diurnal variations in the chemical composition of the Ovens River was conducted, to determine whether time of sampling would likely influence results obtained in the longitudinal study. The diurnal study was conducted at the Porepunkah site, approximately 32 km from the upper most (Harrietville) site. This site was chosen as it was reasoned this would be close to the last site sampled during the campaign. The study of diurnal variation in physical and chemical characteristics of Ovens River water was conducted over a 20 hour period, with samples collected every 4 hours from 4 pm on the 16th of May 2012 to 12 pm on the 17th of May 2012. At the time of sampling the Ovens River was close to base flow, with 60 days since the last significant (>20 mm) rain event (Australia Bureau of Meteorology, Australian Government Webpage http://www.bom.gov.au/climate/data/stations/, accessed 1st October 2012).

No significant trends were observed in the measured parameters over the 20-hour period (Figure S4.2), particularly with respect to day or night. On this basis, we concluded that sampling time during a single day would unlikely influence the results of the longitudinal field surveys.

4.3.3 Longitudinal study field sampling

Longitudinal samples were collected on two occasions; once in winter during a receding flood event (20th August 2012) and once in summer at base flow (2nd February 2014) (Figure 4.2). Samples were filtered (0.45 µM pore size; cellulose-acetate) for analysis of dissolved organic carbon (DOC), total dissolved nitrogen (TDN), inorganic N-containing nutrients (oxides of nitrogen (NO_x; NO_2-N and NO_3-N)) and ammonium (NH_4^+). Filtered

samples were also collected for dissolved combined amino acids (DCAAs). At each site physico-chemical, parameters (temperature, dissolved oxygen (DO) (mg/L), pH, DO saturation (%) and turbidity) were recorded using a QUANTA hydrolab. All sampling was carried out over a 4-5 hour period in the morning, which combined with the pilot study, gave us assurance we were not detecting daily changes in nutrient concentrations.

4.3.4 Chemical analyses

Total dissolved nitrogen (TDN), oxides of nitrogen (NO_x; NO_2-N and NO_3-N) and ammonium (NH_4^+) were measured by flow injection analysis (FIA) (Lachat Quikchem 8000 Flow injection analyser; TDN range: 0.02 – 5.0 mg-N/L; oxides of nitrogen and ammonium ranges: 0.006 – 2.5 mg-N/L). TDN samples were digested using a (NaOH-$K_2S_2O_8$) persulfate oxidation procedure at 120 ºC for one hour (Hosomi & Sudo, 1986). NO_x produced was analysed by FIA as described above. DOC was measured by either wet oxidation using an OI Analytical model 1010 Total Organic Carbon (TOC) Analyser or by combustion using an Analytik Jena 3100 N/C Analyser (DOC range: 0.3 – 250 mg/L). Cross-comparison between these two instruments confirmed practically identical values for the same samples. All procedures were carried out following the quality control and assurance of Australian National Association of Testing Authorities. Procedures included method blanks and quality controls to check against potential sample contamination and calibration drift.

An analytical error in the base flow measurements of DOC in reaches 1-2 gave implausibly high concentrations (~8 mg/L) (Figure S4.3). Previous work has established a strong correlation between the concentrations of DOC derived from *E. camaldulensis*, the major tree on floodplains of south eastern Australia, and absorbance at 245 nm (Baldwin, 1999). For these implausibly high samples the DOC concentrations were subsequently

determined by UV/VIS spectroscopy (described above), using the samples from reaches 3 and 4, where DOC had been obtained by TOC measurement, as a calibration set.

4.3.5 Total dissolved amino acid analysis

Our analysis involved acid hydrolysis of filtered samples, so we simultaneously detect free amino acids as well as condensed amino acids (peptides and proteins), referred to as dissolved combined amino acid (DCAA) throughout the paper. All amino acids in this study are referred to using their standard three letter abbreviations (see Table S4.2). Amino acid digestions and analyses were conducted using new (pyrolysed) sample vials. For DCAA analysis 3-6 mL of filtered sample was freeze-dried, 200 µL of HCl (containing 0.02 % phenol) added and the samples hydrolysed under an argon atmosphere at 110 °C for 24 hours. HCl was removed by freeze drying, the samples reconstituted and tagged with 80 µL (3:1 borate buffer: Milli-Q water mixture) and 20 µL of 6-aminoquinolyl-N-hydroxysuccinimyidyl carbamate (AQC) reagent (heated at 55 °C for 10 minutes). Tagged samples were diluted with 100 µL of 0.1 % formic acid and centrifuged to remove any solid material (7323g; 5 min). Approximately 150 µL of supernatant was transferred into a limited volume insert (LVI) and placed into an auto sampler vial. Some amino acids are not recoverable following acid hydrolysis of proteins; tryptophan is destroyed and cystine is typically poorly recovered from the hydrolysed samples (Fountoulakis & Lahm, 1998). During acid hydrolysis, asparagine and glutamine are de-aminated to aspartic acid and glutamic acid, respectively, making it impossible to determine the contributions of each of these amino acids individually; these pairs of amino acids are referred to as ASX and GLX (Anders *et al.*, 2003).

Tagged amino acid samples were analysed by liquid chromatography – mass spectrometry (LC-MS/MS). The LC-MS/MS system consisted of a Shimadzu Nexera X2 UPLC coupled to a Shimadzu 8030 triple quadrupole mass spectrometer, operated in

positive ion electrospray ionisation (ESI) mode (Shimadzu corporation Kyoto, Japan). Individual tagged amino acids were detected in multiple reaction monitoring (MRM) mode, with collision parameters optimized individually. Source conditions were: sheath gas temperature 275 °C, nebulizer flow 3 mL min^{-1}, nebulizer pressure 45 psi and capillary voltage 2.5 kV. Separation was achieved using a gradient eluent with a Waters Aquity UPLC BEH C18 column (2.1 x 150 mm; pore size 1.7 µM) maintained at 50 °C. The mobile phase consisted of 0.1 % formic acid (eluent A) (Sigma-Aldrich, St Louis, USA) and 100 % OPTIMA LCMS grade acetonitrile (eluent B) (Fischer Scientific, Pittsburgh, USA) with flow of 0.55 mL/min.

Calibration standards were made using amino acid standard H (Waters Corporation) spiked with glutamine (GLN), asparagine (ASN) and tryptophan (TRP) allowing measurement of all standard proteinogenic amino acids over the range 0.01 – 2 pmol/µL (equivalent to 0.01 – 2 µMol/L). A quality control sample of selected amino acids was prepared at 1 pmol µL containing HIS, ARG, GLN, LYS and ILE. Blanks were included in each sample run, including: (i) a reagent blank consisting of 80 µL borate buffer and 20 µL AQC, (ii) a sample blank comprising 40 µL of Milli-Q water, hydrolysed and derivatized as detailed above and (iii) a 0.1 % formic acid blank. A sample of insulin (bovine pancreas; Sigma Aldrich, St Louis, USA) was also included in every hydrolysis set to check for amino acid recovery (recoveries of individual amino acids ranged from 23 to 96 %; see Table S4.3).

4.3.6 Data analyses

4.3.6.1 Water nutrients

Additive Gaussian models (Generalised additive models, GAMs, with a Gaussian link function) were used to model the impacts of discharge, distance downstream and tributary confluences on concentrations of DON, DOC, NO_x and NH_4. Additive models were

deemed appropriate given we desired models that accommodated (a) non-monotonic responses of the four nutrients with distance, and (b) curvilinear responses that were not neatly defined by a specific nonlinear equation. Discharge, *Q*, was a nominal variable having values low (*L*; summer samples; Figure 4.2) and high (*H*; winter samples; Figure 4.2). Distance downstream, *d*, was a numeric variable (km), while tributary confluences were described by two nominal variables, depending on the hypothesis being tested: T_{all}, a variable containing four values denoting reaches between the three major confluences (Buckland, Buffalo and King), and T_{King}, a variable denoting only two reaches delineated by the confluence of the King alone. Three additive models were fitted to the data for the purpose of testing which of the three hypotheses presented in the Introduction provided the most parsimonious description of the data, these being:

$$R_i = \alpha + f_1(d_i) \cdot ID_{L,i} + f_2(d_i) \cdot ID_{H,i} + \epsilon_i \quad (1)$$

$$R_i = \alpha + f_1(d_i) \cdot ID_{L,i} + f_2(d_i) \cdot ID_{H,i} + T_{all} + \epsilon_i \quad (2a)$$

$$R_i = \alpha + [f_1(d_i) \cdot ID_{L,i} + f_2(d_i) \cdot ID_{H,i}] \cdot T_{all} + \epsilon_i \quad (2b)$$

$$R_i = \alpha + f_1(d_i) \cdot ID_{L,i} + f_2(d_i) \cdot ID_{H,i} + T_{King} + \epsilon_i \quad (3a)$$

$$R_i = \alpha + [f_1(d_i) \cdot ID_{L,i} + f_2(d_i) \cdot ID_{H,i}] \cdot T_{King} + \epsilon_i \quad (3b)$$

where *R* indicates one of the four water nutrient response variables; α is the intercept; f_1 and f_2 denote *Q*-specific smoothing functions and the *ID* vectors are numeric logical vectors indicating which of the R_i's correspond to each *Q* value; $i \in 1,\ldots,55$ (27 samples in winter and 28 in summer). Errors, ϵ_i, are assumed to be normally distributed. All three models are varying coefficient models (Wood, 2017) in that they allow for a unique smoothing function of *d* for each of the two discharge conditions. Generalised cross-validation was used to determine the optimal degree of smoothing. The additional *T* term in Models 2 and 3 allows for tributary confluences to generate discrete discontinuities (vertical shifts) in the *Q*-specific relationship between distance and water nutrient

concentration. There were insufficient data points to fit models accommodating unique reach-specific smoothers (vertical shifts as well as reach-specific curvilinearity). For Models 2 and 3, any vertical shifts in the smoother associated with tributary confluences are constant across *Q* values in a, while Models b allow for *Q*-specific magnitudes of any such shift (Wood, 2017).

Attempts to fit mixed additive models including terms allowing for spatial autocorrelation among samples did not converge. It follows we assumed no substantial spatial autocorrelation among samples; we briefly discuss the implications of this assumption in the results.

Akaike Information Criteria (AIC) were used for model selection (Burnham & Anderson, 1998) and in instances where two models had very similar AIC values we opted for the simpler model (model with fewer parameters). Additive modelling was carried out using the mgcv package in R (Wood, 2017).

4.3.6.2 Amino acids

Amino acid concentrations at each site and time were converted to proportions and we derived a similarity matrix based on Euclidean distances. Principle Co-ordinate Analysis (PCoA) and a Permutational Analysis of Variance (PERMANOVA), based on the similarity matrices, were used to explore the relationships between flow, reach and amino acid composition of the DCAAs. We used a bootstrapping approach to place region estimates around means of the differences in the multivariate variation of amino acid composition. The multivariate statistical routines were implemented within PRIMER7 (PRIMER-E Ltd, Plymouth UK).

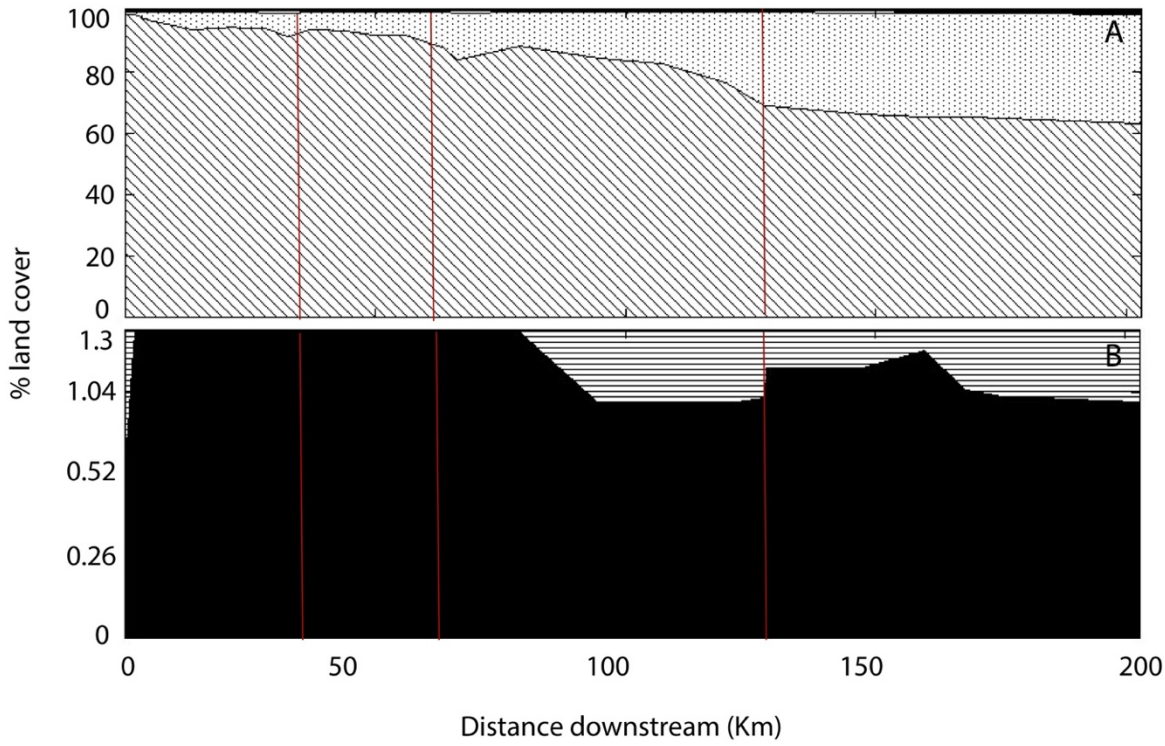

Figure. 4.1 Spatial series plots showing the proportion of land cover along the Ovens catchment. (a) shows the proportion of land use for four categories; trees (sloped lines), agriculture (dots), urban (horizontal lines) and water (black). (b) (note scale) shows the contribution by urban and water and are extracted from upper panel as these components make up a small contribution to overall land cover. Vertical lines divide the longitudinal profile of the Ovens River into the four reaches used in this study. Proportion of land use was calculated during high flow as a season average.

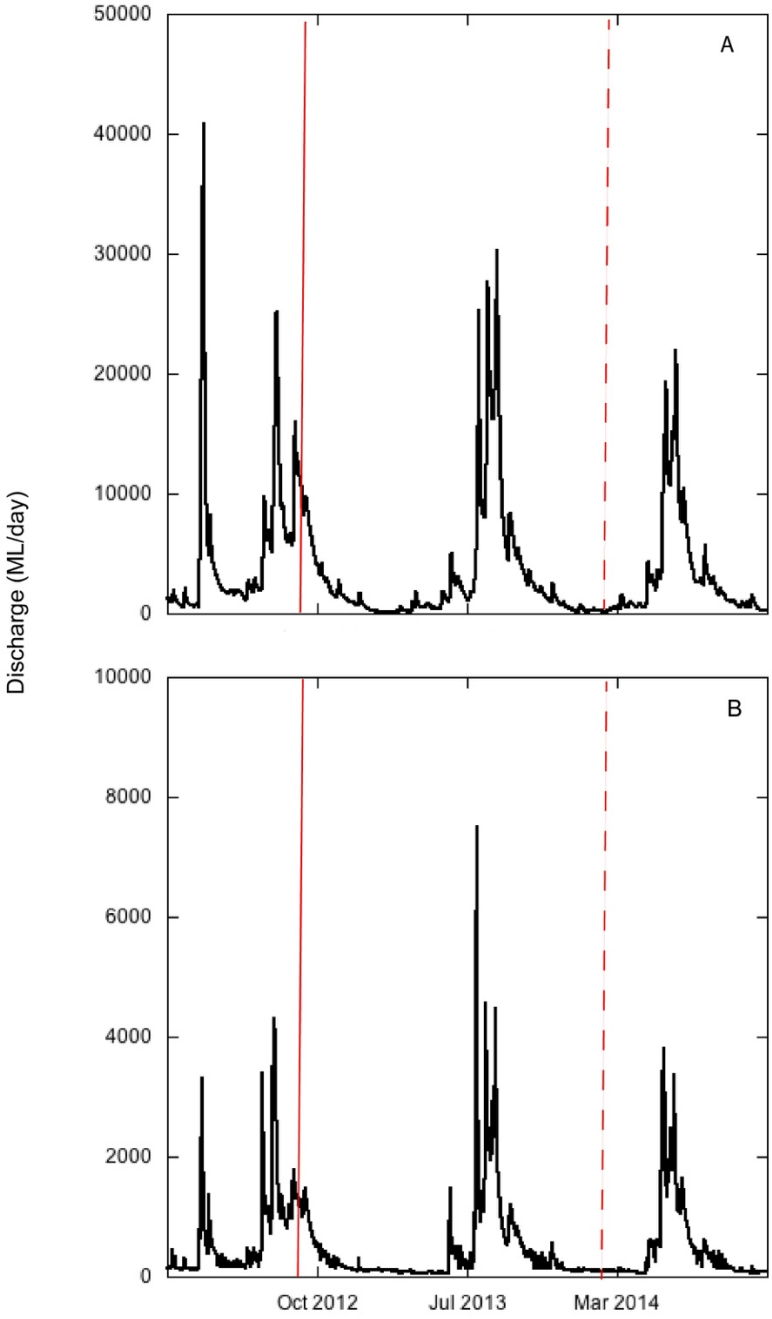

Figure. 4.2 Hydrographs of the Ovens River at Peechelba and the King River at Cheshunt over the period from 1 January 2012 to 31 December 2014. The vertical lines show when sampling for the longitudinal studies occurred: high flow on 20th of August 2012 (solid line) and base flow on 2nd of February 2014 (dashed).

4.4 Results

4.4.1 Physical data

Temperature increased predominantly throughout the first reach for both surveys rising from 6.8 °C at the uppermost site to 9.5 °C at site 2-2 under high flow, and from 15.6 °C to 21.2 °C at base flow (Figure 4.3). A small step in turbidity from 16 to 24 NTU was observed at the confluence with Buffalo River (sites 1-11 and 2-1) and a large step from 39 to 60 NTU at the confluence with the King River (reach 4). Turbidity during the base flow sampling ranged between 0 and 48 NTU with the King River having much less influence than during high flows. The pH ranged from 7.1 to 7.9 at high flow and 6.8 and 8.3 at base flow. DO ranged from 9.82 mg/L to 11.36 mg/L at high flow and between 5.70 mg/L and 9.50mg/L at base flow.

4.4.2 Dissolved nutrients

DOC, DON, NO_x and NH_4^+ concentrations for high flow and base flow studies are shown in Figure 4.4 (a-d), along with the optimal GAMs to describe the observed behavior (discussed below). For all four nutrients Model 3b provided the most parsimonious description of the data (Table 4.1), and so it follows that, given these data, hypothesis 3, '*concentrations increase with discharge and distance downstream with only the King River causing a discontinuity in this trend*', is the most likely of the three presented in the chapter introduction.

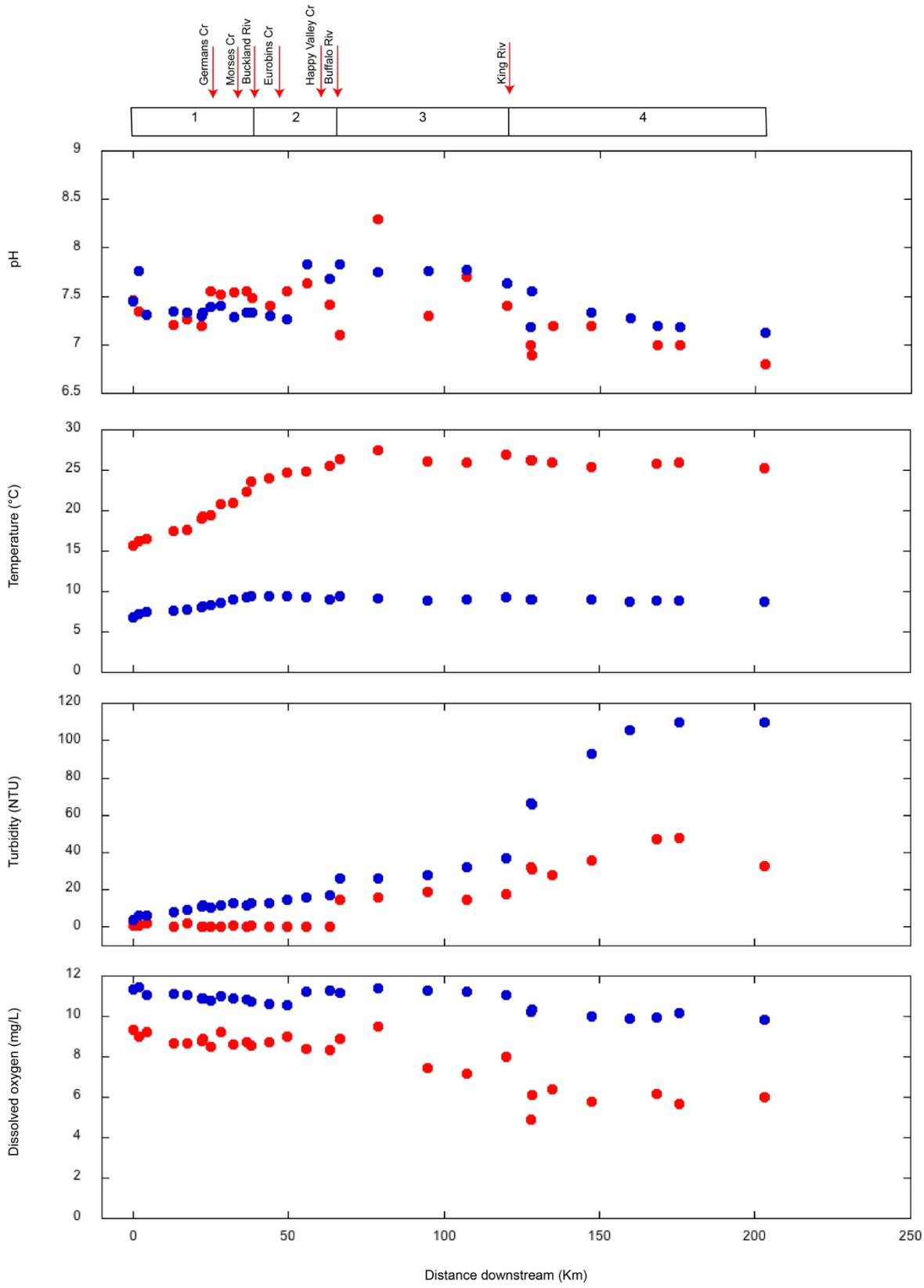

Figure. 4.3 Spatial series plots for pH, temperature (°C), turbidity (NTU) and dissolved oxygen (mg/L) for both high flow (blue) and base flow (red). Study reaches are shown on the top panel, and red arrows indicate major tributaries.

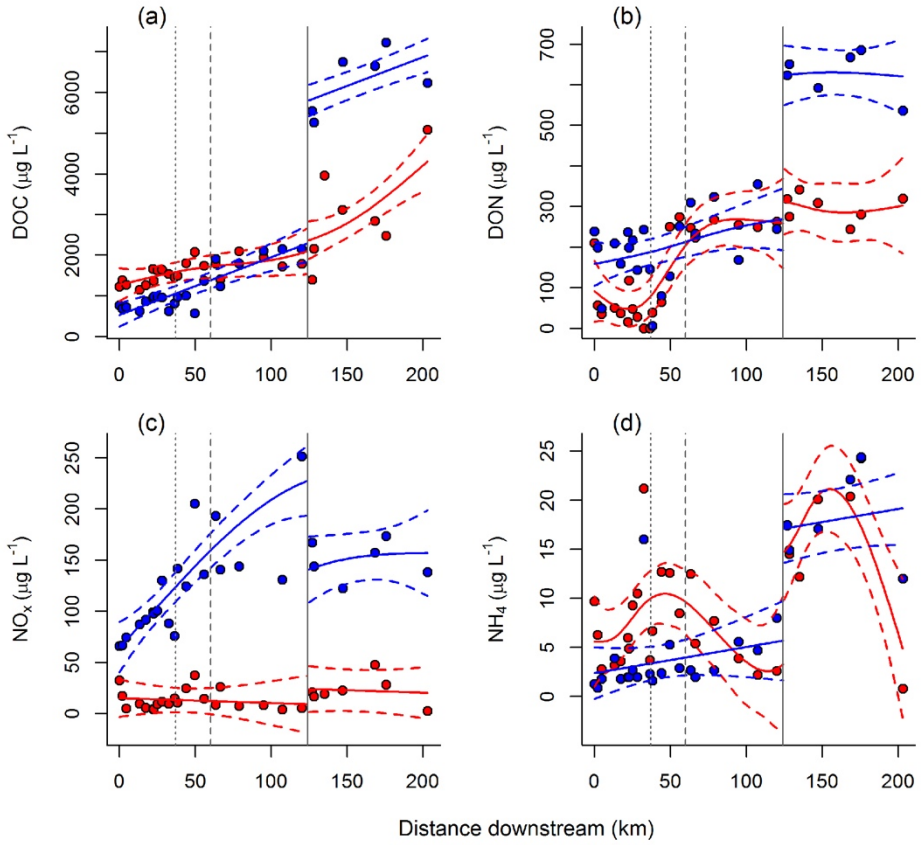

Figure. 4.4 Concentrations of dissolved organic carbon (DOC) (a), dissolved organic nitrogen (DON) (b), oxides of nitrogen (NO_x) (c) and ammonium (NH_4^+) (d) as a function of distance downstream and flow (high flow: blue; base flow: red). Predicted values (solid lines; fitted values of GAMs) ± 95 % confidence intervals (dashed lines) are presented. Vertical lines denote confluence of the Ovens River with major tributaries: Buckland River (dotted); Buffalo River (dashed); King River (solid).

Table. 4.1 Akaike Information Criteria (AIC) of the five models fitted to water nutrient data. The lower the value the more parsimonious the model.

	M1	**M2a**	**M2b**	**M3a**	**M3b**
DOC	856	835	839	844	834
DON	652	638	627	638	633
NO_x	535	539	534	536	530
NH_4^+	323	319	324	317	319

With respect to DOC the deviance explained by Model 3b was 94 %. DOC significantly and strongly increased continuously with distance downstream under both low *Q* (*F* =

5.95; $P < 0.001$; Figure 4.4a) and high Q ($F = 30.04$; $P < 0.001$; Figure 4.4a). The magnitude of the discrete increase in DOC concentration associated with the confluence with the King River was dependent on season as indicated by significant interaction parametric term (interaction $Q{:}T_{King}$: $F = 34.279$; $P < 0.001$; non-interaction terms not significant). The King River confluence was associated with a strong increase in DOC under high discharge conditions, but not under low discharge (Figure 4.4a).

The variation in DON explained by Model 3b was 87 %, with DON exhibiting a significant continuous increase in concentration downstream under low Q ($F = 6.73$; $P < 0.001$; Figure 4.4b), but not under high Q ($F = 1.90$; $P = 0.116$; Figure 4.4b). Under high discharge conditions the significant longitudinal change in the concentration of DON was a discrete increase that was associated with the confluence with the King River (Figure 4.4b). The discrete increase in DON concentration associated with the King River was only significant under high Q (Figure 4.4b; interaction $Q{:}T_{King}$: $F = 8.86$; $P = 0.005$; non-interaction terms not significant).

Model 3 also explained a large proportion of the variance in the NO_x data (88 %). NO_x exhibited a significant and strong continuous increase in concentration with distance downstream under high Q ($F = 19.72$; $P < 0.001$; Figure 4.4c), but not under low Q ($F = 0.08$; $P = 0.769$; Figure 4.4c). Again there was a strong and significant interaction between T_{King} and Q, whereby the confluence with the King River is associated with a strong decrease in NO_x, but only under high Q (Figure 4.4c; interaction $Q{:}T_{King}$: $F = 8.60$; $P = 0.005$). Unlike DOC and DON, the model indicated a significant overall discharge effect on NO_x concentrations, with NO_x being significantly higher under high Q, irrespective of distance downstream and tributaries (Figure 4.4c; Q: $F = 74.02$; $P < 0.001$).

Model 3 explained 73 % of the variance in NH_4^+. The smoothing terms indicated significant continuous changes in NH_4^+ under low Q ($F = 3.51$; $P = 0.006$; Figure 4.4d), but there were no significant continuous changes under high Q ($F = 1.29$; $P = 0.263$; Figure 4.4d). Although continuous, the smooth changes measured in NH_4^+ under high Q were not significant over the first three reaches, but there was a significant discrete increase in NH_4^+ associated with the King confluence under both high and low Q (main term T_{King}: Figure 4.4d; $F = 5.32$; $P = 0.026$). Unlike the other three nutrient variables modelled, the interaction $Q{:}T_{King}$ was not significant, indicating that the magnitude of increase in NH_4^+ associated with the King confluence was similar under both high and low Q (Figure 4.4d).

As stated in the methods we assumed no spatial autocorrelation of samples due to the difficulty we encountered fitting mixed-effect GAMs (no convergence). Some spatial autocorrelation may be present in the data (note subtle clustering of contiguous samples either above or below fits; Figure 4.4). Given the strength of the effects we reported above it is most unlikely that inclusion of random autocorrelation terms would affect any of our inferences, and so we do not discuss this further in the discussion. Future studies on landscape-scale trends in water nutrient concentrations within rivers should aim for larger sample sizes such that more complex mixed-effects models could be fitted to data that may contain spatially correlated errors.

4.4.3 Dissolved combined amino acids

At base flow, the concentration of DCAAs was nearly constant along the river (range 10 – 40 µg-N/L) (Figure 4.5). However, this was not the case at high flows. In the upper catchment, DCAA concentrations were lower than at base flow, while in the lower catchment DCAA concentrations were higher than at base flow (10 – 70 µg-N/L). A significant step in concentration was detected during high flows between reaches 3 and 4.

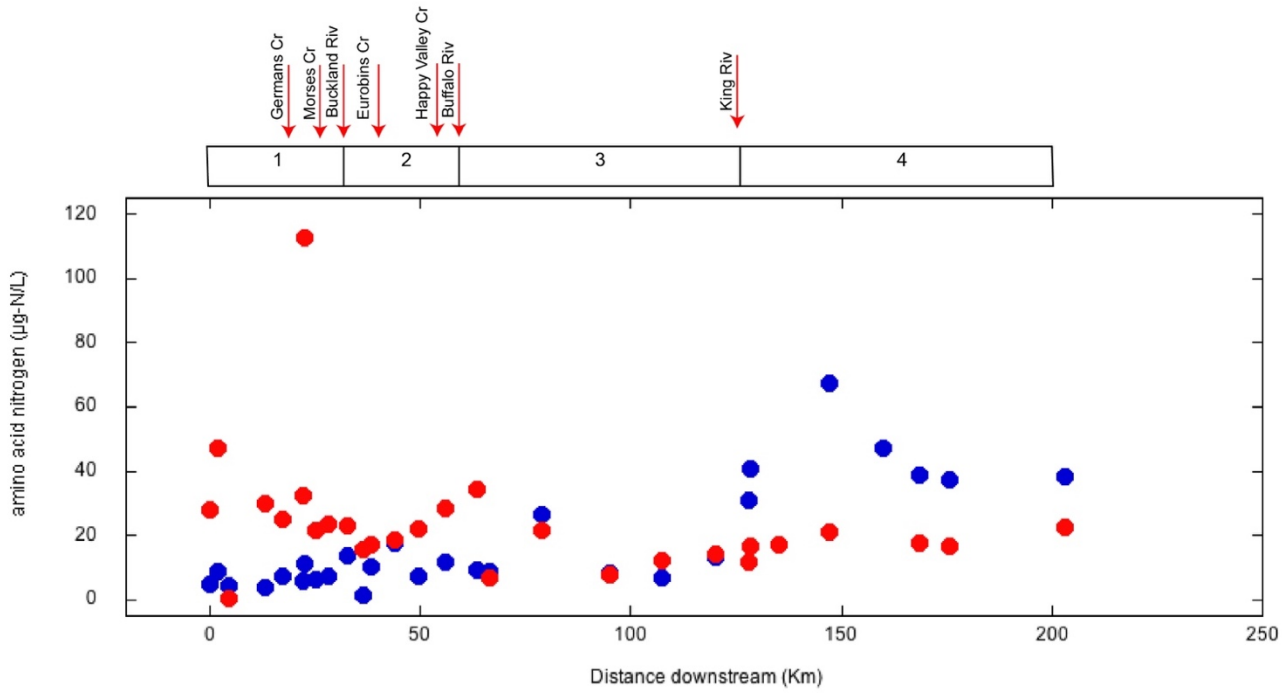

Figure. 4.5 Spatial series plot showing the concentrations of nitrogen in the form of dissolved combined amino acids (DCAA-N) for both high flow (blue) and base flow (red). Study reaches are indicated on the top panel and red arrows indicate major tributary confluences.

The amino acids making the greatest contribution to the overall DCAAs, across the four reaches during the high flow study were GLY (19.3 %), SER (13.7 %), ASX (12.7 %), ALA (11.6 %) and GLX (10.2 %). While the same amino acids dominated at base flows, their proportions were different: GLY (17.3 %), SER (14.6 %), GLX (11.4 %), ALA (9.7 %) and ASX (8. 4 %). Individual longitudinal profiles for most amino acids follow a similar pattern to amino acid nitrogen (aa-N) showing a peak in reach 4.

The composition of the DCAAs were significantly different between the two flow events and in different reaches (PERMANOVA, $p = 0.0019$ and 0.0001 respectively) with the interaction (reaches × flow) showing a strong trend ($p = 0.0572$, Figure 4.6). Pairwise comparison of individual reaches between the two events showed only reaches 1 and 2 to have different compositions ($p = 0.0001$, and 0.0306 respectively Figure 4.6). Notably, the composition at reach 4 was not different between flow events ($p = 0.467$, Figure 4.6).

The contribution of amino acids to overall DON was highly skewed during the base flow (Figure 4.7). While the median proportions during base and high flow were similar (7 and 5 % respectively), the mean percentages were 23 % and 5 % respectively, with the 25th and 75th percentiles 5 – 44 % during base flow and only 3 – 6 % at high flow.

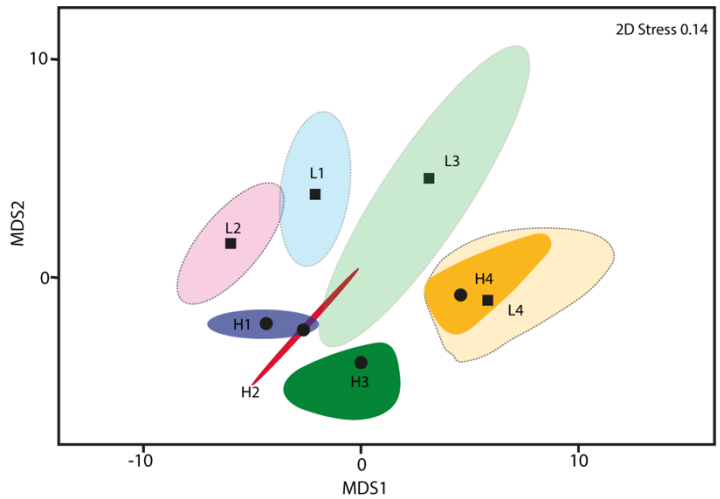

Figure. 4.6 Metric multi-dimensional scaling plot of bootstrap regions (95 %) and averages for DCAA composition during high flow and base flow. L1, L2, L3, L4 show the 95 % confidence intervals for low flow at reaches 1– 4 respectively and black squares the respective bootstrap average. H1, H2, H3 and H4 show the 95 % confidence intervals for high flow at reaches 1– 4 respectively and black circles the respective bootstrap average.

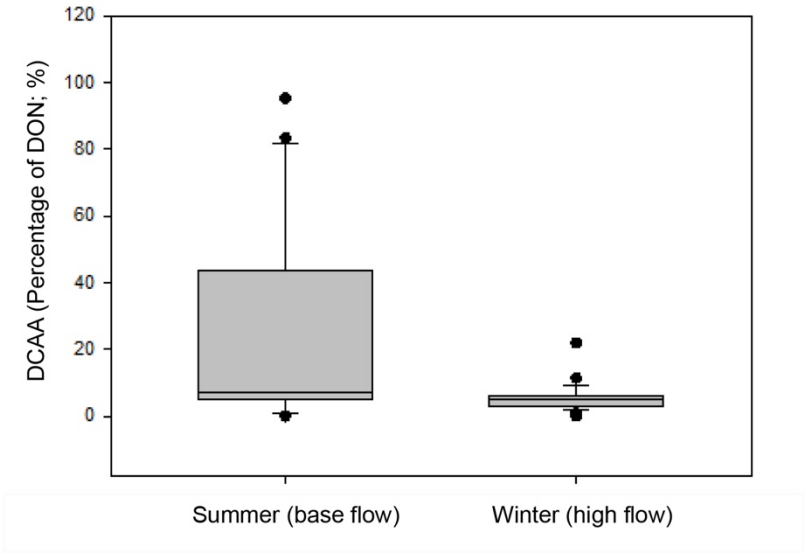

Figure. 4.7 Box and whisker plot showing the proportion of the DON that is in the form of DCAA at high flow and base flow. The box shows the 25th and 75th percentiles, the horizontal line shows the median, bars are the 5th and 95th percentiles and dots are outliers.

4.5 Discussion

Previous studies on the longitudinal relationships of nutrient composition in rivers have often focused on river regulation and adjacent land use and shown that adjacent and catchment wide land practices can influence the type and quantity of nutrients reaching the river channel. (Hadwen *et al.*, 2010; Lu *et al.*, 2014; Mattsson *et al.*, 2015; Petrone, 2010). While some studies have examined DON concentration and bioavailability (Bernal, Butturini, & Sabater, 2005; Kaushal & Lewis, 2005), only limited effort has examined compositional aspects of DON and in particular, DCAAs (Stepanauskas, Laudon, & Jørgensen, 2000). In our work, we examined longitudinal trends in nutrients and DON at two seasons, involving two different flow regimes, we characterized the portion of the DON that are present as DCAAs and examined changes in longitudinal concentration and composition with relation to land use and major tributaries. In doing this, we provide a strong context for studying DCAAs as an organic-N source in rivers and enhance our understanding of both DON composition and its relationship to flow, season and land use.

4.5.1 Nutrient concentration trends

Nutrient concentrations increasing downstream during base and high flow is a feature of unregulated lowland river systems (Brookshire *et al.*, 2005). While we found some similarities with previous work, there were some obvious differences: (i) high flows greatly enhanced the role of a major tributary (King River) in driving the DOC and DON concentrations and (ii), the longitudinal patterns of NO_x concentration were remarkably different during the two flow events, leading to quite different responses in the inorganic nitrogen species.

Two possible explanations can account for the response of NO_x to discharge. The elevated NO_x could be due in part to the occurrence of a wildfire in January 2013 that

burnt 15,353 ha of Alpine Ash (*Eucalyptus delegatensis*) forest Harrietville (reach 1) (Bassett *et al.*, 2015). 88 % of this fire occurred within the Alpine national park which is the major source of runoff to the Ovens River at Harrietville (Bassett *et al.*, 2015). Our sampling excursion occurred after the peak flow and it is possible that the strong continuous increase in concentration represents a NO_x pulse travelling down the river. However, apart from the King River, the tributaries did not lead to any discontinuities, as might be expected if fire alone was the contributing factor to elevated NO_x. Furthermore, NH_4^+ levels in soil have also been shown to increase post-fire due to the presence of residual ash; for example two fold increases in NH_4^+ have been observed within 12 months of a fire event (Knoepp & Swank, 1993; Kutiel & Naveh, 1987). No equivalent increase in NH_4^+ occurred with NO_x, however, NH_4^+ concentrations can vary depending on the time and intensity of the fire (Knoepp & Swank, 1993; Kutiel & Naveh, 1987), thus the NH_4^+ effect may not be as long-lived as NO_x. While urban and agricultural activities are not extensive in the upper reaches, the increasing NO_x during high flow is consistent with observations in a series of streams in the USA, where NO_x increased with human land use, Stanley and Maxted (2008) also attributed to overbank connection and runoff from those regions of the catchment (Divers, Elliott, & Bain, 2014; Wagner *et al.*, 2008).

The different responses of DON to the two flow events presents some insight to possible sources of DON. DON in streams can be derived from autochthonous production or allocthonous inputs, its quantity as well as its bioavailability can vary substantially depending on the source (Martin & Harrison, 2011); Seitzinger, Sanders, and Styles (2002) also showed bioavailability of DON to differ in relation to land use, with storm water runoff having the highest proportion of available DON forms (59 ± 11 %), and agricultural pastures and forests having less (30 % ± 14 % and 23 % ± 19 %,

respectively). At low discharge, a significant and continuous increase in DON concentration was measured over the length of the river and is most likely to have been autochthonous. Under high flow, there was a major discontinuity in DON concentration between reaches 3 and 4, coinciding with the King River confluence. However, it is important to recognize that the regional city of Wangaratta is located immediately downstream of this confluence, somewhat confounding the contribution by the King River. Our first sampling site was 300 m downstream of the confluence and although we cannot identify any major point source within those 300 m, it is possible that there is some effect of urbanization through diffuse sources. Be that as it may, there is documented evidence of the effects of tributaries on nutrient composition (Kiffney *et al.*, 2006; Liu *et al.*, 2003), supporting our third hypothesis, that concentrations increase with discharge and distance downstream with only the King River causing a discontinuity in this trend. Although the King River contributed approximately 49 % of the daily flow in the Ovens River on the day of sampling, discharge from the tributary alone did not account for the major step in nutrients during the high flow study, as the Buffalo River contributed a similar proportion to the daily flow at its confluence (http://data.water.vic.gov.au/monitoring.htm).

4.5.2 Dissolved combined amino acids as a component of in stream nitrogen

Here we have directly measured the dissolved peptides and protein (DCAA), allowing us to resolve at least some of the nitrogen species that constitute the DON fraction. While we have not carried out specific bioavailability experiments in our work, peptides and proteins are presumed to be bioavailable, following breakdown by extracellular proteases (Findlay & Sinsabaugh, 2003). DCAAs were equal to, or in excess of NH_4^+ concentrations, irrespective of flow. DCAAs were of similar concentrations to NO_x at base flow, and only in high flow, did the NO_x exceed nitrogen contained in amino

acids. Taking these data, we suggest that at base flow conditions, the DCAA should be considered as an important contributor to the overall nitrogen budget. Only at higher flows would inorganic nitrogen (NO_x) become the dominant form of instream nitrogen,

With respect to organic nitrogen, dissolved combined amino acid nitrogen (DCAA-N) comprised a mean of 5 % of total DON in the high flow study and 23 % during the base flow study. The upstream sites contributed to the large variation during base flow and in particular two outlying data points. While theoretically feasible, given the unusually high value of the outliers, we suggest such variation may be due to the approach used to calculate DON. DON is calculated by difference and as concentrations approach levels of detection, small errors can manifest as large proportions of the total material present. Lusk and Toor (2016) found 5.1 – 7.1 % of DON in two Florida streams to be biologically available, chemical analyses showing the bioavailable materials to be protein or lipid-like molecules. Stepanauskas, Laudon, and Jørgensen (2000) reported DCAAs comprised between 5 and 18 % of the total DON in two Swedish streams, values similar to our study. However, in contrast to our findings, the proportion of DCAA in DON increased during a flood event. We showed the proportional contribution of DCAAs to DON to be greater at base flow in summer and if a similar degree of bioavailability exists would also suggest DCAA to be the dominant N-source under these conditions.

4.5.3 Land use and dissolved combined amino acid composition.

We have shown that DCAA composition changes along a longitudinal gradient, coinciding with changing land use, and reflecting different proteins and peptides present in the river. The DCAA composition within each reach differed strongly between the two sampling campaigns, except for the lowest reach where we measured no significant difference in composition. This result is somewhat surprising given the observation of a major discontinuity between reaches 3 and 4 at high flow and suggests that Ovens River

water composition in reach 4 is either controlled by processes and inputs within reach 4, or by the King River.

As noted previously, in addition to the King River confluence, reach 4 also has inputs from Wangaratta, and the DCAA composition is likely influenced by storm water and treated wastewater inputs. Further, floodplain forests occur extensively through the lowest reach of the Ovens River. In addition to overbank flooding, there is extensive floodplain-channel connection in the lower region of the Ovens River through a network of ephemeral channels, where mobilization of organic material occurs during flooding (Gessner, 1991; Junk, Bayley, & Sparks, 1989). The lower Ovens floodplain is dominated by *Eucalyptus camaldulensis* which are known to leach between 20 – 40 % of their total leaf mass within a few days of submersion, thus a large proportion of dissolved organic material in the Ovens Rivers originates from plant material deposited directly into the river or indirectly through floodplain interactions (Baldwin, 1999; Baldwin *et al.*, 2013). Furthermore, DCAAs comprise up to a third of the DON released during 24 hours immersion of *E. camaldulensis* (Harris *et al.*, 2016). The similarity in the DCAA composition in reach 4 across the two events suggests one or multiple sources having a strong influence on composition, either directly supplying protein and peptide, or stimulating secondary metabolism by microbes, which drive dissolved protein composition.

4.5.4 Dissolved organic nitrogen as a nitrogen source in freshwater ecosystems

The common perception is that nitrogen loss from catchments is in the form of NO_3^- and when nutrient budgets for nitrogen losses in agricultural systems are constructed, DON is typically not part of this accounting (Ghani *et al.*, 2007). Although detected as a decrease in its proportion of total dissolved nitrogen, due to major increase in nitrate, an extensive study of European systems showed that DON increases in concentration along a

gradient from oligotrophic to eutrophic water (Durand *et al.*, 2011). Concentrations upward of 9 mg-N/L of DON have been reported (Johnes & Burt, 1991). A growing body of evidence is demonstrating that DON is leached from agricultural land and can account for large nitrogen losses, particularly in agricultural plots that receive farm yard manure (Curtin *et al.*, 2014; Murphy *et al.*, 2000; Petrone, 2010). DON leaching losses from agricultural land can be highly variable, ranging from 0.3 kg N $ha^{-1}yr^{-1}$ in a grassy clover plot (Saarijärvi *et al.*, 2004) to a maximum of 127 kg N $ha^{-1}yr^{-1}$ in a pasture after urine and manure was applied as a fertiliser (Wachendorf, Taube, & Wachendorf, 2005). Nitrogen export from the landscape is likely further controlled by soil types due to adsorption processes that control mobility; understanding the importance of these processes to nitrogen migration and export will require detailed examination and understanding of DON (and DCAA) adsorption onto soil mineral surfaces.

Here we have shown that while a portion of DCAAs may be a source of bioavailable nitrogen it is unlikely that DCAAs are as important as DIN in satisfying nitrogen demand. DCAAs contribute less than 25% of the nitrogen sourced from DIN and are readily adsorbed to inorganic particles in stream which can lead to DCAA-N becoming dormant and unavailable. However, the expansion of DON speciation to include the amino acid composition provides considerably greater power in discriminating between different land uses and will likely be a valuable tool in managing the nutrient levels in surface waters interacting with different land use types. It would therefore be advisable that both DON and more specifically DCAAs are considered when constructing nitrogen budgets, monitoring aquatic nitrogen levels or studying nutrient dynamics in river systems where land use is an important consideration. While we can account for part of the DON pool, there remains a large fraction that does not report to the

DCAAs measured in this work. The further identification of these species will likely assist in our understanding of nitrogen availability in freshwater systems.

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4.8 Supporting information

Longitudinal trends in concentration and composition of dissolved organic nitrogen (DON) in a largely unregulated river system.

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Summary of the supporting information

- Number of pages: 14 (including this page)
- Number of figures: 5
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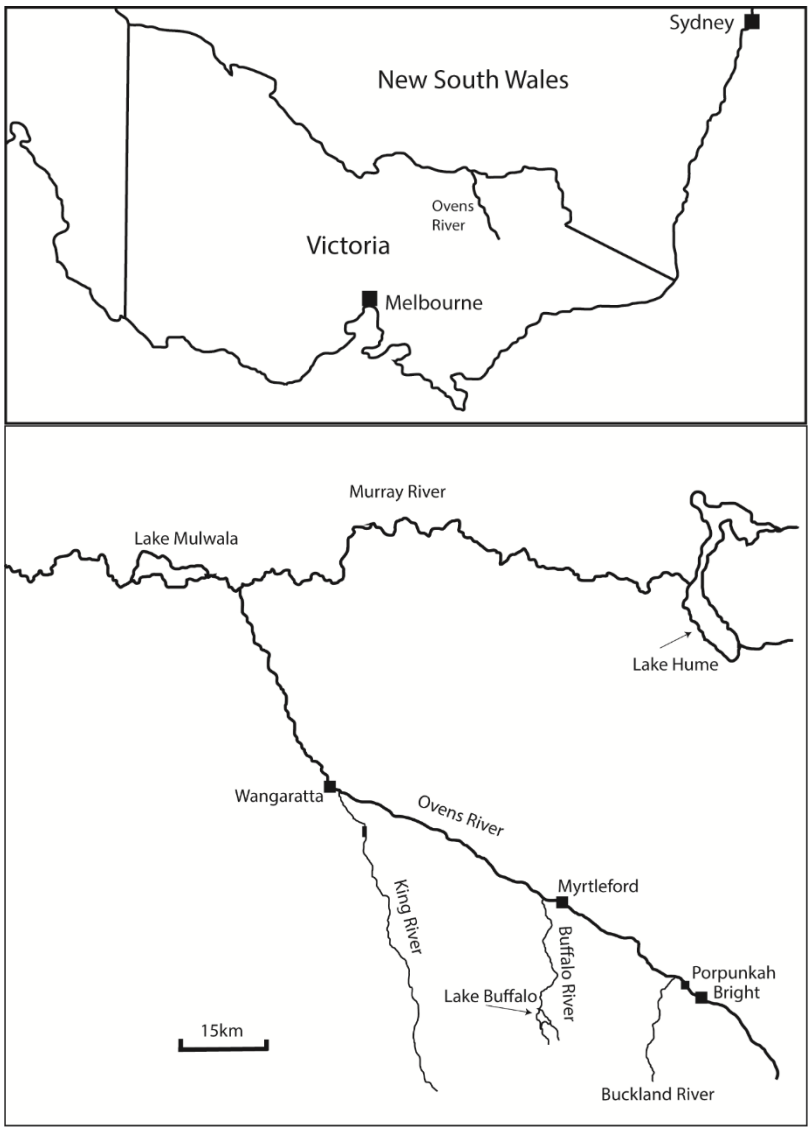


Figure. S4.1 Maps showing the location of the Ovens River in north-east Victoria (top panel), and details of urban settlements and major tributaries (lower panel).

Table. S4.1 Site list detailing the four study reaches, sample sites, their designated common name, and geographical location.

Reach	Site number	Site Name	Latitude	Longitude
	1	Log Park	S 36˚53’23.2”	E 147˚03’53.7”
	2	Howard’s bridge	S 36˚52’21.8”	E 147˚03’47.8”
	3	Trout farm	S 36˚51’20.6”	E 147˚04’38.8”
	4	Freeburgh	S 36˚47’26.1”	E 147˚02’28.6”
	5	Freeburgh bridge	S 36˚45’25.4”	E 147˚02’25.2”
	6	Pine plantation A	S 36˚43’38.8”	E 146˚ 59’51.0”
	7	Pine plantation B	S 36˚43’33.3”	E 146˚ 59’32.5”
	8	Bright	S 36˚43’33.1”	E 146˚59’32.6”
	9	Canyon walk	S 36˚43’05.4”	E 146˚56’40.1”
	10	Porepunkah	S 36˚41’57.7”	E 146˚54’34.4”
1	11	Big 4 caravan park	S 36˚40’55.1”	E 146˚52’50.4”
	1	Cavedons lane	S 36˚40’32.8”	E 146˚52’13.0”
	2	Deer farm	S 36˚38’17.5”	E 146˚51’0.55”
	3	Fernydale	S 36˚37’00.5”	E 146˚48’16.4”
2	4	Seltzers Ln	S 36˚35’49.1”	E 146˚45’34.7”
	1	Mid Myrtleford	S 36˚34’08.3”	E 146˚43’04.2”
	2	Lower Myrtleford	S 36˚33’07.4”	E 146˚42’02.3"
	3	Bowman's forest	S 36˚29'58.8"	E 146˚36'10.7"
	4	Markwood	S 36˚26'28.7"	E 146˚31'34.3"
	5	Tarwingee River Rd	S 36˚24'39.9"	E 146˚27'13.9"
3	6	Freeway	S 36˚22'39.0"	E 146˚21'50.1"
	1	Ulah Park	S 36˚21'04.9"	E 146˚19'43.1"
	2	Wangaratta	S 36˚20'47.8"	E 146˚19'22.7"
	3	Wangaratta 3	S 36˚20'57.1"	E 146˚19'10.0"
	4	Warby range Rd	S 36˚16'56.1"	E 146˚16'01.8"
	5	Frost Rd	S 36˚11'21.9"	E 146˚14'07.6"
	6	Peechelba	S 36˚09'35.9"	E 146˚13'58.2"
4	7	Bundalong	S 36˚04'05.6"	E 146˚11'53.7"

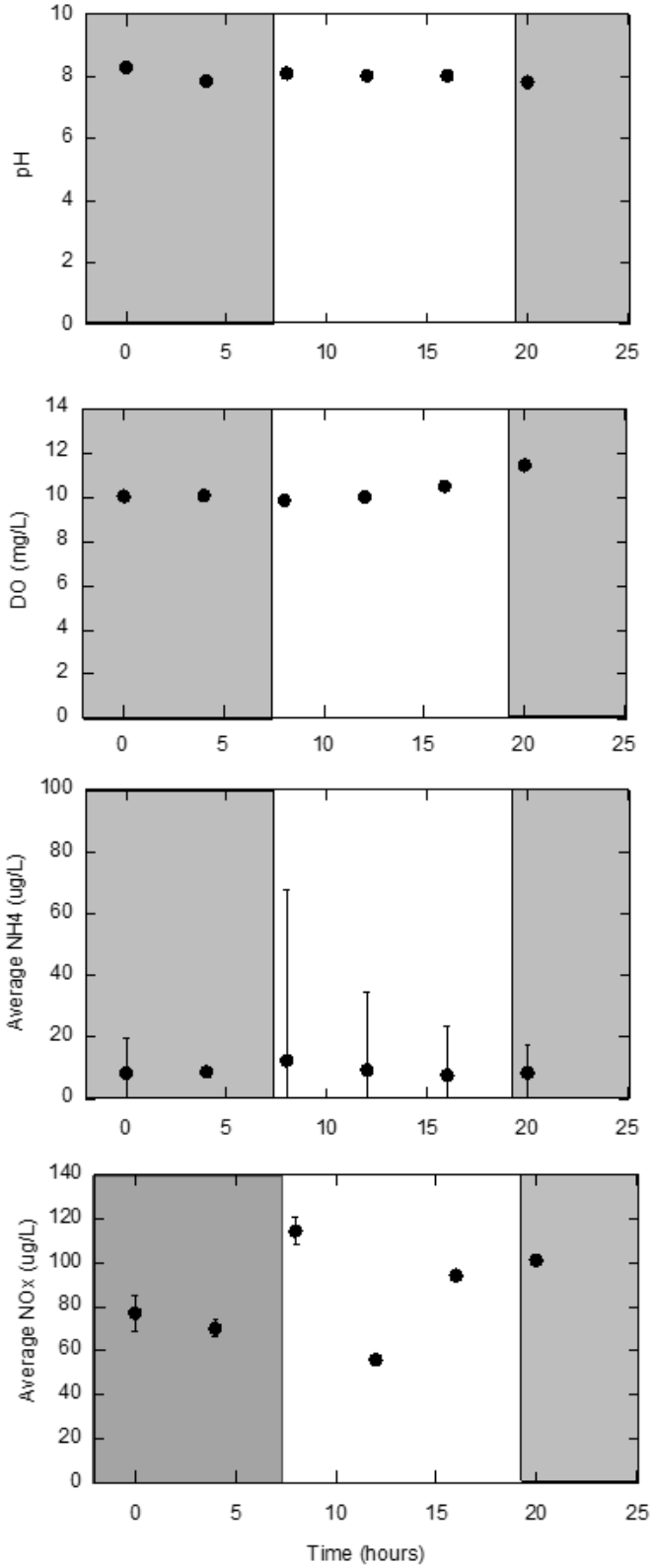


Figure. S4.2 Diurnal time course measurements showing average pH, dissolved oxygen content (DO) and average concentrations of nutrients ammonium (NH_4^+) and oxides of nitrogen (NO_x) at the Porepunkah caravan park site (10) with time zero occurring at 1600 hours and the grey area representing post-meridian time. Data represent the means of three replicate samples ± 2 SE.

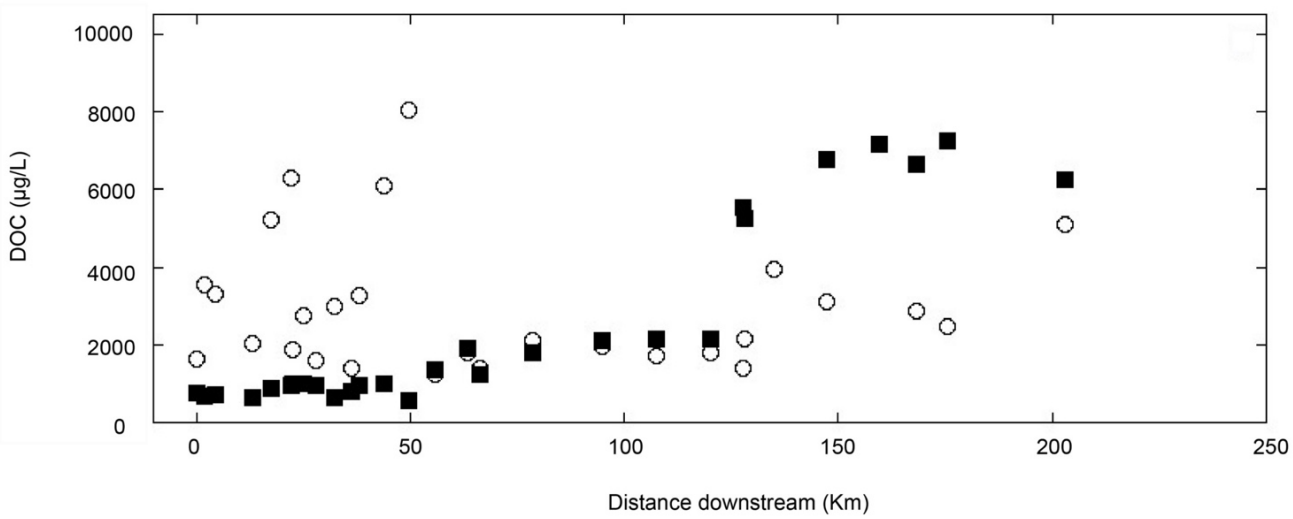


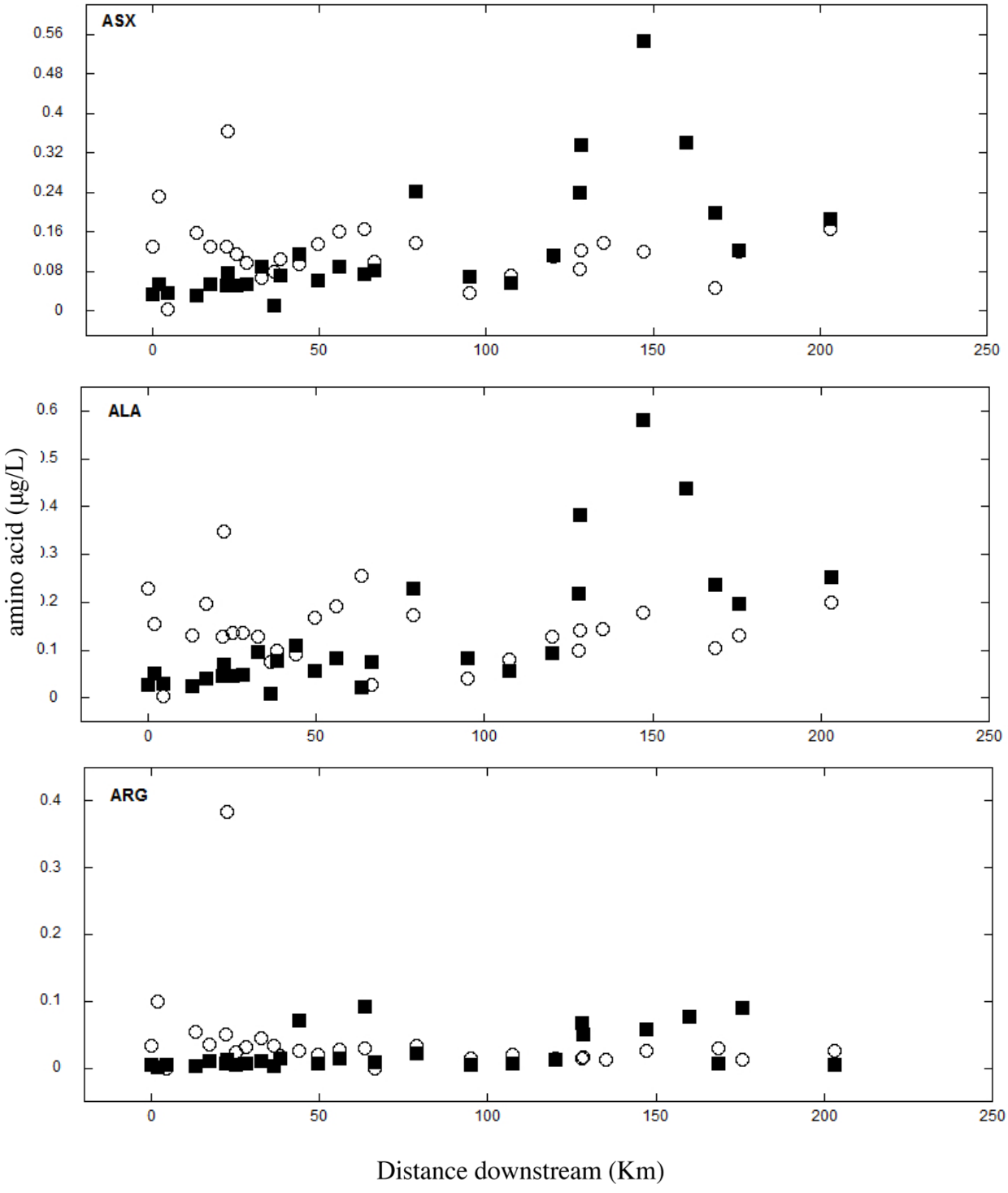
Figure. S4.3 Original DOC values showing anomalously high values in Reaches 1 and 2 before correction using UV/VIS spectroscopy.

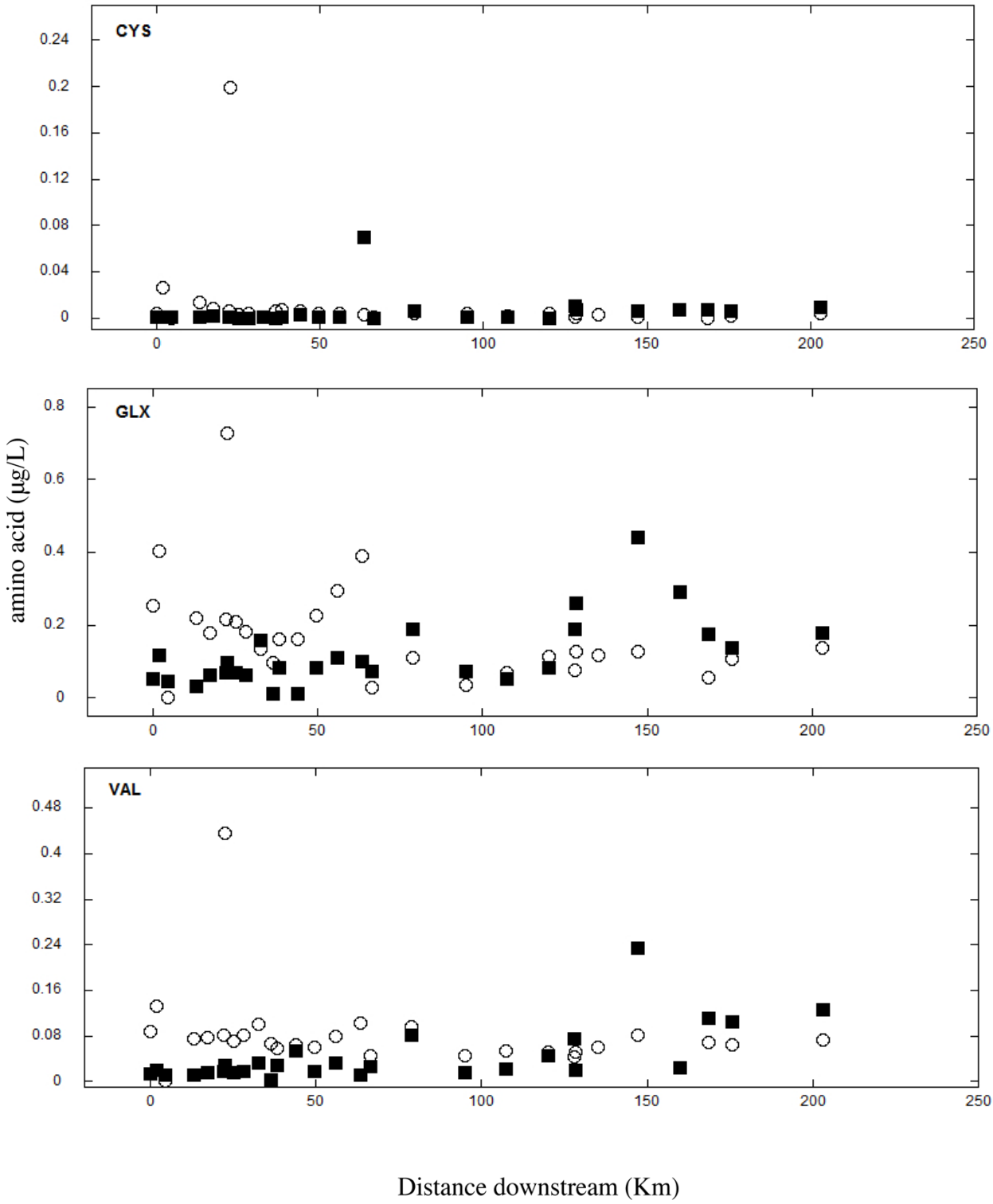
Table. S4.2 Three letter abbreviations for amino acids described in our study. Note that glutamine and asparagine are de-aminated during acid hydrolysis, thus are detected as glutamic and aspartic acid respectively. Hence our use of the abbreviation ASX and GLX.

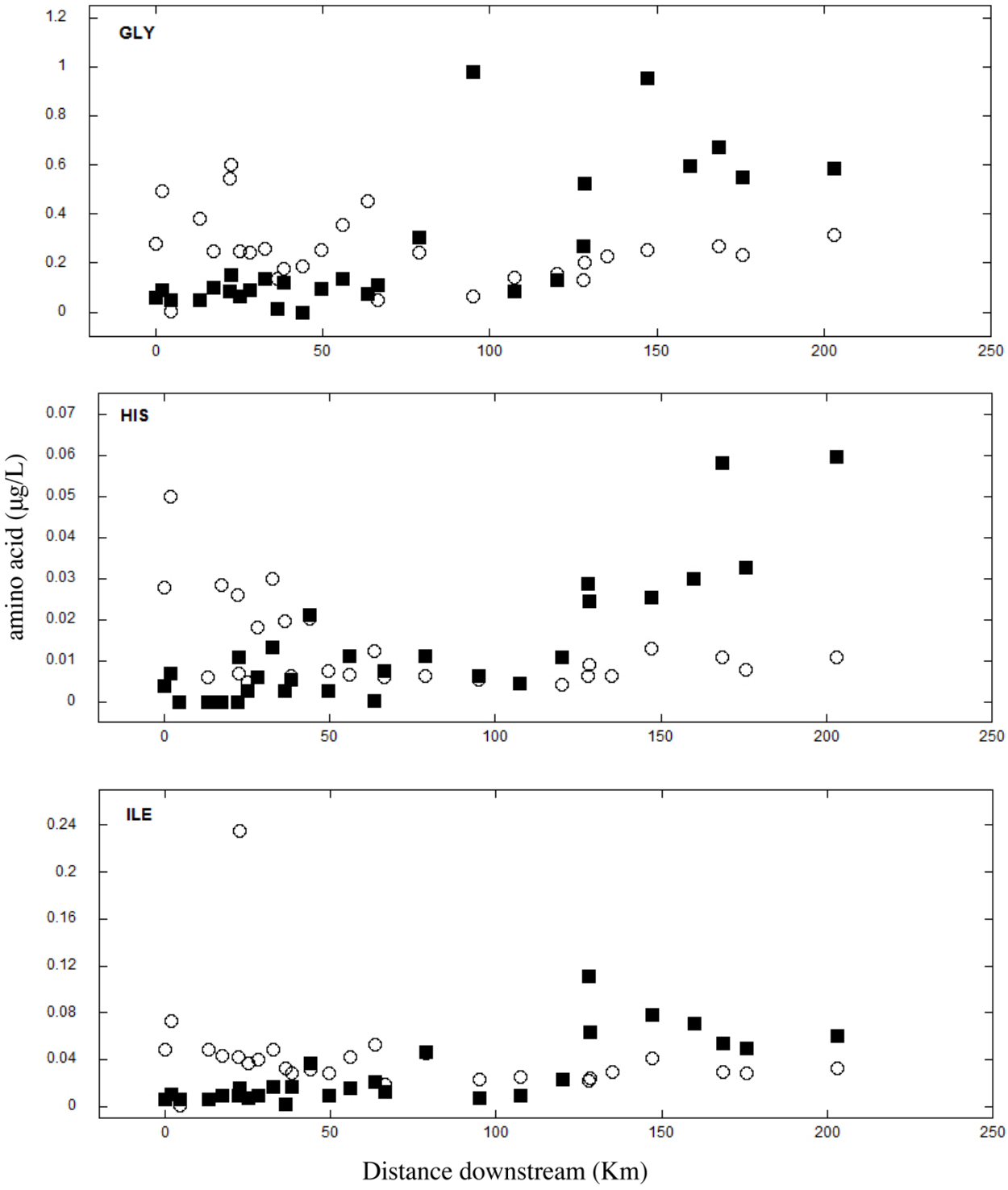
Amino acid	**Three letter abbreviation**	**Amino acid**	**Three letter abbreviation**
Aspartic acid and asparagine	ASX	Cystine	CYS
Serine	SER	Tyrosine	TYR
Glutamic acid and Glutamine	GLX	Valine	VAL
Glycine	GLY	Methionine	MET
Histidine	HIS	Lysine	LYS
Arginine	ARG	Isoleucine	ILE
Threonine	THR	Leucine	LEU
Alanine	ALA	Phenylalanine	PHE
Proline	PRO	Tryptophan	TRP

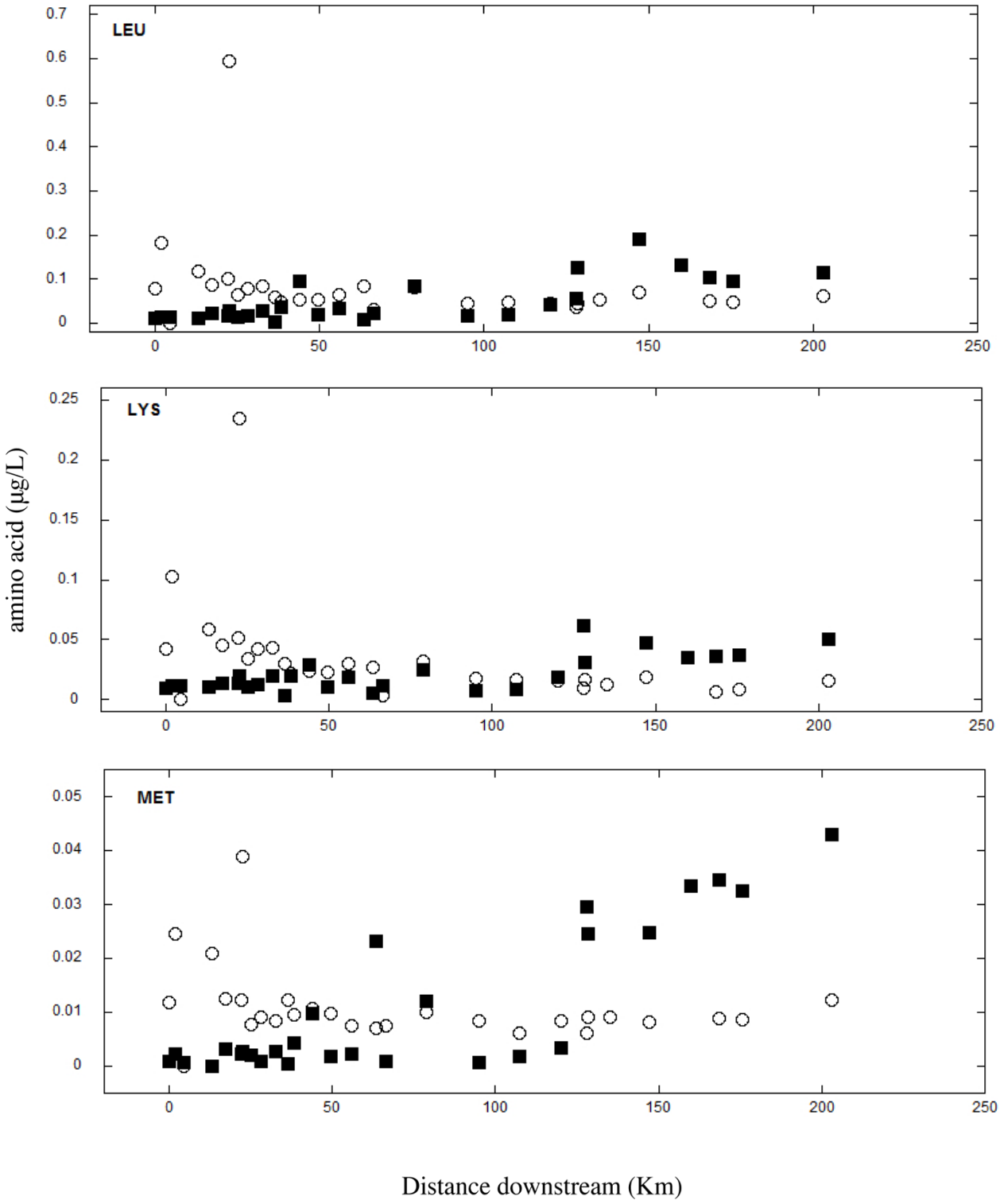
Table. S4.3 Individual amino acid recoveries for bovine insulin standard.

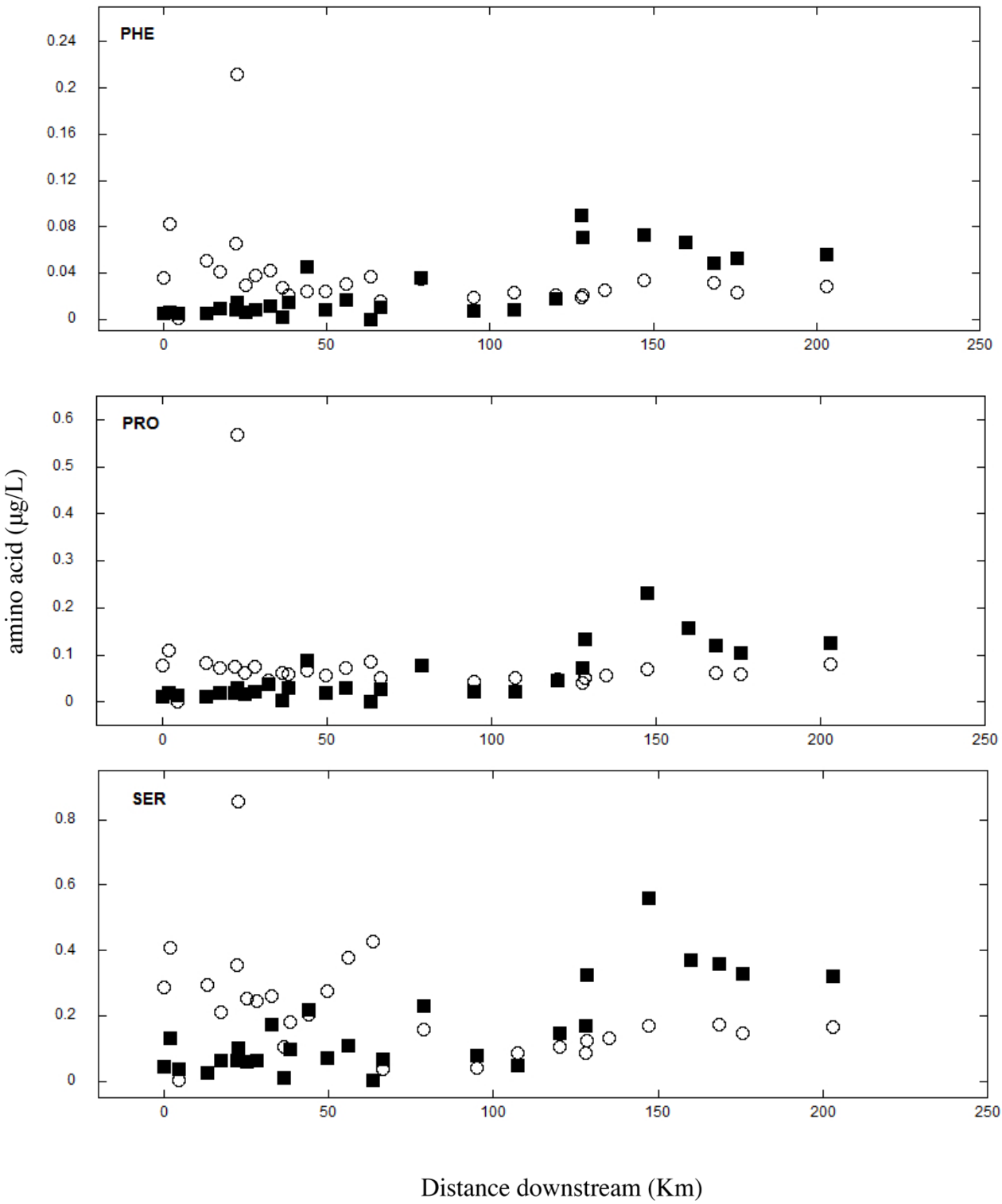
Amino Acid	Theoretical concentration (pmol/ μL)	Average measured concentration (pmol/ μL)	Recovery (%)
HIS	0.57	0.21	37.54
ARG	0.29	0.21	73.62
SER	0.86	0.69	80.00
GLY	1.14	1.10	96.09
ASX	0.86	0.75	87.21
GLX	2.00	1.61	80.59
THR	0.29	0.19	65.64
CYS	0.43	0.81	94.45
ALA	0.86	0.68	79.83
PRO	0.29	0.17	60.15
LYS	0.29	0.20	71.63
TYR	1.14	0.63	55.58
VAL	1.43	0.56	39.41
ILE	0.29	0.06	23.00
LEU	1.72	0.93	54.09
PHE	0.86	0.70	81.09


ASX
ALA
ARG
amino acid (µg/L)
Distance downstream (Km)


CYS
GLX
VAL
amino acid (µg/L)
Distance downstream (Km)


GLY
HIS
ILE
amino acid (µg/L)
Distance downstream (Km)


LEU
LYS
MET
amino acid (µg/L)
Distance downstream (Km)


PHE
PRO
SER
amino acid (µg/L)
Distance downstream (Km)

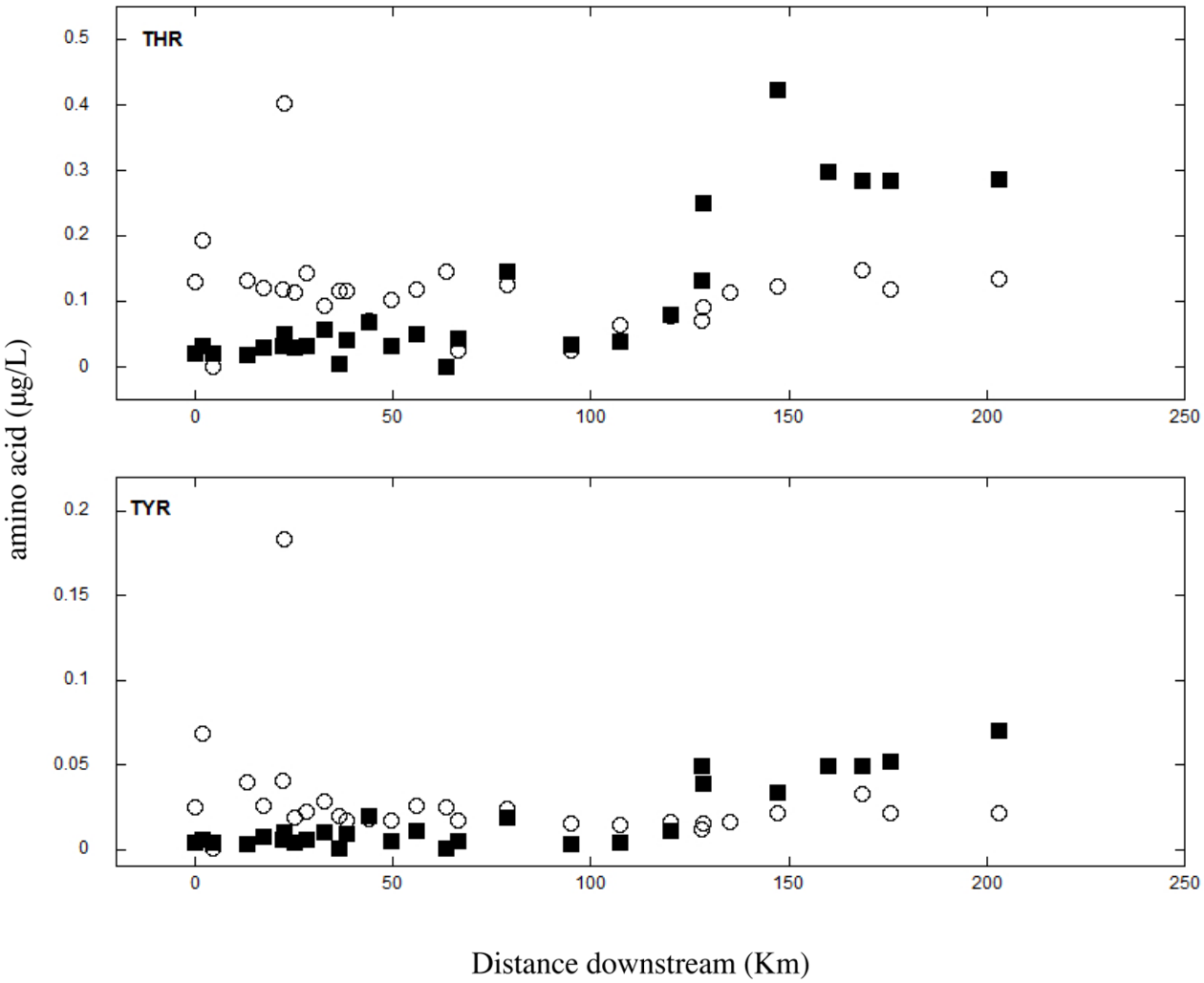


Figure. S4.4 Spatial series plot for individual amino acids at high flow (■) and base flow (○).

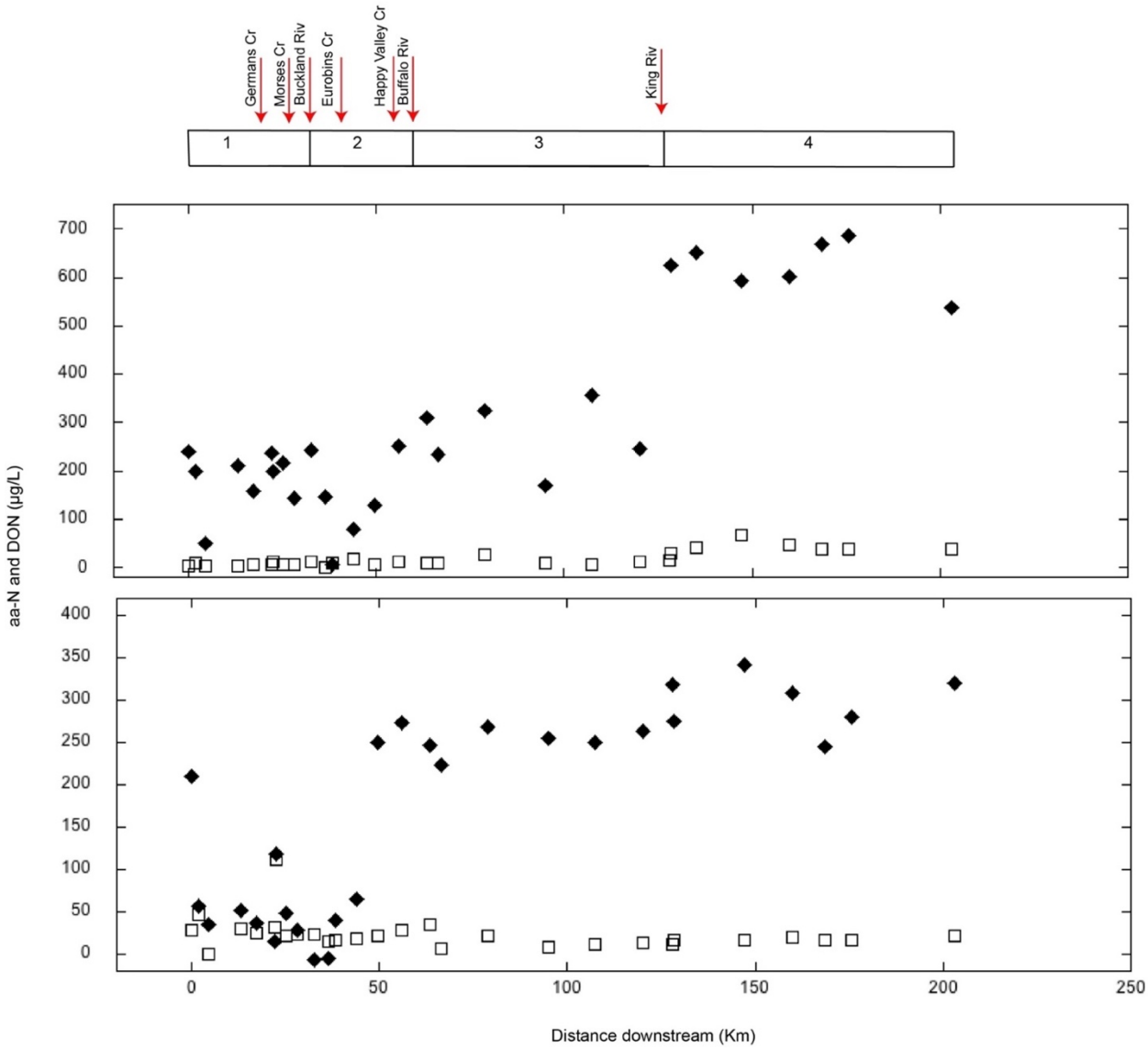


Figure. S4.5 Spatial series plots of dissolved organic nitrogen (DON) (♦) and amino acid nitrogen (aa-N) (□) at high flow (panel A) and base flow (panel B).

Linking Narrative: Chapter 4 to 5

In the previous chapter dissolved organic nitrogen (DON) and dissolved combined amino acid (DCAA) concentration and proportion was studied along a longitudinal gradient in relation to land use, floodplain connection and major townships and confluences. While outside influences such as major confluences and major townships are determined to influence DON and DCAA concentration and composition it is unknown what processes are occurring in-stream that could potentially act as a sink for DON and DCAAs. The following chapter investigates the adsorption of DCAAs onto common substrates found in Australian rivers (kaolinite mineral and a natural stream sediment).

Chapter 5 Determining the extent of adsorption of dissolved combined amino acids onto naturally occurring substrates: a potential sink of DON

Authorship statement

The following chapter is in preparation for submission to *(yet to be decided)*.

Author contribution: I and ES collected the data and I analysed the data with advice from ES, GR and JP. I prepared the manuscript for publication with editing advice from both ES and GR (as per the normal supervisor role).

Determining the extent of adsorption of dissolved combined amino acids onto naturally occurring substrates: a potential sink of DON

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5.1 Abstract

Dissolved organic nitrogen (DON) can comprise up to 80 % of the dissolved nitrogen pool in riverine ecosystems and often encompasses a broad range of organic nitrogen-containing materials. Some forms of DON such as dissolved combined amino acids (DCAAs; proteins, peptides and free amino acids) are highly available sources of nitrogen but may be lost or kept dormant in stream due to adsorption processes. We conducted 24 hour adsorption experiments using two substrates; a natural stream substrate collected from a local river and kaolinite, a naturally abundant clay mineral in Australian freshwater streams. We conducted experiments at pH 5, 7 and 9 to understand the effects of both substrate and pH on the adsorption of DCAAs. Both substrates were capable of adsorbing DCAAs from a stock *Eucalyptus camaldulensis* leaf leachate and adsorption characteristics were similar between substrates at similar pH. Both kaolinite and the natural stream substrate adsorbed a large proportion of DCAAs at ~pH 6 – 7 (95 and 85 % respectively). Two amino acids (HIS and ARG) showed a strong adsorption on both substrates and at all pH conditions. Kaolinite adsorbed all DCAAs strongly at pH 6.38 however, only weakly adsorbed polar netural amino acids (SER, THR and GLY) and non polar neutral amino acids (ALA, VAL, ILE, PRO and LEU) at pH 4.76 and 5.82. Natural sediment was also found to strongly adsorb both polar neutral and non polar neutral amino acids at pH 6.64. By statistically comparing non-pH adjusted and pH adjusted (5, 7 and 9) amino acid controls that were not exposed to substrate we show that not only adsorption but pH adjustment played a role in the concentration of DCAAs measured in samples. Part of the difference in concentration between pH adjusted and non-adjusted controls may be due to a portion of the protein in solution being protected from hydrolysis via association with dissolved organic carbon. The concentration and selectivity of adsorbed DCAAs throughout this experiment indicates that while adsorption is occurring

at ~pH 6, decreases in DCAA concentration may be partly due to an association of DCAAs with humic material in the leaf leachate

5.2 Introduction

5.2.1 Dissolved organic nitrogen and dissolved combined amino acids from leaves

Dissolved organic nitrogen (DON) is a major constituent of the total dissolved nitrogen (TDN) pool in many aquatic systems (Campbell & Fuchshuber, 1995; Vitousek *et al.*, 1979). However, DON is often overlooked as an important nitrogen component due to its variety of sources and the difficulty associated with analysis (Campbell *et al.*, 2000; Giller, Giller, & Malmqvist, 1998). Consequently, DON is often determined by difference (TDN minus inorganic N-containing nutrients) (Campbell *et al.*, 2000; Frankovich & Jones, 1998). Some recent work has identified a significant component (~40 %) of DON released from *Eucalyptus camaldulensis* leaves after 24 hours of immersion occurs in the form of dissolved combined amino acids (DCAAs) (Harris *et al.*, 2016).

During the 24 hour time course leaching experiments conducted by Harris *et al.* (2016) an apparent adsorption of DCAAs was observed after 10 hours. As these leaching experiments were carried out in acid-washed glassware with Milli-Q water, microbial assemblages are unlikely to have established within 24 hours. Therefore, this temporary decrease in DCAA concentration is unlikely a result of microbial consumption but may be attributed to adsorption of DCAAs onto the leaf substrate or biofilm existing on the leaf surface. While the biofilm in these leaching experiments are presumed to be dead due to leaf desiccation, a study by Armstrong and Barlocher (1989) shows that adsorption of DCAAs onto biofilm occurs as a result of biofilm surface properties and is not dependant on whether biofilm is living. As biofilm cover most submerged surfaces, adsorption onto stream or wetland substrates is potentially an important sink of organic-N that may remain dormant for long periods of time and possibly play a role in conditioning surfaces for further biofilm growth.

Biofilms that grow and colonize on submerged river and floodplain material (including *E. camaldulensis* leaves) display adsorptive properties (Baldwin, Whitworth,

& Hockley, 2014; Flemming, 1995; Scholz & Boon, 1993) due to their cell walls, cell membranes and cytoplasm serving as adsorption sites (Flemming, 1995; Späth, Flemming, & Wuertz, 1998). These living biofilm also contain microbial assemblages of autotrophic and heterotrophic organisms such as bacteria, algae and fungi that can readily consume low molecular weight, bioavailable dissolved organic matter (DOM) in freshwater environments (Romaní *et al.*, 2013). However, biofilms are not the only potential sink for DCAAs in the aquatic environment, clay minerals and stream substrates can also act as a sink for DCAAs (Dashman & Stotzky, 1982).

5.2.2 Composition of natural particles in streams

Naturally abundant clay minerals such as montmorillonite, smectite, illite and kaolinite are a class of layered aluminosilicates abundant throughout the Murray-Darling Basin, Australia (Gingele & De Deckker, 2004, 2005). Clay minerals possess strong adsorptive qualities when they come in contact with biomolecules such as DNAs, RNAs and proteins in the natural environment (Dashman & Stotzky, 1982; Yu *et al.*, 2013). A study by Specht, Kumke, and Frimmel (2000) examined adsorption of natural organic matter (NOM) onto kaolinite and montmorillonite and found that large, hydrophobic molecules showed the strongest affinity towards the clay surfaces with smaller aromatic compounds showing little to no adsorption. Adsorption of these biomolecules by clay minerals occurs via a variety of physical and chemical interactions such as cation exchange, van der Waals forces, electrostatic interactions, hydrogen bonding and hydrophobic affinity (Yu *et al.*, 2013). In general, it is believed that positively charged DCAAs can be strongly adsorbed to clay minerals in stream, due to ion exchange reactions with negatively charged clay surfaces (Dashman & Stotzky, 1982).

5.2.3 Adsorption of proteins onto minerals and stream particles

There are various factors that influence protein adsorption onto clays; the type and surface area of a clay mineral plays a critical role in the amount of protein it can adsorb. Kaolinite and illite only have external adsorption sites due to their non-expanding layers while clays such as montmorillonite are expanding layer silicates and have large surface areas where adsorption can occur (Dashman & Stotzky, 1982; Yu *et al.*, 2013). The structure, molecular size and amino acid sequence of proteins also dictates the affinity of adsorption onto clay minerals and adsorptive properties of proteins can change with pH (Dashman & Stotzky, 1982). However, previous work by Fischer, Ingwersen, and Kuzyakov (2010) has also shown that microbial uptake may out compete adsorption with uptake of low molecular weight organic substances (including dissolved free amino acids) being more than 10 times faster than adsorption rates to a soil matrix.

Research has shown that the suspended sediment in rivers is dominated by composite materials that are also known as flocs and aggregates (Woodward & Walling, 2007). These composite materials can contain a variety of clay minerals as well as biofilm and other organic material which has been shown to readily adsorb proteins and amino acids onto their surface within a natural pH range of 4 – 9 (Hedges & Hare, 1987; Woodward *et al.*, 2002; Woodward & Walling, 2007). The composition of suspended materials and stream substrate is mostly dependant on the characteristics of parent soils and rocks within the catchment (Gingele & De Deckker, 2005).

5.2.4 This study

This study investigates the adsorption of the dissolved combined amino acid (DCAA) portion of DON leached from *Eucalyptus camaldulensis* leaves onto different mineral substrates (kaolinite and natural river sediment) across a range of pH levels (5, 7 and 9). In doing so, we examine which substrate is most effective at adsorbing amino acids and

aim to answer three main questions. (i) Do substrates within freshwater streams act as a potential sink for dissolved combined amino acids (DCAAs)? (ii) does pH level influence the adsorption of DCAAs? And (iii) does the partitioning of DCAAs between surfaces and water vary between different substrate types?

5.3 Methods

5.3.1 Pure mineral source

Pure kaolinite mineral was ordered from The Clay Minerals Society, Chantilly VA, USA and was sourced from Warren County, Georgia, USA.

5.3.2 Preparation of natural sediment

Natural sediment cores were collected from the Kiewa River at Killara Reserve (-36.1386°S, 146.9520°N) Wodonga, Victoria, Australia on the 3rd of January 2017. Sediment cores were collected from the river's edge to a depth of 4 cm with a Terumo 50 mL syringe. Approximately 150 g portions of collected river sediment were sifted through a 0.2 mm mesh before being transferred to HDPE Nalgene bottles and washed three times with 150 mL Milli-Q water for 15 minutes upon an orbital shaker. Between washes sediment was left to settle for an hour and supernatant poured off. To remove any organic carbon already adsorbed to substrate the washed sediment portions were treated with 150 mL 3 % hydrogen peroxide (adjusted to pH 2 with HNO_3), incubated at 50 °C for 24 hours and shaken every hour for the first 6 hours (Hedges & Hare, 1987).The sediment was left to settle, supernatant poured off and the peroxide treatment repeated. Sediment portions were then washed three times with 150 mL Milli-Q water and left to air dry.

5.3.3 Total dissolved amino acid analysis

All amino acids in this study are referred to using their standard three letter abbreviations (Table S5.1). Amino acid digestions and analyses were conducted using new (pyrolysed)

sample vials. One mL of filtered sample was freeze-dried, 200 µL of HCl (containing 0.02 % phenol) added and the samples hydrolysed under an argon atmosphere at 110 °C for 24 hours. HCl was removed by freeze drying, the samples reconstituted and tagged with 80 µL (3:1 borate buffer: Milli-Q water mixture) and 20 µL of 6-aminoquinolyl-N-hydroxysuccinimyidyl carbamate (AQC) reagent (heated at 55 °C for 10 minutes). Tagged samples were diluted with 100 µL of 0.1 % formic acid and centrifuged to remove any solid material (7323 g; 5 min). Approximately 150 µL of supernatant was transferred into a limited volume insert (LVI) and placed into an auto sampler vial. Some amino acids are not recoverable using this method, tryptophan is destroyed and cystine is often difficult to determine from the hydrolysed samples (Fountoulakis & Lahm, 1998). During acid hydrolysis, asparagine and glutamine are deamidated to aspartic acid and glutamic acid, respectively, making it impossible to determine the contributions of each amino acid individually; thus are referred to as ASX (ASN+ASP) and GLX (GLN+GLU) (Anders *et al.*, 2003).

Tagged amino acid samples were analysed by liquid chromatography – mass spectrometry (LC-MS/MS). The LC-MS/MS system consisted of a Shimadzu Nexera X2 UPLC coupled to a Shimadzu 8030 triple quadrupole mass spectrometer, operated in positive ion electrospray ionisation (ESI) mode (Shimadzu corporation Kyoto, Japan). Individual tagged amino acids were detected in multiple reaction monitoring (MRM) mode, with collision parameters optimized individually. Source conditions were: sheath gas temperature 315°C, gas flow 10 $L.min^{-1}$, nebulizer pressure 45 psi and capillary voltage 3800 V. Separation was achieved using a gradient eluent with a Waters Aquity UPLC C18 column (2.1 x 150 mm; pore size 1.7 µm) maintained at 50 °C. The mobile phase consisted of 0.1 % formic acid (eluent A) (Sigma-Aldrich, St Louis, USA) and 100

% OPTIMA LCMS grade acetonitrile (eluent B) (Fischer Scientific, Pittsburgh, USA) with flow of 0.55 mL/min.

Calibration standards were made using amino acid standard H (Waters Corporation) spiked with glutamine (GLN), asparagine (ASN) and tryptophan (TRP) allowing measurement of all detectable amino acids over the range 0.01 – 2 pmol µL. A quality control sample of selected amino acids was prepared at 1 pmol/µL containing HIS, ARG, GLN, LYS and ILE. Blanks were included in each sample run, including: (i) a reagent blank consisting of 80 µL borate buffer and 20 µL AQC, (ii) a sample blank comprising 40 µL of Milli-Q water, hydrolysed and derivatised as detailed above and (iii) a 0.1 % formic acid blank. An insulin standard (bovine pancreas; Sigma Aldrich, St Louis, USA) was included in every sample set to test for amino acid recovery (recoveries ranged from 27.6 to 89.3 %; see Table S5.2). For comparison with concentrations measured by non-specific methods, amino acid concentrations were converted to µg-N/L.

5.3.4 Pilot adsorption experiments

An initial adsorption experiment was run with kaolinite and natural substrate to determine the adsorption capacity of each substrate based on DOC lost from solution (Jardine, McCarthy, & Weber, 1989). Leaf leachate was prepared using air-dried, terrestrially aged *Eucalyptus camaldulensis* leaves collected from Wonga Wetlands (36.0686°S, 146.8543°E) Albury, NSW during summer leaf fall of 2012. Approximately 0.25 g of dry leaves were left to leach in 1 L of Milli-Q water for 24 hours at a constant temperature of 20 °C. A zero-time (T0) measurement for DCAA was taken from the stock leachate sample at the 24 hour mark. For ease of reading DCAA concentrations throughout this chapter will be expressed in molar form (µM) rather than mass form (µg/g of leaf) as seen previously. Micromolar-nitrogen concentration was calculated for each individual amino

acids post hydrolysis and analysis of sample. Individual nitrogen concentrations were then summed to achieve the total nitrogen concentration for DCAAs within solution.

10 mL of leachate was then added to centrifuge tubes with different amounts of substrate (0.01 0.05, 0.1, 0.5, 1, 2 and 5 g). The centrifuge tubes were placed upon the orbital shaker and left for 24 hours before being centrifuged at 7323 g for 10 minutes. The supernatant was measured for dissolved organic carbon (DOC) content using a spectrophotometer at an absorbance of 254 nm (Baldwin & Valo, 2015).

5.3.5 Adsorption experiments

Adsorption experiments were run using solid concentrations of substrate that adsorbed similar levels of DOC (Figure 5.1) (0.75 g kaolinite and 2 g natural sediment). 5 replicates of each substrate were run at three different pH levels (initially adjusted to 5, 7 and 9) to observe effect of pH upon adsorption properties. Substrate was added to 10 mL of leaf leachate and pH adjusted by slow addition of either 10 mM HCl or 10 mM NaOH (5 replicates x 3pH levels x 2 substrates= 30 samples). 25 controls ((5 replicates at pH 5, 7, and 9, shaken 24 hours), 5 replicates of T0 (no adjustment, not shaken) and 5 replicates T24 (no adjustment, shaken for 24 hours)) were also included with no substrate addition to test the effect of pH adjustment on leaf leachate. Samples were placed on an orbital shaker for 24 hours before being centrifuged at 7323 g for 10 minutes and supernatant removed and stored for amino acid analysis. pH was measured again at the completion of the experiment to detect drift (Table 5.1). pH was found to drift throughout the 24 hour shaking period so final pH values (taken at 24 hours) are used to display and discuss results.

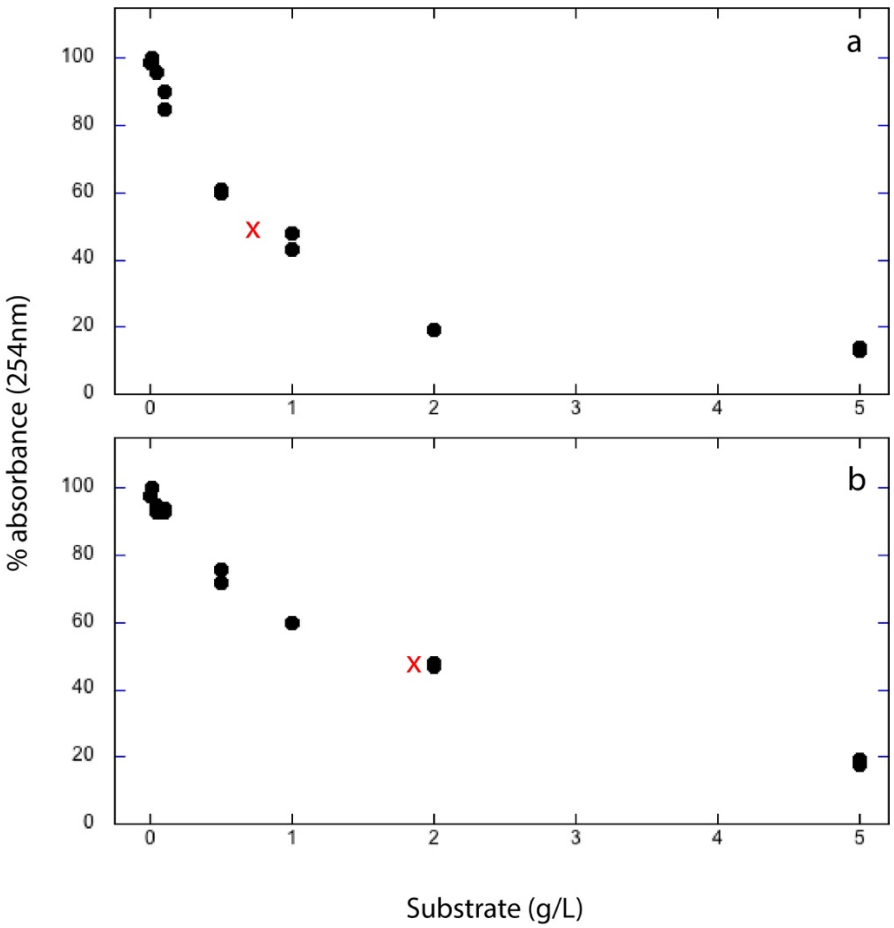

Figure. 5.1 Pilot experiment curves showing the optimum amount of substrate to be used in the adsorption experiments. Curves shown are grams of kaolinite (a) and grams of natural sediment (b).

5.3.6 Data analyses

Two principal coordinate analyses (PCoA) and Permutational Analyses of Variance (PERMANOVA) were carried out to explore the relationship between pH, substrates and individual amino acids (i) all experimental controls; T0 (stock leachate, not shaken), T24 (stock leachate, shaken for 24 hours) and pH adjusted stock leachate (pH 5, 7 and 9 and shaken for 24 hours) and (ii) amino acid concentration in supernatant exposed to substrates and shaken for 24 hours (natural and kaolinite) at pH 5, 7 and 9. Final pH values (taken at 24 hours) are used to display and discuss results. The multivariate statistical routines were implemented within PRIMER7 (PRIMER-E Ltd, Plymouth UK).

Table. 5.1 pH drift detected in kaolinite, natural sediment and stock leachate samples after 24 hours. Values are average of 5 replicate samples ± 2 SE.

Treatment	Adjusted pH	Average pH after 24 hours
Kaolinite	5	4.76 ± 0.08
	7	5.82 ± 0.08
	9	6.38 ± 0.04
Natural sediment	5	5.04 ± 0.04
	7	6.64 ± 0.04
	9	8.06 ± 0.14
Stock leachate (T0)	5	4.9 ± 0.06
	7	6.66 ± 0.12
	9	7.08 ± 0.04

5.4 Results

5.4.1 Leachate controls

Both T0 (stock leachate, not shaken) and T24 (stock leachate, shaken for 24 hours) controls were analysed to examine whether shaking for 24 hours had any effect upon DCAA concentration and composition within sample vials. T24 controls were found to be not significantly different to T0 controls (Figure 5.2, PERMANOVA, $p = 0.6091$). When calculating concentration of amino acids adsorbed by substrates the largest single source of error was the T0 stock leachate controls (Table S5.3) therefore, T24 stock leachate controls were included in calculating adsorption to reduce this uncertainty. From here on, a combined average of the T0 and T24 controls will be referred to as the '**stock leachate control**'. All sample error is expressed as ± 2 SE except where stated otherwise.

The average concentration of total DCAAs in the stock leachate control samples (T0 and T24 combined) was 0.586 μM. The dominant amino acids were: HIS (11 %, 0.066 ± 0.012 μM), ARG (11.48 %, 0.068 ± 0.018 μM), GLX (12 %, 0.072 ± 0.018 μM) and GLY (13 %, 0.074 ± 0.016 μM) while the least abundant were: LYS (0.2 %, 0.001 ± 0.0002 μM) and MET (1.4 %, 0.008 ± 0.002 μM).

Interestingly, pH adjusted (5, 7 and 9) controls showed considerable pH drift over 24 hours (4.90, 6.66 and 7.08, respectively) and displayed lower concentrations of DCAAs with increasing pH, indicating that the interaction between pH and DCAAs is more complex than originally thought (Figure 5.3). Total DCAA concentrations were found to be 0.810 μM at pH 4.90, 0.551 μM at pH 6.66 and 0.127 μM at pH 7.08. Comparison of DCAA composition between T0 (not pH adjusted) and T24 pH adjusted controls was found be significantly different at pH 9 (7.08) (PERMANOVA, $p = 0.0094$) (Figure 5.2). This was also the case when comparing DCAA composition of T24 (not pH adjusted) leachate controls and T24 controls adjusted to pH 7.08 (PERMANOVA, $p = 0.0087$) (Figure 5.2).

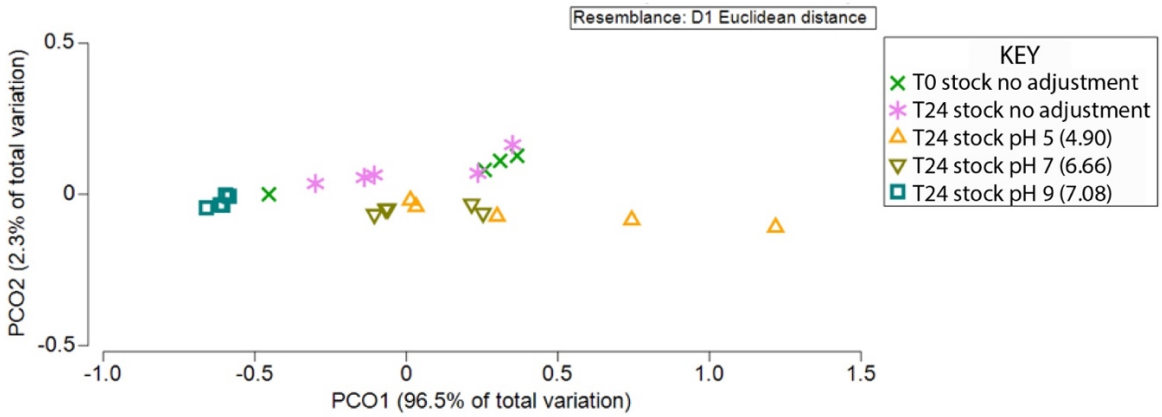

Figure. 5.2 Principal coordinate analysis (PCoA) of amino acid composition across stock leachate at zero hours (T0) with no pH adjustment, stock leachate at 24 hours (T24) with no pH adjustment and stock leachate at 24 hours that was pH adjusted.

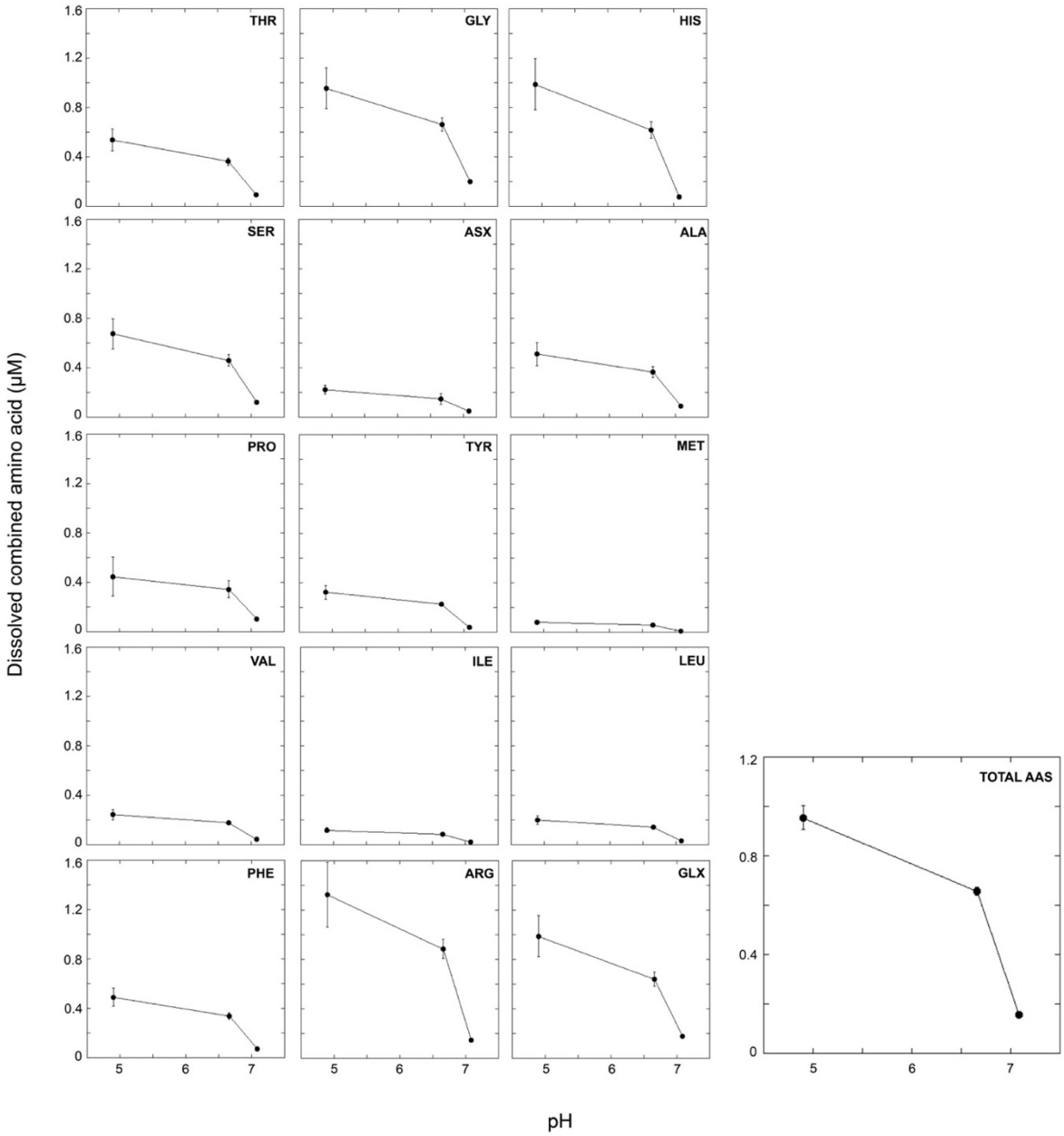

Figure. 5.3 Concentration of individual amino acids and total dissolved combined amino acids (DCAAs) within pH adjusted controls measured at pH 4.90, 6.66 and 7.08. Values are average of 5 replicate samples ± 1 SE.

5.4.2 Total dissolved combined amino acid adsorption

Both substrates were capable of adsorbing DCAAs from leaf leachate and displayed similar adsorption characteristics at similar pH (Figure 5.4). Overall, when compared to the average concentration of the stock leachate control, the percentage DCAAs adsorbed on both kaolinite and natural substrates increased with pH (Figure 5.4). Similar adsorption was observed between kaolinite at pH 4.76 and natural sediment at pH 5.04 (17 and 29 % total DCAAs respectively) as well kaolinite at pH 6.38 and natural sediment 6.64 (95 and 85 % total DCAAs respectively). Adsorbed concentrations were as follows; kaolinite: pH 4.76 = 0.104 ± 0.006 μM DCAAs, pH 5.82 = 0.180 ± 0.006 μM DCAAs, pH 6.38 = 0.554 ± 0.004 μM DCAAs and natural substrate: pH 5.04 = 0.172 ± 0.010 μM DCAAs, pH 6.64 = 0.500 ± 0.004 μM DCAAs, pH 8.06 = 0.540 ± 0.004 μM DCAAs.

Adsorption increased significantly for both substrates at over the pH range 6 – 7. With the addition of natural sediment 85 % (0.500 ± 0.004 μM DCAAs) were adsorbed at pH 6.64 and with the addition of kaolinite 95 % (0.554 ± 0.006 μM DCAAs) were adsorbed at pH 6.38. The concentration adsorbed onto both kaolinite and natural sediment at ~pH 6 – 7 was much greater than the decrease of 0.036 μM DCAAs observed when the stock leachate control was adjusted to pH 6.66. This indicates that adsorption is in fact occurring at ~pH 6 – 7.

Figures 5.4 and 5.5 show that pH adjustment and adsorption had a significant effect upon DCAA concentration and composition between samples (PERMANOVA, $p = 0.0002$). A pairwise comparison shows that both kaolinite and natural samples initially adjusted to pH 9 are significantly different in DCAA composition to the T0 leachate and all other pH adjusted samples regardless of substrate (PERMANOVA, $p < 0.05$). Of all amino acids, HIS and ARG are the two amino acids that are strongly and consistently adsorbed by both substrates across all pH conditions.

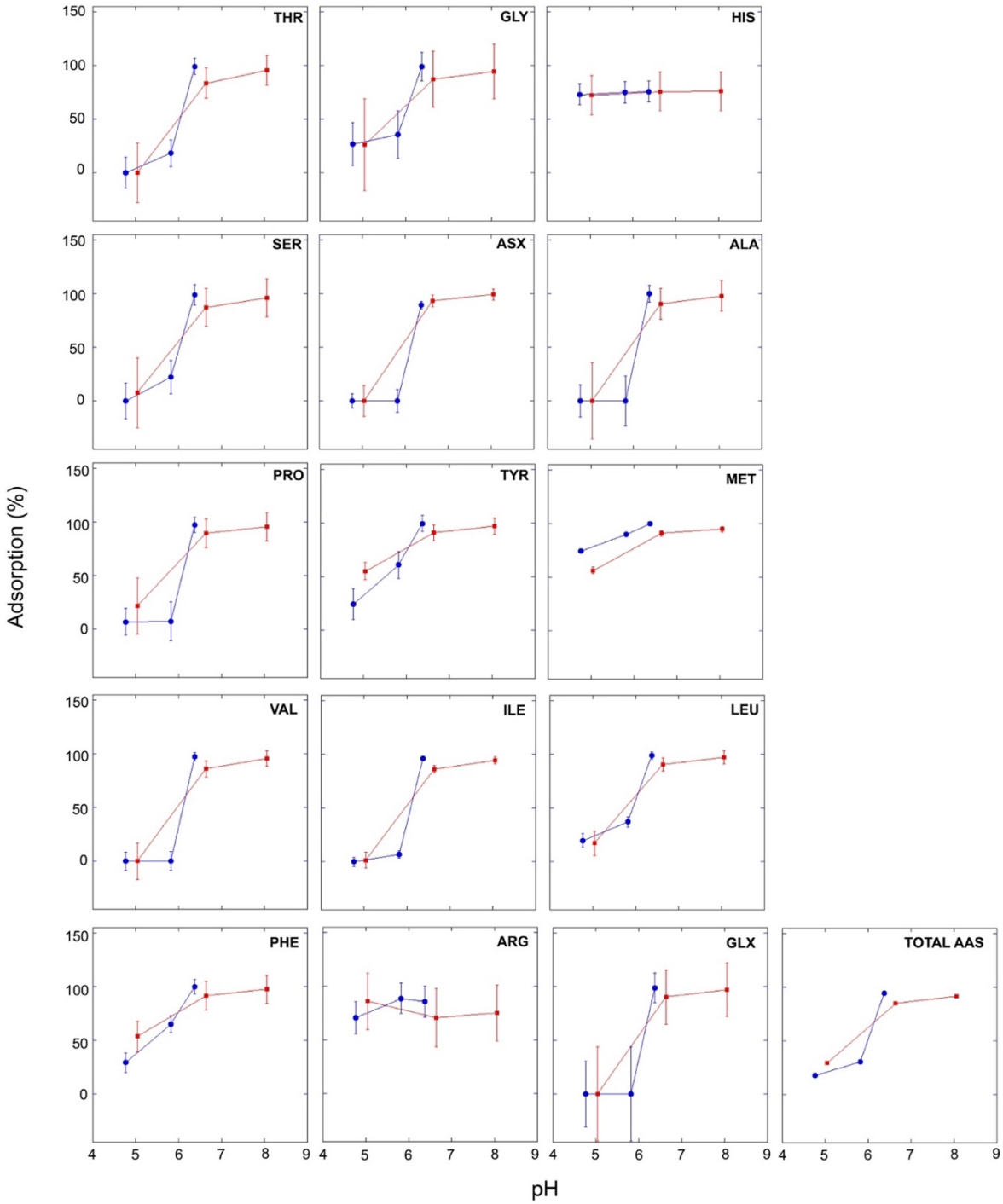

Figure. 5.4 Adsorption of individual amino acids on kaolinite (•) and natural substrate (▪) after 24 hours. Values are average of 5 replicate samples ± 1 SE.

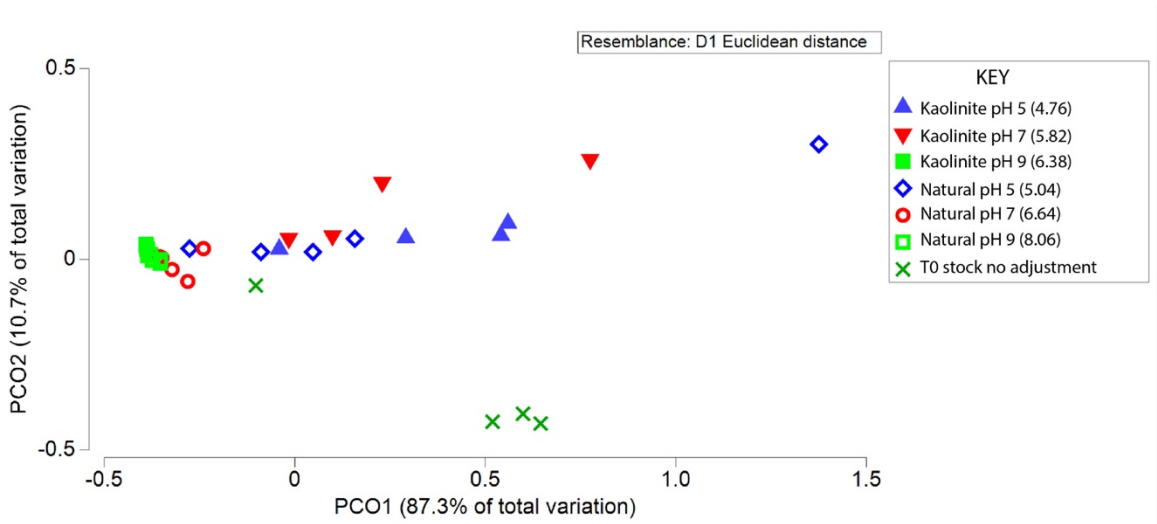

Figure. 5.5 Principal Co-ordinate analysis of amino acid composition showing both substrate types (kaolinite and natural) and final pH values.

5.4.3 Individual amino acid adsorption

All DCAAs exposed to natural sediment were adsorbed to a higher extent as pH increased apart from ARG and HIS which remained relatively constant across all pH treatments. HIS as a proportion of total adsorbed DCAAs decreased dramatically from 27.7 % at pH 5.04 to 10.0 % at pH 6.64 and to 9.4 % at pH 8.06 however, concentrations of HIS adsorbed remained relatively constant (0.050 ± 0.014 – 0.052 ± 0.012 µM). ARG as a proportion of total adsorbed DCAAs also decreased from 37.6 % at pH 5.05 to 10.9 % at pH 6.64 and 10.7 % at pH 8.06 however, the concentration of ARG also remained relatively constant (0.054 ± 0.020 – 0.064 ± 0.018 µM).

All DCAAs exposed to natural sediment bedsides ARG showed lowest adsorption at pH 5.04, most of these amino acids (ASX, SER, GLX, GLY, ALA, PRO, VAL, ILE, LEU, and THR) displayed between 0 – 25 % adsorption while MET, TYR, HIS and PHE were adsorbed to a higher extent (54 – 72 %). Most amino acids (ASX, SER, GLX, GLY, ALA, PRO, TYR, VAL, ILE, LEU, PHE, MET and THR) showed a large increase (between 34 – 94 %) in adsorption between pH 5.04 and 6.64 and a smaller increase in

adsorption (6 – 12 %) between pH 6.64 and 8.06. HIS showed a small increase in amount adsorbed between pH 5.04 and 6.64 of 4 % (0.024 ± 0.002 μM) and remained the same at pH 8.06. Interestingly, of all DCAAs exposed to natural sediment ARG was the only amino acid to decrease in adsorption (15 %, 0.01 ± 0.028 μM) from pH 5.04 to 6.64 however, the decrease in adsorption is much smaller than the associated uncertainty. Individually, the DCAAs that were most strongly adsorbed between pH 5.04 and 8.06 were SER (67 %), GLX (97 %) and GLY (68 %).

Many amino acids (HIS, ASX, GLX, PRO and ALA) were adsorbed to a similar extent on kaolinite at pH 4.76 and 5.82. SER, VAL, ILE, LEU, THR, and GLY displayed a small increase in adsorption (8 – 33 %) between pH 4.76 and 5.82 and a large increase in adsorption (58 – 95 %) between pH 5.82 and 6.38. MET also displayed this behaviour across pH treatments but exhibited much higher adsorption (75 %, 0.006 ± 0.002 μM) at pH 4.76 than the other DCAAs. GLX, ASX, ALA and PRO display a small decrease in adsorption (0.5 – 16 %) between pH 4.76 to 5.82 and a large increase in adsorption (85 – 100 %) between pH 5.82 and 6.38. Similar to the natural substrate ARG and HIS remained high and constant in percentage adsorbed (73 – 89 %) however, ARG adsorption was highest at pH 5.82. PHE, MET and TYR displayed a near linear relationship between increasing pH and concentration adsorbed, all reaching 100 % adsorption at pH 6.38. Individually, the DCAAs that changed the most between pH 4.76 and 6.38 were ALA (100 %), GLX (99 %), VAL (97 %) and ILE (96 %).

5.5 Discussion

Historically, many studies have examined amino acid and protein adsorption onto clay minerals in various environmental systems (marine, freshwater and soil) (Gao *et al.*, 2017; Wang & Lee, 1993). It is known that adsorptive interactions of organic molecules can often modify their bioavailability (Gao *et al.*, 2017) as well as mobilise organic molecules that may be attached to suspended sediment (Campbell *et al.*, 2000; Kretzschmar, Sticher, & Hesterberg, 1997).

This study examined amino acids as a source of dissolved organic nitrogen (DON) that may be readily adsorbed by substrates in stream. Previous work has identified dissolved combined amino acids (DCAAs) as a variable (3 – 44%), yet important portion of the DON pool within freshwater ecosystems (Harris *et al.*, 2018). This study aims to answer the following questions; (i) Do substrates within freshwater streams act as a potential sink for dissolved combined amino acids (DCAAs) (ii) does pH level influence the adsorption of DCAAs and (iii) does the partitioning of DCAAs between surfaces and water vary between different substrate type?

Interestingly, Figure 5.2 shows stock leachate samples that were adjusted to pH 9 at the outset of the experiment but not exposed to substrates to be significantly different in DCAA composition from both T0 and T24 stock leachate samples that were not pH adjusted. DCAA concentrations in pH adjusted controls decreased as pH increased, suggesting that the apparent concentration of DCAAs within the stock leachate is directly affected by pH adjustment. Figure 5.4 shows that DCAAs are capable of being adsorbed by both substrates at certain conditions. It is also possible that a small portion of DCAAs are adsorbed to experimental glassware in addition to substrates. However, we cannot be certain that this is not partly due to pH adjustment alone..However, the DCAA concentration in the T24 control adjusted to pH 7 (6.66) is very similar to the

concentration of the stock control leachate (not adjusted) (0.551 and 0.587 μM DCAAs respectively). This provides strong evidence for adsorption occurring at ~pH 6 – 7 for both substrates as the changes in DCAA concentrations in the presence of these substrates are much larger than that observed for pH adjustment alone.

Assuming that adsorption is occurring across all pH treatments, kaolinite adsorbed amino acids most strongly at pH 6.38. All DCAAs were adsorbed most weakly at pH 4.9, similar to the results of Hedges and Hare (1987) that found kaolinite to weakly adsorbed both polar netural amino acids (SER, THR and GLY) and non polar neutral amino acids (ALA, VAL, ILE, PRO and LEU) at ~pH 5. Natural sediment was also shown to most strongly adsorb DCAAs, including both polar neutral and non polar neutral amino acids at ~pH 6 (6.64).

As previously mentioned, asparagine and glutamine are deamidated to aspartic acid and glutamic acid making both ASX and GLX acidic. We observed almost no adsorption of proteins/peptides containing ASX and GLX onto kaolinite below pH 6.38. Ikhsan *et al.* (2004) found no adsorption of free aspartic acid onto kaolinite to occur anywhere within the range of pH 3 – 10. This may indicate that the presence of aspartic acid in peptides and proteins influences ability to adsorb regardless of substrate and pH.

TYR and PHE behaved similarly when exposed to substrates, both displayed a linear relationship with increasing pH when exposed to kaolinite and very similar concentrations were adsorbed when exposed to natural sediment. TYR and PHE are both aromatic amino acids and a study by Keiluweit and Kleber (2009) found that the presence of an aromatic ring within organic molecules influences their adsorption behaviour. Tsvetkov and Mingelgrin (1987) also found aromatic amino acids to behave in a similar way when adsorbed onto montmorillonite clay complexes. This is due to the π electrons

of the aromatic ring being attracted to the positive surface of the clay mineral (Tsvetkov & Mingelgrin, 1987).

Similar to the results of Hedges and Hare (1987) HIS, and ARG are strongly removed from solution regardless of pH level and substrate type. A study conducted by Laird, Martens, and Kingery (2001) examined carbon sequestration in agricultural soils and the ability of clay soils to adsorb humic materials, ARG was found to be the most abundant amino acid adsorbed to the clay soils. Soil nitrogen was measured by a thermal combustion method and it was found that 83 – 100 % of soil nitrogen was sourced from amino acids and amino sugars and that 50 – 66 % of amino sugar nitrogen was sourced from ARG adsorbed to the soil (Laird, Martens, & Kingery, 2001). This is because ARG is the most basic of the 20 α-amino acids in biological systems and with a positively charged guanidinium group (pKa = 12.48) ARG bonds directly to the negative charge sites on both of the substrates used in this study (Laird, Martens, & Kingery, 2001). The surface of kaolinite is also known to become increasingly negative with more basic pH achieving an isoelectric point at pH 4.8 (Kretzschmar, Sticher, & Hesterberg, 1997). As the lowest pH used in our experiment was a pH of 5 this explains why adsorption of ARG to kaolinite was strong throughout all pH treatements.

The similarity in adsorption profiles of dissolved combined amino acids between substrates is possibly influenced by: (i) a high proportion of kaolinite (or similar clay minerals) in the natural sediment, (ii) the pH under which adsorption is occuring and (iii) the fact that amino acids are not adsorbing independantly but are a part of larger proteins and peptides. Kaolinite is known to be a common mineral within Australian rivers and as the natural sediment is a composite material it is likley that a proportion of the natural sediment is kaolinite (Gingele & De Deckker, 2005). However, Figure 5.2 shows that pH adjustment alone (i.e. in the absence of substrate) has some effect on DCAA

concentrations. Tomaszewski, Schwarzenbach, and Sander (2011) conducted a study showing that protein encapsulation by natural organic matter occurs in freshwater aquatic environments at varying degrees with different pH. They examined the encapsulation of a lysozyme protein in a mixture of humic acids across a pH range of 5 – 8. In doing so, they found that the lysozyme protein was encapsulated by one humic acid across the entire pH range (pH 5 – 8) and by seven humic and fulvic acids from terrestrial aquatic sources between pH 5 and 6.

Additional studies such as Knicker and Hatcher (1997), Leinweber and Schulten (2000) and Sharpley and Smith (1995) have reported high proportions of non-hydrolysable nitrogen closely associated with, and protected by humic materials, sourced from clay minerals and sedimentary organic matter. Knicker and Hatcher (1997) conducted a study on algal sediment from Mangrove Lake, Bermuda. They used ^{15}N Nuclear Magnetic Resonance (NMR) spectroscopy to investigate protein content within water associated with the sediment before and after hydrolysis with 6M HCl. The ^{15}N NMR spectra at -256ppm (representative of peptide-N) survived the hydrolysis treatment, indicating that a portion of protein-N is protected from hydrolysis. Therefore, it is possible that a portion of the peptide nitrogen in both the pH adjusted samples and standards is associated with humics from the leaf leachate and protected from hydrolysis with 6M HCl. The DCAA profiles of the pH adjusted samples (Figure 5.3) indicate that the protection of DCAAs from hydrolysis increases with higher pH.

Assuming DCAAs are adsorbed, is it possible for them to be later desorbed? Further experimental work would need to be done to confirm this. Some previous work (Ding & Henrichs, 2002; Grace & Bianchi, 2010) that examined adsorption and desorption of proteins to common marine sediments (illite, goethite and montmorillonite). They found that proteins were rapidly and almost completely adsorbed to marine

sediment however, desorption was much more difficult with only 2.3 – 13.5 % of proteins desorbing from the mineral surface after 2 hours. Another study by Grace and Bianchi (2010) examined amino acid adsorption onto particulate material from the Mississippi River at different levels of salinity. Basic amino acids were found to adsorb more readily whereas acidic amino acids remained within solution and changing ionic conditions changed the rate at which individual amino acids were adsorbed and later desorbed.

It would be of benefit to examine adsorption rates in comparison with rates of microbial uptake. Microbial uptake rates for low molecular weight organic material (including dissolved free amino acids) are known to be up to ten times higher than rate of adsorption (Fischer, Ingwersen, & Kuzyakov, 2010).

This study has implications for explaining changes in DON and DCAA composition of in-stream environments. Potentially, under certain conditions minerals within soil or stream substrates can act as a sink of DON in the form of DCAAs. With substrate often changing longitudinaly along the length of a stream due to land use, erosion and flooding it is likely that DCAAs are lost through physiochemical process to differing degrees throughout a catchment (Costigan *et al.*, 2014; Hadwen *et al.*, 2010; Williams, 1971).

However, it is likely that the observed decrease in DCAAs in sample is due in part to both adsorption and pH adjustment. We show that both adsorption and pH adjustment had a real effect upon measured DCAA concentration of samples between pH 6 – 7. This has implications for real systems such as the Ovens River (Chapter 4) that generally have a pH of ~7. If adsorption is rapid and can account for 100 % of DCAAs (Figure 5.4) and desorption is slow or does not occur there is potential for DCAAs to be unavailable to aquatic and heterotrophic organsisms in real systems. However, to confirm the impact of adsorption and pH adjustment at the other pH treatments further research is required.

Suggestions for future work include investigation into the association of DCAAs with other humic materials in leaf leachate. If we are able to confirm that this is happening and to what extent, better inferences can be made about the adsorption potential of DCAAs onto substrates. If we are seeing adsorption happening in the present study re-adjusting the pH of samples from basic to acidic will test whether DCAAs are desorbed or become unassociated with humic materials as pH decreases. It would also be useful to analyse mineral composition of the natural sediment used within this study to confirm whether natural sediment contains kaolinite mineral or whether other minerals in the natural sediment display similar adsorptive properties to kaolinite. Innoculation of a second set of samples with riverine bacteria would also allow comparison between rates of adsorption and microbial uptake in stream.

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5.8 Supporting information

Determining the extent of adsorption of dissolved combined amino acids onto naturally occurring substrates: a potential sink of DON

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Summary of the supplementary information

- Number of pages:3 (including this page)
- Number of figures: 0
- Number of tables: 2

Table. S5. 1 Three letter abbreviations for amino acids described in this study. Note that glutamine and asparagine are largely de-aminated during acid hydrolysis, thus are mainly detected as glutamic and aspartic acid respectively. Hence the use of the abbreviation ASX and GLX.

Amino acid	**Three letter abbreviation**	**Amino acid**	**Three letter abbreviation**
Aspartic acid and asparagine	ASX	Cystine	CYS
Serine	SER	Tyrosine	TYR
Glutamic acid and Glutamine	GLX	Valine	VAL
Glycine	GLY	Methionine	MET
Histidine	HIS	Lysine	LYS
Arginine	ARG	Isoleucine	ILE
Threonine	THR	Leucine	LEU
Alanine	ALA	Phenylalanine	PHE
Proline	PRO	Tryptophan	TRP

Table. S5. 2 Individual amino acid recoveries for bovine insulin standard**.**

Amino acid	Theoretical concentration (pmol/μL)	Average measured concentration (pmol/μL)	Recovery (%)
HIS	0.57	0.24	42.9
ARG	0.29	0.21	73.5
SER	0.86	0.64	74.2
GLY	1.14	1.01	89.3
ASX	0.86	0.67	77.7
GLX	2.00	1.5	75.0
THR	0.29	0.21	69.8
CYS	0.86	0.63	73.3
ALA	0.86	0.67	79.8
PRO	0.29	0.16	56.2
LYS	0.29	0.22	76.6
TYR	1.14	0.58	50.6
VAL	1.43	0.55	38.2
ILE	0.29	0.08	27.6
LEU	1.72	0.90	51.2
PHE	0.86	0.44	50.6

Table S5. 3 Concentration and ±2SE of T0 and T24 stock leachates used to calculate the 'stock leachate control'.

	T0 Stock leachate		T24 Stock leachate	
Amino acid	**T0 Concentration**	**Standard error (±2SE)**	**T24 Concentration**	**Standard error (±2SE)**
HIS	0.344	0.119	0.321	0.079
ARG	0.373	0.174	0.308	0.108
SER	0.262	0.117	0.227	0.072
GLY	0.400	0.170	0.356	0.104
ASX	0.090	0.036	0.079	0.023
GLX	0.387	0.166	0.334	0.111
THR	0.221	0.091	0.195	0.062
CYS	0.015	0.008	0.012	0.004
ALA	0.210	0.094	0.192	0.065
PRO	0.202	0.087	0.185	0.059
LYS	0.007	0.003	0.004	0.001
TYR	0.126	0.049	0.112	0.298
VAL	0.111	0.047	0.100	0.011
ILE	0.054	0.022	0.05	0.016
LEU	0.094	0.040	0.084	0.027
PHE	0.215	0.086	0.187	0.05

Chapter 6 Concluding remarks and prospective directions for future study

6.1 Summary of results

Dissolved organic nitrogen (DON) can constitute up to 94 % of the total dissolved nitrogen pool in rivers however, there is still limited knowledge about the components that comprise DON, its sources and bioavailability. This thesis set out to explore some of these poorly-understood aspects. Throughout this thesis *Eucalyptus camaldulensis* leaves were studied as a source of DON. *E.camalduelnsis* leaves were chosen as they are a common riparian and floodplain species in Australia and form up to 60,000ha of forest in total across the Murray-Darling Basin (Reid & Quinn, 2004). *E. camaldulensis* is the most widely distributed Eucalypt in Australia and is adapted to long-term fluctuations in water availability (Doody *et al.*, 2015). This allows individuals to survive in river-floodplain systems characterised by periods of low base flow in summer followed by periods of episodic flooding during winter-spring (Doody *et al.*, 2015).

Previous studies have shown that when *E. camaldulensis* leaf litter is inundated, between 20 and 30 % of total leaf mass is leached within a few days of submersion (Gessner, Chauvet, & Dobson, 1999; O'Connell *et al.*, 2000). Leaf decomposition studies in Australian aquatic systems have focused on the potential of lateral links between the river and floodplain to mobilise large amounts of dissolved organic matter (DOM) and the rapid release of DOC, but little attention has been paid to DON (Baldwin, 1999; Briggs & Maher, 1983; O'Connell *et al.*, 2000).

This thesis examines the relationship between DON leached from terrestrially aged *E.camaldulensis* leaves, influences upon DON concentration and composition (floodplain connection, agricultural activities, major townships and river confluences) along a longitudinal gradient and potential sinks of DON, including adsorption of DCAAs

to stream substrates and metabolic consumption (shown in Figure 6.1) to gain a better understanding of DON in Australian river systems.

In the first experimental chapter (Chapter Two) a suite of five microbial inhibitors were tested to determine the best inhibitor for time course (24 hour) leaf leaching experiments and whether the chosen inhibitor influences the composition of dissolved combined amino acids (peptides and proteins; DCAAs) released from the leaf during leaching. While many microbial inhibitors are available it is not always possible to cease microbial activity without some undesired analytical effects. Overall, no single inhibitor was entirely ideal for the measurement of all N-containing nutrients and no inhibitor was suitable for the measurement of DON. DCAAs could however, be measured in the presence of both mercuric chloride ($HgCl_2$) and sodium azide (NaN_3). When compared to the control experiment, total recovery of DCAAs were similar in range to NaN_3. NaN_3 was also better able to recover DOC than $HgCl_2$ and was therefore deemed to be the best possible compromise for experiments of this nature.

In chapter three the initial stage of leaf decomposition (leaching) was studied and protein and peptide (dissolved combined amino acid (DCAA)) and dissolved free amino acid (DFAA) contribution to DON leached from *Eucalyptus camaldulensis* leaves over 24 hours was examined. Two leaching experiments were carried out, one with the addition of the chosen microbial inhibitor (sodium azide) and a control experiment with no microbial inhibitor. Fourier-transform infrared (FTIR) microspectroscopy was carried out at the Australian Synchrotron to determine the likely source of DCAA within the leaf tissue. DFAAs were found to be below the detection limit in all leachate samples however, DCAAs were found to be a significant component (38.5 %) of DON leached from leaf tissue in 24 hours. FTIR microspectroscopy showed that over 90 % of leaf protein remained in the leaves after 24 hours. Leaf protein was highly partitioned to the fungal

colonized palisade cells in the leaf mesophyll, with evidence for depletion of this material after leaching. Comparison of leaching kinetics in the presence and absence of a microbial inhibitor suggests that microbial uptake or adsorption commences within the timescales of these leaching experiments.

The aim of chapter four was to gain some understanding of how DON concentrations existed in a real world situation. To this end, the longitudinal trends in DON were explored along a largely unregulated river, namely the Ovens River, Victoria. Changes in DON composition and DCAA proportions were investigated along the length of the river in relation to major confluences, townships, floodplain connectivity and land use activities. The Ovens River has identifiable regions of native vegetation (including *E. camaldulensis*), agricultural activity and floodplain connection. Two longitudinal studies were carried out; one in winter during a period of high flow and one in summer during a period of stable base flow. DON concentrations were found to be higher than dissolved inorganic nitrogen under both base flow and high flow conditions. Under base flow conditions DON exhibited a continuous increase in concentration downstream (ranging from 50 to 300 µg/L), compared to a larger increase under high flow (150 to 600 µg/L). During high flow a major tributary (The King River) contributed a major discrete increase of ~ 350 µg/L DON. DCAA concentrations varied less strongly longitudinally at base flow but increased with distance downstream at high flow. The proportion of DON that was in the form of DCAAs was reasonably uniform during high flow (3 – 6 %) but highly variable at base flow (5 – 44 %) and the amino acid composition of the DCAA varied along the river and differed between flow regime. The exception to this was below the King River confluence where DCAA composition under the two flow conditions converged. This chapter provides evidence that DON provides a variable, but potentially large component (4 – 81 %) of the total dissolved N pool and given that 5 – 23 % is in the

form of DCAA, DON represents an important and potentially bioavailable source of nitrogen.

Finally, the last experimental chapter (Chapter Five) builds upon the possible adsorption process that was observed in chapter three. Two adsorption experiments are carried out using DCAAs sourced from *Eucalyptus camaldulensis* leaf leachate onto different, naturally occurring substrates (kaolinite clay mineral and a natural sediment). Both substrates were capable of adsorbing DCAAs from stock leaf leachate with adsorptive ability being similar at similar pH. Overall, when compared to the average concentration of the stock leachate control, total DCAAs for both kaolinite and natural substrates were adsorbed at a higher extent as the pH rose. Both kaolinite and natural sediment exhibited similar adsorption at ~pH 6 (92 and 94 %).HIS and ARG were strongly and consistently removed from solution in the presence of either substrate across all three pH conditions.

Interestingly, stock leachate samples that were pH adjusted but not exposed to substrates were found to be significantly different from each other in DCAA concentration and composition. This is likley due to high proportions of DCAAs becoming non-hydrolysable through close association with other humic materials in the leaf leachate.

6.2 *Eucalyptus camaldulensis* as a source of dissolved organic nitrogen

As this thesis has demonstrated, DCAAs are a substantial component of DON and a very likely bioavailable portion of the dissolved nitrogen pool in freshwater river systems. To confirm bioavailability of DCAAs it would be useful to conduct further experiments exposing DCAAs to riverine bacteria over the same time scale as the adsorption experiments. This would allow comparison between microbial uptake and adsorption of DCAAs to substrates which may force DCAA-N into dormancy.

For the terrestrially aged leaves used in these experiments over a third (38.5 %) of DON released occurred in the form of protein and peptides (dissolved combined amino acids (DCAAs)). DCAA nitrogen accounted for 57 % of total leaf nitrogen while dissolved inorganic nitrogen (DIN) only accounted for 2 % of total leaf nitrogen (based on the amount of DIN leached). DCAAs are widely thought to be a bioavailable form of DON to bacteria and algae (Stepanauskas, Laudon, & Jørgensen, 2000; Zhang *et al.*, 2015) while the remainder (41 %) is likely associated with other organic- N containing molecules that are either recalcitrant or require further microbial breakdown before becoming available.

Similar to Suter *et al.* (2011) who examined fungal colonisation in *Eucalyptus pauciflora,* staining of *E.camaldulensis* leaf material with lacto-phenol cotton blue (LPCB) also showed extensive colonisation of fungal material within both the mesophyll and midvein sections of the leaf before and after leaching. Fungal cell walls are a polymer of N-acetyl glucosamine, a source of DON and therefore, may constitute a portion of the uncharacterised DON.

DON leached from *E. camaldulensis* leaves has relevance not only in floodplain environments but also in rivers that receive a nutrient pulse during flood. Longitudinal studies worldwide have often focused on river regulation and adjacent land use practices that can influence the type and quality of nutrients reaching the river channel. Predominantly, studies have been carried out on DIN species and the role they play in satisfying nitrogen demand in aquatic systems (Howarth, 1988; Seitzinger, 1988; Vitousek *et al.*, 1979). Some longitudinal studies have investigated DON (Bernal, Butturini, & Sabater, 2005; Kaushal & Lewis, 2005) however, only limited work has looked at DON composition (Stepanauskas, Laudon, & Jørgensen, 2000).

6.3 The influence of adjacent land use practices on dissolved organic nitrogen composition

DCAA was found to vary in composition along the length of the Ovens River between base and high flow studies except below the King river confluence (Reach 4) where the DCAA composition converged. The King river valley is a large agricultural valley and likely, has a substantial impact upon DCAA composition. Reach 4 also receives stormwater runoff from Wangaratta, a major township on the Ovens River and displays extensive *Eucalyptus camaldulensis* floodplain-channel connection where mobilization of organic material occurs during flooding. Flooding occurs most often during winter months and as we do not see a difference between high flow and base flow DCAA composition the similarity in Reach 4 suggests that the King River or the township of Wangaratta exerts a strong influence on water quality.

Many previous studies have examined DIN concentrations in relation to land use (Divers, Elliott, & Bain, 2014; Stanley & Maxted, 2008; Wagner *et al.*, 2008) however, no studies in Australia have examined DCAAs in relation to adjacent land activity.. Seitzinger, Sanders, and Styles (2002) conducted a study in New Jersey, USA on the bioavailability of DON in streams draining natural (forested), agricultural and urbanized land. Overall, the streams that had the highest proportion of available DON forms were those receiving urban/stormwater runoff (59 % ± 11) followed by agricultural pastures (30 % ± 14) and forests (23 % ± 19). As there is extensive floodplain connection and both agricultural and urban runoff in reach 4, it is difficult to be certain which source had more influence on DCAA composition. However, the analytical approach used in this study is a powerful approach to examining nutrients composition in a river system. For this reason, it would be of use to carry out further longitudinal studies and adopt a hypothesis driven approach to examining the impact agricultural areas, tributaries, urban settings and floodplain connections have towards nutrient concentration and composition.

6.4 Adsorption of dissolved combined amino acids; a potential sink of dissolved organic nitrogen

While it is difficult to make inferences about the extent of the adsorption potential of DCAAs in Australian rivers without knowing the mineral composition of substrates, this thesis has demonstrated that under neutral pH conditions, typical of that found in Australian river systems, DCAAs are either adsorbed from solution or associated with other humic materials in the leaf leachate. Previous studies (Benetoli *et al.*, 2007; Wang & Lee, 1993) have shown that DCAAs adsorb readily onto clay minerals with adsorption behaviours changing at different pH levels. Often, desorption of this material is much more difficult, if possible at all, and many mineral substrates act as a sink for potentially bioavailable forms of DON. On the other hand, given particles of sediment also have aggregations of bacterial cells (Bigambo & Mayo, 2005), it could be that nitrogen cycling occurs within this microscopic biofilm. The biochemical aspects of this hypothesis require testing.

Biofilms that grow and colonize on submerged river and floodplain material (including *E. camaldulensis* leaves) also display adsorptive properties (Baldwin, Whitworth, & Hockley, 2014; Flemming, 1995; Scholz & Boon, 1993). Biofilms adsorb proteins and amino acids due to their cell walls, cell membranes and cytoplasm serving as adsorption sites (Flemming, 1995; Späth, Flemming, & Wuertz, 1998). As the leaves used in the study were terrestrially aged it is safe to assume that biofilms were present on the leaf surfaces. While the biofilm in our leaching experiments are presumed to be dead due to leaf desiccation, a study by Armstrong and Bärlocher (1989) shows that adsorption of DCAAs onto biofilm occurs as a result of biofilm surface properties and is not dependant on whether biofilm are living or dead.

This strong affinity for adsorption of DCAAs to both clay mineral and biofilm surfaces may explain some of the observations within this thesis. Firstly, in chapter three

leaching profiles observed for DON, DCAA and DOC in the absence of a microbial inhibitor suggest a re-adsorption of organic material at the after approximately 10 hours. It is possible that this material is being adsorbed back onto the leaf surface or onto biofilm on the leaf surface. Secondly, in chapter four there is no observable effect of floodplain connection on DCAA composition in Reach 4, this may be due to contributions from the King River, urban runoff or floodplain material however, it may also be due in part to adsorption and metabolism. Much like the potential adsorption effect observed in the chapter three leaching profiles, a similar process could be happening on the floodplain. It is possible that DCAAs leached from floodplain vegetation are being re-adsorbed to surfaces (vegetation, biofilm and various clay/substrate minerals) or metabolised soon after they are released. This thesis has shown that while a portion of DCAAs can potentially provide bioavailable organic nitrogen it is unlikely that DCAAs are as important as DIN in satisfying N-demand. Chapter 4 found DCAAs to comprise between 5 and 23% of DON during high flow however, inorganic nitrogen concentrations are almost as high as total DON. Unlike DON all DIN is readily available and as shown in Chapter 5, adsorption to substrates can remove up to 100% of total DCAAs in solution leading to DCAA-N becoming dormant and unavailable.

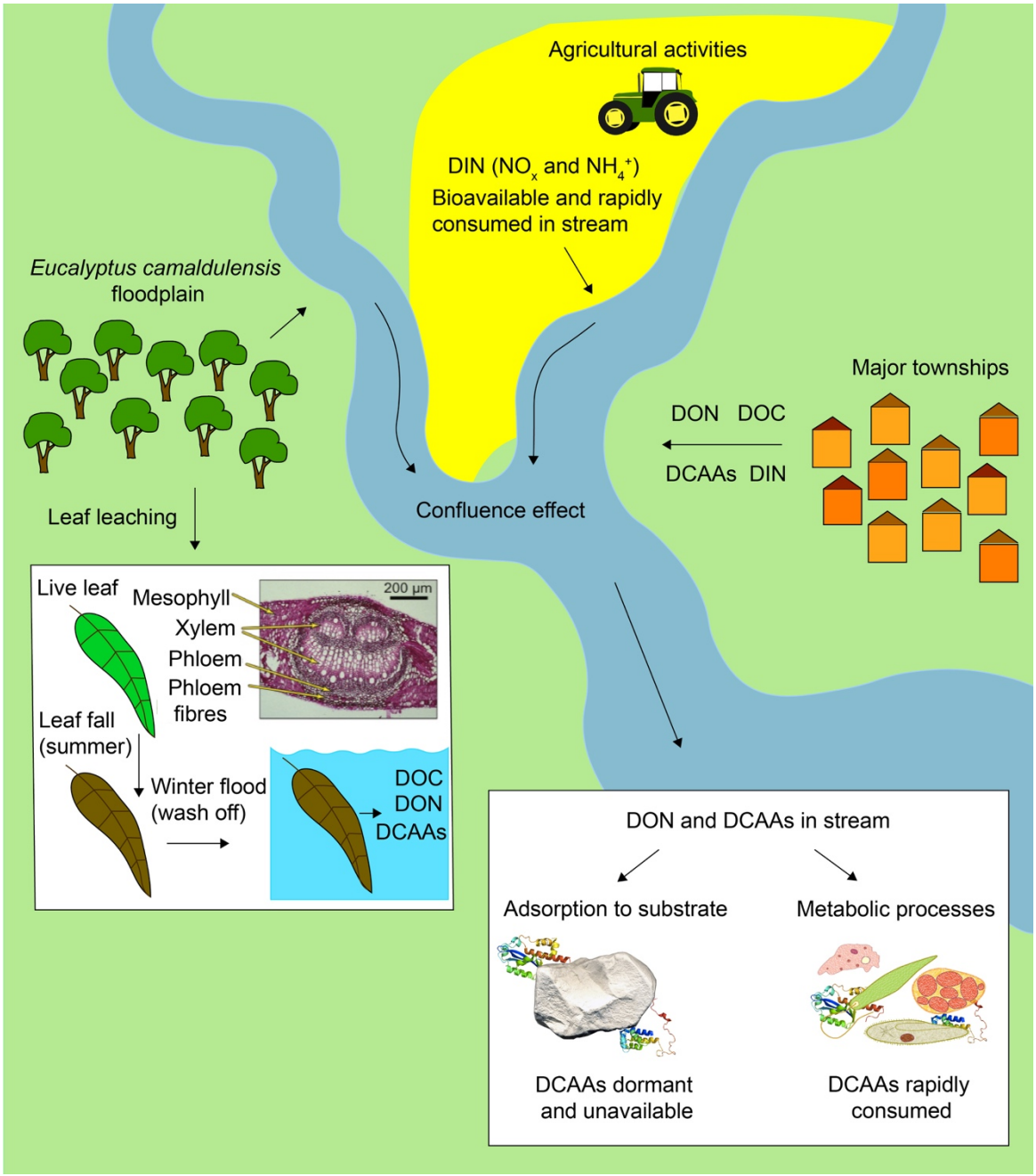

Figure 6.1 Conceptual diagram showing the relationship between dissolved organic nitrogen (DON) sourced from *Eucalyptus camaldulensis* leaves, influences (confluences, major townships and adjacent land use practices) upon DON and in-stream sinks (adsorption and metabolism) within a river system.

6.5 Future perspectives

6.5.1 Understanding the composition and sources of dissolved organic nitrogen

The large number of potential DON sources makes understanding the composition of DON particularly difficult. Sources can vary due to land-use, floodplain interactions, riparian vegetation and several other influencing factors. However, a more in depth understanding of composition could be obtained from detailed speciation of DON along the length of the Ovens River, especially where river-floodplain interactions, deposition of leaf material, major townships and major confluences occur. By characterising the DON pool in detail at these interfaces we could develop a better understanding of the influence of floodplain connection, urban runoff, adjacent land use, riparian vegetation and direct deposition on the composition of the DON pool.

6.5.2 Bioavailability of dissolved organic nitrogen sources

Further work needs to be done to better understand the bioavailability of individual DON sources. While it is generally accepted that DCAAs and DFAAs are readily bioavailable it is less clear to what extent larger biomolecules such as N-acetylglucosamine, lignin and melanin satisfy N-demand in aquatic ecosystems. As this thesis has shown, there is a high rate of fungal colonisation in terrestrially aged *E. camaldulensis* leaves and it would be useful to better understand the role N-acetylglucosamine plays in satisfying N-demand. It would be of benefit to (i) develop a method to analyse the amount of N-acetylglucosamine leached from leaf tissue over 24 hours and (ii) if this material is not released, better understand the time and mechanisms needed to break down fungal cell walls and release DON as an available form. This will help to further characterise the DON pool as well as increase understanding of the role of aquatic fungal material in freshwater ecosystems.

While this thesis identifies various sources of DON, it would also be of benefit to understand which DON sources are preferentially consumed within the aquatic environment. To better understand the bioavailability of various DON components experiments could be run to expose organisms to different DON sources over various time periods. This would provide further insight into how quickly components of DON are consumed and whether DCAAs have a role in satisfying N-demand in aquatic systems.

6.5.3 Fate of dissolved organic nitrogen in stream

While this doctorate examines adsorption potential of DCAAs onto some common substrates much more could be investigated in this area. Within stream and wetland environments there are a large range of substrates that could potentially adsorb different nitrogen containing molecules. Characterising the mineral composition of the substrates as well as the DON speciation at river wetland interfaces would allow inferences to be made about adsorption potential and therefore, loss of available DON at these sites. Adsorption and desorption potential of different DON sources should then be tested in the presence of identified substrates (clay minerals and biofilms) to better understand the fate of DON in stream. It would also be of benefit to better understand the association of DCAAs with other humic materials in leaf leachate. If we are able to understand to what extent association is occuring better inferences can be made about the adsorption potential of DCAAs onto substrates

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