School of Earth and Planetary Science

Functional ecology of calcrete aquifers in arid zone Western Australia

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This thesis is presented for the Degree of

Doctor of Philosophy

Of

Curtin University

January 25th, 2020

DECLARATION

I hereby declare that this thesis is my own work, except where otherwise stated with respect to manuscript contributions, and contains no material published prior to or outside of the PhD enrollment period. I also certify that this thesis provides original and significant contribution to the field of groundwater ecology.

This work has not been submitted in any form for another degree or diploma at any other tertiary institution.

Information derived from the published and unpublished work of others has been acknowledged in the text.

Details on authors' contributions, per each one of the published and submitted manuscripts composing this thesis is detailed after each chapter title.

Signed,

Mattia Saccò

January 25th, 2020

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ABSTRACT

Groundwaters host vital resources - 97% of unfrozen freshwater worldwide - playing a key role in the near future of humanity. Stygofauna (subterranean obligate aquatic invertebrates), together with microbes, are crucial actors in shaping and maintaining the organic matter cycles in these environments, which are characterized by low energy and scarce carbon availability. However, our knowledge about how these ecosystems function is limited, and subterranean environments are increasingly exposed to anthropogenic impacts and climate change-related processes. In order to dig into groundwater ecological dynamics, we investigated arid zone calcrete stygofaunal and microbial patterns linked with contrasting rainfall periods (low rainfall (LR), dry season; high rainfall (HR), wet season), through an interdisciplinary design composed of multivariate analyses, hydrology, isotopic ecology and genetics. Our results from multivariate investigations indicate that stygofaunal niches are closely linked to the hydrodynamic conditions influenced by different rainfall regimes (LR and HR). Isotopic evidence depicted stygofaunal tendencies towards opportunistic and omnivorous habits, typical of an ecologically tolerant community, shaped by bottom-up controls linked with changes in carbon flows. Biochemical data suggest that the inflow of rainfall under HR is responsible for increased nutrient concentrations in the system and dissolved organic carbon pulses. Metabarcoding data confirmed that the HR regime, and its subsequent terrestrial carbon incorporation, triggers a cascade effect driven by microbes (organic matter processors), copepods and amphipods (biofilm grazers), which is finally transferred to the aquatic beetles (top predators). This study provides baseline biochemical and ecological data for stygofaunal interactions in calcretes. Further long-term investigations, incorporating broader ecological perspectives, will help to understand the impacts associated with climate change and anthropogenic pressures on one of the most threatened and underrated ecosystems in the world.

ACKNOWLEDGEMENTS

Curtin University sits on the traditional land of the Whadjuk Noongar people, and I would like to acknowledge their continuing connection to the land, waters and culture of their country. I offer my respects to their Elders, past, present and emerging.

This thesis is the result of an unforgettable journey that could not have been possible without the support of many people.

First, I would like to thank my main supervisor Alison Blyth for 'bringing' me down under and giving me a unique opportunity to grow both personally and professionally. Thank you, Alison, for putting up with my loudness and messiness. Your guidance played a crucial role in achieving this professional milestone in my life. I would also like to thank my other supervisor Bill Humphreys. Thank you, Bill, for your constant willingness to give me your priceless help and scientific wisdom. My other two supervisors Bill Bateman and Kliti Grice also greatly contributed to my research with support and advice.

I gratefully acknowledge the Australian Research Council and the Australian Institute of Nuclear Science and Engineering for supporting this research. Special thanks go all the coauthors of the published (and submitted) manuscripts and all the colleagues, technicians, professors, consultants that contributed to this research. You all made it possible that this interdisciplinary study came to reality.

I would like to thank my friends spread all around the world for making me feel at home wherever I travelled and lived. You have been a crucial pillar during the last four years. Foremost, I specially thank my Italian and Australian families for their love. This thesis is dedicated to you. Thank you for always being there, for encouraging me daily and for bearing with me during hard times.

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Thesis overview

Chapter 1 provides an introductory analysis on the research background and overall significance of the thesis. First, a review on the ecological mechanisms - including spatial, temporal and functional dynamics - shaping groundwater ecosystems is presented. Second, methodological and analytical challenges in groundwater functional studies are outlined. Finally, research gaps are pinpointed and overarching aims of the thesis are depicted.

Chapter 2 presents a literature review focused on the current state of the art in groundwater ecology, combined with an integrative overview on analytical designs able to bring new light to subterranean environmental studies. The first section of this chapter gives an overview of stygofaunal studies in groundwater environments considering three main topics: stygofaunal diversity, ecotoxicology and biochemical pathways. Next, knowledge gaps are identified, and a multidisciplinary design to widen perspectives in the field of groundwater functional ecology is outlined. This chapter was published in the scientific journal *Science of the Total Environment* in January 2019.

In **Chapter 3**, this research looks at the calcrete ecological niche dynamics of stygofauna under two contrasting rainfall periods corresponding to the dry and wet seasons. Here, I elucidate the linkages between rainfall events and subterranean community assemblages. Environmental gradients and community patterns are analysed through conventional multivariate statistical tools, allowing investigation of seasonal shifts in niche occupations both at community and species levels. Insights into hydrogeochemical patterns and associated biotic adaptations are also presented and discussed in detail. This study provides baseline information - filling some of the preliminary research gaps that emerged in chapter 2 - for the understanding of the ecological mechanisms regulating subterranean aquatic invertebrates in calcretes. This chapter was published in the scientific journal *Ecohydrology* in September 2019.

Chapter 4 explores food web interactions of the stygofaunal community at the calcrete through some of the isotopic techniques outlined in the chapter 2. This chapter involves the deciphering of stygofaunal trophic dynamics under contrasting rainfall regimes. Here, the information gathered in the chapter 3 is expanded by the application of a combination of conventional (bulk tissue stable isotope analysis) and novel (compound specific stable

isotope analysis) isotopic analyses to unravel energy flows, trophic level characterizations and dietary proportions within stygofauna. Analysis of shifts in organic matter incorporations and feeding preferences related to the different rainfall periods is then coupled with abundance data to infer broad ecological functioning of the system. This chapter was published in the scientific journal *PLOS ONE* in October 2019.

Chapter 5 and 6 focus on the study of the biogeochemical flows from a 'bottom-up' perspective and bring to practice the multidisciplinary approach advanced in the chapter 2. These chapters elucidate the biogeochemical functioning of the ecosystem by tracking the path of carbon across the biotic communities of the calcrete. The 'top-down' ecological insights provided by the findings of chapter 3 are complemented with further analysis of organic flows in the calcrete. Chapter 5 centers on the analysis of carbon inputs with an interdisciplinary design combining carbon isotope investigations (carbon stable isotopes and radiocarbon analyses) and fluorescence tests in water, and metabarcoding analyses on the microbial community from the calcrete. Insights into the linkages between rainfall-driven carbon inputs and metabolic responses from the microbial community are provided. Chapter 6 expands this research approach to the stygofaunal community to unravel a complex web of interactions between primary and secondary consumers, and predators. Overall, both studies provide novel untangling of the ecological mechanisms sustaining biota in arid zone subterranean environments, and final diagrams depicting the main biogeochemical pathways are presented at the end of the sections.

Chapter 7 presents the main conclusions of the thesis and, finally, **Chapter 8** provides final reflections on the work, suggests future research avenues and concludes the thesis with a poetic license from the author.

Chapter 1| Background and significance

1.1 Ecological dynamics in groundwaters

1.1.1 *Groundwater habitats*

Groundwater constitutes the largest reservoir of liquid freshwater in the world (Griebler et al., 2009). Within the continental earth, 97% of all unfrozen freshwater is subsurface, whereas lakes and rivers represent less than 2% (Gibert et al., 1994). Groundwater provides a life-sustaining source that plays a central role in many aspects: it supplies water to billions of people, plays a central part in irrigated agriculture and influences the health of many ecosystems (Gleeson et al., 2012). However, both the growing demand, the declining availability and quality are generating threatened systems (Millennium Ecosystem Assessment, 2005). In contrast to their value, subsurface environments are one of the least explored on earth (Griebler et al., 2014).

From a purely ecological perspective, groundwaters are considered as 'extreme habitats' (Danielopol et al., 2000). The intrinsic aphotic nature of subterranean habitats triggers a substantial lack of primary production responsible for truncated trophic webs characterized by the dominance of primary and secondary consumers (Hancock et al., 2005). As a result, groundwaters are typically low in sources of energy (Huppop, 2000), and biota principally rely on energy from allochthonous organic matter which is transported to groundwater habitats by water flow, percolation, gravity or animals, in the form of coarse and fine particles (Deharveng & Bedos, 2000).

Groundwaters contain vertical stratifications which are unique to each aquifer (Ingebritsen & Sanford, 1999) and highly dependent on local hydrogeological patterns (Kresic, 2006). However, the environmental conditions within different zones can be very stable, especially if compared to their surface counterparts (Humphreys, 2009). The border areas between surface, unsaturated zones, aquatic sediments, and the saturated sub-superficial environments harbour a great biodiversity, responsible for the transfer of nutrients, particles, organisms and energy between zones (Danielopol & Griebler, 2008; Gibert et al., 2009). Chemoautotrophic production has also been found to regulate biological diversity in

aquifers (e.g. Hutchins et al., 2016) and caves (e.g. Engel et al., 2007). As a result, a composite range of chemical and physical factors play a key role in shaping the biotic communities (microbes, stygofauna and troglofauna) from both pristine (e.g. Culver & Sket, 2000; Humphreys, 2001) and contaminated (e.g. Hartland et al., 2011; Korbel et al., 2014) aquifers. Understanding the inter-relationship of these dynamics and their interactions with groundwater biota is essential to better understand these ecosystems.

1.1.2 *Temporal and spatial patterns in groundwater ecosystems*

Precipitation patterns play a key role in shaping essential inputs of nutrients and organic matter to groundwater ecosystems (Reiss et al., 2019). Rainfall-driven recharge processes have been found to trigger changes in community assemblages that reflect the response to newly available resources such as increased dissolved oxygen levels (Dole-Olivier et al., 2009) and dissolved organic matter (DOM) (Datry et al., 2005; Brankovits et al., 2017). Microbially-processed DOM is then transferred to higher trophic levels of subterranean biota by higher primary consumers (Simon et al., 2003; Hancock et al., 2005). Overall, these ecological dynamics shape fundamental energy flows sustaining biota in groundwater environments (Foulquier et al., 2011; Stegen et al., 2016).

Distributions of subterranean communities usually have low temporal variability (Culver, 1982), a commonly accepted assumption in groundwater ecology (i.e. Crouau-Roy et al., 1992: Poulson et al., 1995; Lunghi et al., 2017). In a long-term study on terrestrial cave communities, Di Russo et al. (1997) found that the species composition of resident cave invertebrate community was stable over time, and similarly, Farnleitner et al. (2005) reported little to no temporal variability in microbial structure from karst springs, suggesting stable ecological patterns across seasons. In line with these findings, Venarsky et al. (2012) found no effect of seasonality on the macroinvertebrate community structure and litter breakdown rates from cave streams.

However, the vast majority of current investigations focus on changes in community assemblages rather than shifts in ecological function, such as food web interactions. Moreover, the few functional studies available present short-term approaches (i.e. Francois et al., 2015; Hartland et al., 2011; Hutchins & Schwartz, 2013). Incorporation of medium to

long-term plans (e.g. Olivier et al., 2019) is needed to allow monitoring of how groundwater food webs change over time and to model whole system ecosystem functioning.

The direct influence of spatial factors on subterranean food web dynamics is largely unexplored. Investigations focusing on changes in diversity patterns suggest that spatial trends are linked to shifts in food web assemblages (e.g. Hahn & Fuchs, 2009; Tobin et al., 2013). Local geological features such as porosity and permeability influence ecohydrological dynamics, faunal movement through aquifers, and affect evolutionary and biodiversity patterns in groundwaters (e.g. Dole-Olivier et al., 2009), but there is only limited understanding of their relationship to functional ecology. Malard et al. (2009) reported that European porous aquifers host more diverse stygofaunal communities when compared to karst systems, suggesting that greater hydrological connectivity might trigger higher colonisation rates by competitively superior species. Voids and interstices also provide potential refugia for the subterranean communities, easing dispersion of key consumers (i.e. copepods and amphipods, Hutchins et al., 2016; Hyde et al., 2018), triggering differential ecological niche occupations (Kozel et al., 2019) and driving changes in food web assemblages (Hose et al., 2017).

Among surficial spatial drivers influencing groundwater biotic communities, vegetation, land cover and land use are thought to play a key role in shaping the variability of community assemblages in groundwaters (e.g. Baker et al., 2000; Boulton et al., 2008; Español et al., 2017). Site-specific organic pollution episodes have also been found to trigger shifts of groundwater functional community assemblages in several subterranean ecosystems (Venarsky et al., 2014 and references therein).

Given the subterranean lightless environment limits autochthonous carbon production (Humphreys, 2006), the incorporation of nutrients and organic matter (e.g. surface plants, carbon from the surface sediment, organic matter stored in the hyporheic zone) is frequently dependent on the characteristics of the hyporheic and surface frameworks (Aldous et al., 2014). As a result, subterranean invertebrate trophic web dynamics are influenced by the biogeochemical interactions between surface and subsurface environments at a site-specific level (Foulquier et al., 2011). Concurrently, top-down effects exerted from spatially subsidised consumers can affect availability of *in situ* resources and occasionally trigger trophic cascades (Polis et al., 1993).

At a larger scale, the categorisation of groundwater ecoregions ('stygoregions', as defined by Stein et al., 2012) indicates that region-specific patterns can drive different functional community structures. Nonetheless, to date the role played by space in shaping groundwater food web interactions follows a rather descriptive approach, with very few studies focusing on the functional changes at a local scale (e.g. Simon et al., 2003; Brankovitz et al., 2016; Francois et al., 2016;). Improved understanding of the effect of spatial dynamics over time on subterranean food web interactions will provide vital guidance for management and conservation plans in groundwaters (Maurice & Bloomfield, 2012).

1.1.3 *Subterranean biotic communities*

The discovery of the first subterranean species, the salamander *Proteus anguinus* (Laurenti, 1768), dates to the late 18th century in the Dinaric Alps of Slovenia and northeast Italy. Almost three centuries after, technological advances have allowed remote aquatic sampling processes at cave outflows as well as in springs and wells, leading to a rapidly expanding groundwater taxonomy (Delamare Deboutteville, 1960), increasing the importance of ecology to the wider field of groundwater research.

Subterranean fauna are commonly referred to as blind and transparent. All categories are recognised by their regressive attributes, especially a lack of eyes and pigment plus a range of morphological traits that may include vermiform shape, elongate appendages and elaborate extra-optic sensory structures (Schmidt & Hahn, 2012) (Figure 1.1).

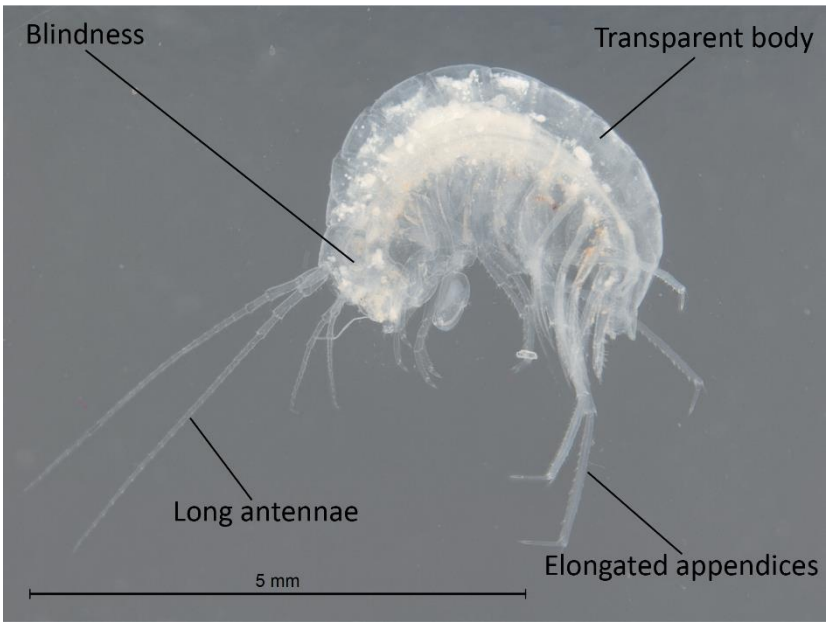

Figure 1.1. Groundwater morphological traits highlighted on a specimen of *Scutachiltonia Axfordi* (King 2012).

Groundwater fauna can be broadly separated into two categories - stygofauna and troglofauna - according to their life cycles and ecological niche occupation. Stygofaunal specimens - i.e. beetles, amphipods, copepods, etc.- belong to obligate aquatic species inhabiting interstices and voids in within the groundwater matrix. Troglofauna - i.e. snails, spiders and scorpions - live in caves, cavities and fractured rock between the water table and the superficial soil layer. Both groups play a key role in regulating the nutrient cycles and energy flows shaping groundwater ecosystems (e.g. Simon et al., 2003; Vernarsky et al., 2014). However, occurrence of subterranean species was recognised well before their ecological importance. Subterranean systems are now accepted as comparable in complexity to surface ecosystems (Rouch, 1977; Gibert et al., 1994), but globally it is only in the last decades that the magnitude of biodiversity present in subterranean waters has been accepted and given prominence (e.g. Rouch & Danielopol, 1997; Sket, 1999; Culver & Sket, 2000; Danielopol et al., 2000; Wilkens et al., 2000).

Resources in groundwater habitats are patchily distributed and often scarce (Poulson, 2012). Subterranean species show a range of evolutionary adaptations to these oligotrophic environments such as low metabolic rates and generalist feeding habits (Gibert & Deharveng, 2002). Concurrently, high resistance to starvation (Hervant & Renault, 2002) and omnivorous strategies (Huppop, 2000) allow maximum use of varied and unpredictable resources (Levinton, 1972).

As mentioned earlier, subterranean communities are considered as basally truncated by the lack in situ photosynthesizers (Hancock et al., 2005), and this functional deficiency is a vital co-driver in shaping less diverse biotic assemblages when compared to their surface counterparts (Boulton et al., 2008). Simultaneously, the scarcity of predators at the top of the food web triggers redundancies in feeding strategies linked with frequent overlaps between niche occupations of consumers and predators (e.g. Mammola et al., 2016). As a result, the predominant paradigm of groundwater trophic ecology studies refers to short and simplified food chains (e.g. Culver, 1994; Tobler, 2008).

However, this assumption has been rarely empirically tested, mainly due to several methodological challenges (e.g. Hervant et al., 1999; Korbel et al., 2017), and recent studies based on novel analytical approaches advocate for a substantial review of this classic archetype. Francois et al. (2016) described high degrees of trophic specialization on sedimentary biofilms in two species of detritivorous cave isopods. In contrast with the idea of high degrees of trophic plasticity within low-productivity cave environments, the authors suggest that evolutionary forces trigger targeted feeding preferences on the most abundant resources available. Hutchins et al. (2014) provided evidence of horizontal stygobiotic trophic diversity and overall increased food chain length, fostered by chemolithoautotrophic processes in a karst aquifer. Bradford et al. (2014) reported species-specific trophic behaviours among three top predator subterranean diving beetles of an arid zone calcrete. However, limited evidence of trophic niche partitioning was provided, with beetles observed to be both scavengers and predators.

Overall, these results suggest that there are composite pathways for the incorporation and transfer of organic matter in groundwaters. Besides the tendency towards simple trophic assemblages, subterranean trophic interactions seem to be shaped by a combination of opportunistic behaviours and evolutionary driving forces (Venarsky et al., 2014). Investigation is required into the role of different factors in regulating groundwater energy flows, food web interactions, and the connection to the maintenance of groundwater assemblages.

1.2 Methodological challenges and novel approaches

Trophic ecology research in groundwater systems faces obstacles in terms of the cryptic nature and lack of accessibility of subterranean environments. Our current knowledge of subterranean food web interactions is a composite of conventional studies using morphological trait-based analyses (e.g. Fryer, 1964; Schram & Lewis, 1989), gut content studies (e.g. Humphreys & Feinberg, 1995; Weitowitz et al., 2019) and isotopic/genetic approaches (Simon et al., 2003; Bradford et al., 2014). The former technique makes use of the morpho-behavioural concept of functional feeding groups (FFGs) (Cummins & Klug, 1979), an approach widely employed in surface freshwater ecology studies (e.g. Brodersen et al., 1998; Tomanova et al., 2006). However, this categorization in groundwater investigations is frequently challenging due to the plastic and opportunistic trophic habits that stygofauna experience (Stoch, 1995). Gut content analyses, while useful for preliminary investigations on trophic habits, are based on a qualitative approach, providing a rather descriptive and simplified interpretation of the groundwater energy flows and food web interactions.

Regarding isotopic and genetic designs, recent advances in investigations from other research fields such as marine biology (Close, 2019 and references therein), archaeology (e.g. Jarman et al., 2017; Jaouen et al., 2019;) freshwater (e.g. Liew et al., 2019; Holtvoeth et al., 2019) and terrestrial (e.g. Gómez et al., 2018; McMahon et al., 2015) ecology are creating new perspectives into the study of food web interactions, and this thesis argues for and demonstrates the transfer of these to subterranean environments. During the last 50 years, investigations of trophic dynamics from a functional perspective have mainly focused on the use of isotopes (Boecklen et al., 2011 and references therein). To date, the limited set of isotope ecology studies in groundwaters focuses on bulk carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope analysis (SIA). However, these proxies are complicated by biochemical signal mixing from different compound groups (i.e. amino acids, lipids) and fractionation pathways, which can distort interpretations of dietary proportions (Steffan et al., 2013). Application of $\delta^{13}C$ and $\delta^{15}N$ compound specific stable isotope analysis (CSIA) of amino acids can help to refine trophic analyses and overcome some of the above issues (Ishikawa, 2018), but has not previously been applied in groundwater ecosystems.

$\delta^{13}C$ CSIA pinpoints potential carbon sources down to the bottom of the trophic chain (Larsen et al., 2013). $\delta^{15}N$ CSIA distinguishes between compounds reflecting the source isotopic signal, and that enriched with each trophic step, thus providing crucial information on prey-predator interactions (Chikaraishi et al., 2007; Takano et al., 2017). A combination of both techniques in a dual CSIA ($\delta^{13}C$ and $\delta^{15}N$ CSIA) analysis provides a powerful tool to unravel energy fluxes and trophic relationships within food webs (Pomerlau et al., 2017; Thorp & Bowes, 2017; Pollierer et al., 2019). However, issues can arise with respect to sample size, and the cost of the technique, with rates quoted between $150 and $3000 AUD per sample (Blyth pers. comm).

Additional to stable isotopes, radiocarbon data (^{14}C) can provide useful information on carbon flows and organic matter incorporation within biotic communities (e.g. Briones et al., 2005; Fernandes et al., 2013; Keaveney et al., 2015). While now employed in freshwater ecology (Larsen et al., 2018 and references therein), and used in groundwater to characterize the age of water bodies (Cartwright et al., 2020), the implementation of this proxy is almost unexplored in groundwater ecology. Given the low energy balances characterising groundwater environments, ^{14}C has the potential to form a key tracer in untangling carbon incorporation and trophic pathways, allowing vital improvement of the understanding of biogeochemical forces sustaining groundwater biota (McMahon et al., 2019).

Integration of geochemical approaches with semi-quantitative genetic techniques would provide further refining of groundwater food web dynamics. Highly reliable molecular techniques such as DNA metabarcoding on faeces (e.g. Guillerault et al., 2017), gut content (e.g. Krehenwinkel et al., 2017) or whole organisms (e.g. Deagle et al., 2017) have the potential to bring light to processes like cannibalism or trophic niche partitioning, which are otherwise hard to detect in groundwater through just one method. Nonetheless, the lack of a robust datasets, together with the usually low metabolic rates that stygofauna and troglofauna experience (Malard & Hervant, 1999), provide major challenges to the field.

1.3 Research gaps and thesis question

1.3.1 *Research gaps in groundwater ecology*

Subterranean ecosystems are vital components of the global water cycle and play a key role in the maintenance of the ecological balance between aquatic and terrestrial environments (Griebler et al., 2014). However, groundwaters are increasingly subjected to (over)exploitation and anthropogenic contamination, triggering high levels of stress and affecting both groundwater abundance and quality (Richey et al., 2015). Increased rates of habitat fragmentation and desertification (e.g. Mace et al., 2018; Mantyka-Pringle et al., 2015; Huang et al., 2016) coupled with global modelling predictions of sea level rise (Neumann et al., 2015), snow cover contraction (Mudryk et al., 2017) and more frequent heat waves coupled with cyclonic precipitations even in temperate climate regimes (Mann et al., 2017), represent serious threats to the future of biota worldwide (Ripple et al., 2017). In groundwaters, artificial warming has found to be linked to increased subterranean biodiversity loss rates (Brielmann et al., 2009), and increased rainfall variability is likely to affect recharge patterns (Rosenberg al., 1999).

Despite the environmental importance of subterranean ecosystems and the advances that the discipline has experienced during the last 20 years, the field groundwater trophic ecology is still in its infancy. As a result, as underlined in the previous sections, a number of research gaps emerge. For instance, substantial lack of knowledge still exists on the role played by seasonality shaping stygofaunal trophic interactions. In fact, while a number of studies have focused on the geochemical mechanisms shaping the input of nutrients and subterranean habitats (i.e. Bryan et al., 2016), our knowledge on the linkage between the abiotic framework and the biotic community (microbes and styfofauna/troglofauna) is still sparse. Moreover, the majority of the limited studies available in the literature focus on shifts in community assemblages and do not explore functional patterns. To unravel the convoluted dynamics shaping groundwater environments, interdisciplinary approaches are required to link together usually disconnected disciplines such as isotope ecology, genetics and hydrology, amongst others. This multi-proxy approach has the potential to lead a vital transition from purely descriptive to functionally-based investigations in groundwaters, providing wider, and urgently needed, perspectives to the field.

1.3.2 *Groundwater ecology in Australia*

Groundwater ecology studies in Australia gained prominence after the discovery of a vast number of highly diverse species during the early 90's (Guzik et al., 2011). Almost 30 years later, we now know that Australia hosts a plethora of subsurface habitats and associated faunas both in karst and non-karst environments (Eberhar & Humphreys, 2003). These studies, in parallel with the research carried out in Europe and the US, highlighted the importance of stygofauna in maintaining the energy flows and environmental conditions in groundwaters (Boulton et al., 2008; Neville et al., 2010). To date, several bioecological and ecotoxicological investigations have been carried out across the Australian continent (e.g. Humphreys, 2008; Korbel & Hose, 2011; Bradford et al., 2014), with the western half being the focus of the majority of them due to its high taxonomic diversity (e.g. Humphreys, 2001; Hancock & Boulton, 2008). Specifically, the Western Australian regions of the Yilgarn and the Pilbara have some of the highest subterranean faunal diversity worldwide, with research aided by the environmental impact requirements of mining explorations in the area (Eberhard et al., 2005; Hyde et al., 2018)

However, over 80% of the Western Australian groundwater fauna remain undescribed, a figure that is not surprising considering the size of the region, and the extent of suitable habitats (Guzik et al., 2011). Considering the high level of endemism in subterranean ecosystems and recent advances in molecular techniques such as eDNA or DNA metabarcoding (i.e. White et al., 2020), the number of described taxa is likely to increase in the near future.

In Australia, high rates of biodiversity loss and habitat fragmentation are predicted through global warming due to the strong influence of El Nino- Southern Oscillation (ENSO) and Indian Ocean Dipole (IOD) (Horwitz et al., 2008). These impacts, once linked with increased anthropogenic pressures such as over-extraction and contamination, put at risk the preservation of the delicate ecological balance sustaining groundwater biota (Spangler & Hahn, 2018). As a result, the understanding of the ecological dynamics sustaining Australian subterranean ecosystems is not only imperative for obvious conservational reasons, but can also provide crucial guidelines for a more sustainable management of one of the biggest diversity hotspots worldwide.

1.3.3 *Thesis question: Functional ecology of calcrete aquifers in arid zone Western Australia*

The Sturt Meadows calcrete in Western Australia (located in the Yilgarn region) is one of the most studied subterranean ecosystems in the world, with 15 years of genetic investigations (e.g. Cooper et al., 2008; Guzik et al., 2009; Bradford et al., 2010; Bradford et al., 2014). This makes it an ideal site for research addressing some of the knowledge gaps identified above.

This thesis addresses the question of how biogeochemical factors influence the ecological functioning of groundwater ecosystems in a shallow calcrete environment. In doing so it also provides proof of concept data for the implementation of interdisciplinary research designs integrating geology, hydrology, conventional ecology, isotopic ecology and genetics within groundwater environments and including the application of the novel techniques highlighted in the section 1.2.

The thesis has five overarching aims:

I. refine groundwater functional ecology studies through the application of novel techniques widely employed in other research fields
II. understand the role played by rainfall conditions in shaping ecological niche occupations amongst the stygofaunal community from an arid zone aquifer
III. elucidate calcrete energy flows and stygofaunal food web interactions under contrasting rainfall periods
IV. unravel the biogeochemical mechanisms shaping changes in local organic source inputs and microbial metabolic shifts
V. investigate the rainfall-driven ecological dynamics characterizing potential trophic cascades across the subterranean biota of the calcrete

The significance of aim I is the urgent need to integrate new techniques in the field of groundwater ecology to better understand not just the composition, but the functioning of these ecosystems, a crucial step in understanding the environmental, social and economic role played by subterranean ecosystems. Aim II relates to the need for comprehension of the hydrological mechanisms shaping subterranean biotic communities. The baseline information generated will be useful for ecological modelling and the understanding of the impact of global change on aquifer recharge processes. Aims III, IV and V expand the extent of the previous objective by incorporating functional aspects, a keystone step to elucidating the ecosystem functioning of the Sturt Meadows calcrete. Overall, this study provides a thorough ecological investigation on the biogeochemical flows regulating subterranean

biota from a Western Australian aquifer. Insights into stygofaunal seasonal shifts, food web interactions, microbial dynamics and nutrient cycles are gained by the application of a multidisciplinary research design. This knowledge will then be able to shape future investigations in a wider range of groundwater and surface water environments.

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Chapter 2 | New light in the dark - a proposed multidisciplinary framework for studying functional ecology of groundwater fauna

Published as a review in *Science of the Total Environment* 662, 963–977 (2019).

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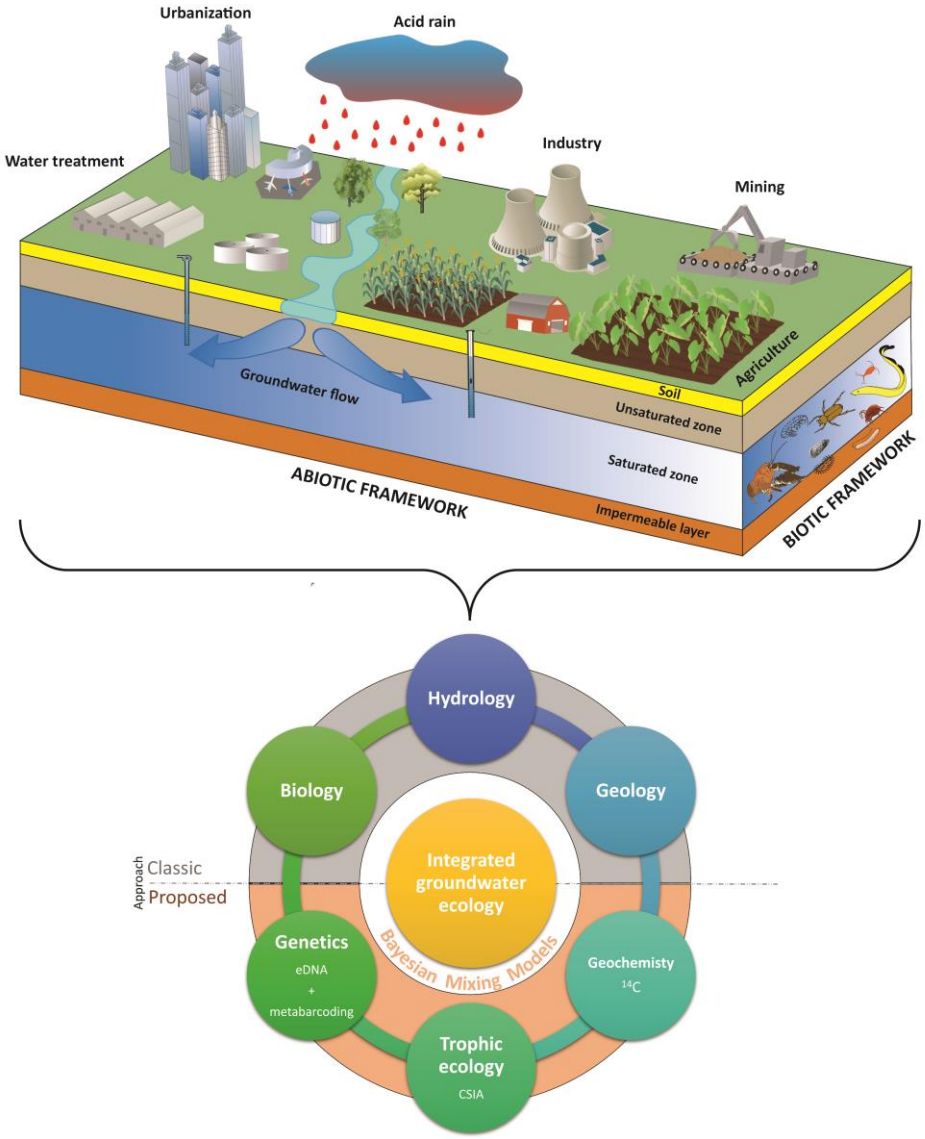
Abstract

Groundwaters provide the vast majority of unfrozen freshwater resources on the planet, but our knowledge of subsurface ecosystems is surprisingly limited. Stygofauna provide vital ecosystem services, but we know little about how their ecosystems function. The cryptic nature of groundwaters, local endemism and site-specific adaptations, represent major obstacles for the field. To overcome these challenges, requires a holistic design drawing on classical ecology, taxonomy, molecular ecology and geochemistry. This study presents an approach based on the integration of existing concepts in groundwater ecology with three more novel scientific techniques: compound specific stable isotope analysis (CSIA) of amino acids, radiocarbon analysis (^{14}C) and DNA analyses of environmental samples, stygofauna and gut contents. The combination of these techniques allows elucidation of ecosystem functions that are often obscured in small invertebrates and cryptic systems. $\delta^{13}C$ and $\delta^{15}N$ CSIA provides a linkage between biogeochemical patterns and ecological dynamics. It allows the identification of stygofaunal food web structures and energy flows based on the metabolic pathway of specific amino groups. Concurrently, ^{14}C provides complementary data on the carbon recycling and incorporation within the stygobiotic trophic webs. Changes in groundwater environmental conditions, and subsequent community adaptations, can be pinpointed *via* the measurement of the radiocarbon fingerprint of water, sediment and specimens. DNA analyses are a rapidly expanding approach in ecology. eDNA is a biomonitoring tool, while metabarcoding of individuals and/or gut contents provides insight into diet regimes. In all cases, the application of the approaches in combination provides more powerful data than any one alone. By combining these quantitative and qualitative approaches *via* Bayesian Mixing Models, linkages can be made between community composition, energy and nutrient sources in the system, and trophic function. This suggested multidisciplinary design will contribute to a more thorough comprehension of the biogeochemical and ecological patterns within these undervalued but essential ecosystems.

Key-words: groundwater, stygofauna, trophic ecology, CSIA, ^{14}C, eDNA, DNA metabarcoding, Bayesian mixing models.

Graphical abstract



2.1 Introduction

Groundwater – water stored underground in the fissures and spaces in soil, sediment and rocks - constitutes 97% of all unfrozen freshwater (Danielopol *et al.,* 2003; Gibert, Danielopol & Stanford, 1994). This subsurface freshwater contributes to the base flow of rivers and provides water to ecosystems, communities, and crops (Gleeson *et al.,* 2012) and supports a wide variety of groundwater dependent ecosystems (Hancock, Hunt, & Boulton, 2009; Mackay, 2006; Merz, Evans & Clifton, 2001). In themselves, groundwaters form unique ecosystems (Gibert *et al.,* 1994b) varying from small, sometimes isolated environments (e.g. a water body in a cave) to extensive, more connected aquifers (e.g. alluvial or karstic systems) (Danielopol *et al*., 2003; Gibert, Danielopol & Stanford, 1994; Gibert & Deharveng, 2002). Many subterranean ecosystems are connected hydrologically as well as to surface waters through springs, parafluvial and hyporheic systems, and therefore can be conceived as a continuum of ecosystems (Freckman *et al.,* 1997).

Several studies (e.g. Brown *et al*., 2011; Eamus & Froend, 2006; Griebler & Avramov, 2014; Kløve *et al.,* 2011) have emphasised the value of ecosystem services provided by groundwater environments. However, it is only recently that the socio-environmental values of these ecosystems have been recognised by countries around the world (Maurice & Bloomfield, 2012). In the USA, law (US Fish and Wildlife Service, 2002) protects some endangered groundwater species. Released in December 2006, the European Union Groundwater Directive states the importance of protective measures for groundwater ecosystems and notes the urgent need for further research (EU-GWD, 2006). The Western Australian Environmental Protection Authority (EPA, 2003) includes limited obligations to investigate the distribution of subterranean species for environmental assessment in case of any subterranean exploitation.

Following these legal frameworks, several research groups have developed integrative approaches for groundwater ecological assessments (Griebler *et al.,* 2010; Griebler *et al.,* 2018; Hahn, 2006; Korbel & Hose, 2017; Stein *et al.,* 2010; Steube, Richter & Griebler, 2009). Stein *et al.* (2012) developed the concept of stygoregions - a biogeographical classification based on stygofaunal pattern distributions for Central Europe - and their study represents the first wide-scale integrative approach for groundwater studies. Cornu, Eme and Malard (2013) followed with a groundwater habitat map of Europe. Heading in the

same direction, a first classification system of groundwater habitats for the UK was recently introduced by Weitowitz *et al.* (2017) .

In spite of the growing awareness of the complexity of groundwater ecosystems, increasing demands for water extraction and the concomitant declining availability and quality continue to generate threats (Millenium Ecosystem Assessment, 2005). Aquifer overexploitation through intense urbanisation and industrial/agricultural developments results in ground water level decline, contamination, sea water intrusion, and subsidence (e.g. Gleeson *et al.,* 2012; Wada *et al.,* 2010); issues that worldwide are leading to the loss of entire aquatic ecosystems, both surface and subsurface (Griebler, Avramov & Hose, 2018; Zektser & Lorne, 2004). Wada and Heinrich (2013) reported that 8% of transboundary aquifers - TBAs; defined as "an aquifer or aquifer system, parts of which are situated in different States" (Article 2c in Stephan, 2009) - globally were under stress, and many TBAs over Africa, Asia and Europe have been under an increasing stress rate for fifty years. Recently, Richey *et al.* (2015) employed remote sensing observations from NASA's Gravity Reco and Climate Experiment (GRACE) to provide the first global observation-based quantification of groundwater resilience and buffer capacity. They concluded that of the world's 37 largest aquifers, 21 had exceeded their sustainable tipping point (aquifer's recharge *via* natural processes like rainfall, snowmelt, etc.), with 13 of them seriously depleted due to little to no recharge (Richey *et al.,* 2015). Many studies at regional and local scales also show declining water tables (Amos *et al.,* 2014; Castle *et al.,* 2014; Love *et al.,* 1993; Scanlon *et al.,* 2012; Shah, 2007; Van der Gun & Lipponen, 2010), indicating that groundwaters are amongst the most vulnerable ecosystems in the world.

During the last decade, groundwater environmental assessment has become crucial in elucidating the ecological quality of subsurface environments, and the development of management plans (e.g. Griebler *et al.,* 2010; Hancock, Boulton & Humphreys, 2005; Korbel & Hose, 2017; Stein *et al.,* 2010; Steube, Richter & Griebler, 2009). Stygobiotic fauna, or stygofauna, inhabiting the interstices and voids in which groundwater is found, are reliable bioindicators for detecting ecological patterns and hydrogeological processes (Dumas, Bou & Gibert, 2001; Malard, Plenet & Gibert, 1996; Maurice & Bloomfield, 2012; Schmidt *et al.,* 2007; Stein *et al.,* 2010). Due to their sensitivity to environmental changes, this biocenosis is the object of many studies (Galassi *et al.,* 2014; Galassi *et al.,* 2009; Griebler *et al.,* 2010;

Hancock, Boulton & Humphreys, 2005; Marmonier *et al.,* 2013; Steube, Richter & Griebler, 2009).

Groundwater environments host relatively simple community assemblages (Poulson & White, 1969) compared with terrestrial and aquatic surface ecosystems, providing useful models for the study of ecological principles (Gibert *et al.,* 2009). The mandatory need to intergrate geology, biology and hydrochemistry in groundwater investigations has been stressed by several researchers (e.g. Gibert *et al.,* 2009; Hancock, Boulton & Humphreys, 2005; Humphreys, 2009; Larned, 2012; Steube, Richter & Griebler, 2009; Wicks & Humphreys, 2011), as has the need to place this interdisciplinary integration within a much broader ecological design (Maurice & Bloomfield, 2012). "The time is ripe for ecologists and biogeographers of subterranean environments to participate in the larger debate toward advancing general community ecology" (Gibert *et al.,* 2009).

This paper provides: (*i*) an integrative examination of the existing knowledge about stygofaunal diversity, ecological patterns and trophic web interactions in groundwater environments; (*ii*) a novel approach to stygofaunal groundwater ecology based on the integration of trophic ecology *via* isotopic indicators and genetics; and (*iii*) a statistical linkage, based on Bayesian mixing models, integrating cross-disciplinary techniques. This proposed combined approach will help shed light to the fundamental key factors that govern groundwater biodiversity, and help understand the ecological functioning of one of the most distinct and fragile ecosystems on Earth.

2.2 Stygofauna vs Environment: diving into groundwater dynamics

Several factors control the occurrence of biota in groundwater ecosystems. Geological and physical conditions (occurrence of interstitial spaces, voids, etc.) determine the abiotic structure (Danielopol *et al.,* 2003); concurrently, hydrological and chemical processes establish the conditions of the environmental network shaping the subsurface community (Galassi *et al.,* 2017). As a result, the biological framework is strongly linked to hydrogeochemical dynamics *via* a complex network of interactions (Madsen & Ghiorse, 1993).

Two aspects make groundwater unique compared with edaphic and surface aquatic environments (Gibert *et al.,* 1994a): the water residence time can vary from several weeks to millions of years, and the lack of photosynthesis limits the organic carbon available for secondary production. Danielopol, Pospisil and Rouch (2000) described these environments as 'extreme' habitats, due to the lack of light and the shortage of organic nutrients. However, although environmental conditions are harsh, they are in most cases stable and predictable. Also, in light of a pronounced structural heterogeneity, i.e. geological and physicochemical stratification, conditions within different zones are likely to be highly constant. The border areas between surface, unsaturated zones, aquatic sediments, and the saturated sub-superficial environments harbour highly adapted communities that serve to transfer nutrients, particles, organisms and energy between compartments (Gibert *et al.,* 1994b; Griebler & Lueders, 2009).

Energy flows are one of the most important factors in the regulation of groundwater environments (Humphreys, 2006), with the dynamics shaped by chemical and physical patterns (Huppop, 2000). Allochthonous organic matter (transferred by water flow, infiltration or gravity) provides one input, especially in shallow subsurface communities (Pabich, Valiela & Hemond, 2001; Shen *et al*., 2015). Bacterial communities either rely on transported organic material, or feed from the rocks *via* chemoautotrophy (Kinkle & Kane, 2000). Organic matter is partly assimilated by microorganisms, and the resulting biomass is thought to constitute the basal food source for stygofauna (Boulton, 2001; Hofmann & Griebler, 2018). Microbes constitute an essential biological component in groundwater ecosystems (Griebler & Lueders, 2009) - in particular with respect to the organic carbon stock, biogeochemical processes and the cycling of elements such as C, N, as well as biodiversity - but due to the size of the research field, are outside the scope of this review.

Stygofauna play a key role in the maintenance of subsurface environmental conditions. They contribute to microbially driven water purification *via* stimulation of microbial processes and consumption of microbial biomass (Murray *et al.,* 2006), while their consumption and excretion of organic matter controls and cultivates the biofilm (Boulton *et al.,* 2008; Mermillod-Blondin & Rosenberg, 2006). Stygofaunal biological activities (grazing, burrowing, bioturbating, etc.) help to maintain the hydraulic connectivity between aquifers and surface environments (Murray *et al.,* 2006; Stumpp & Hose, 2017) (Figure 2.1).

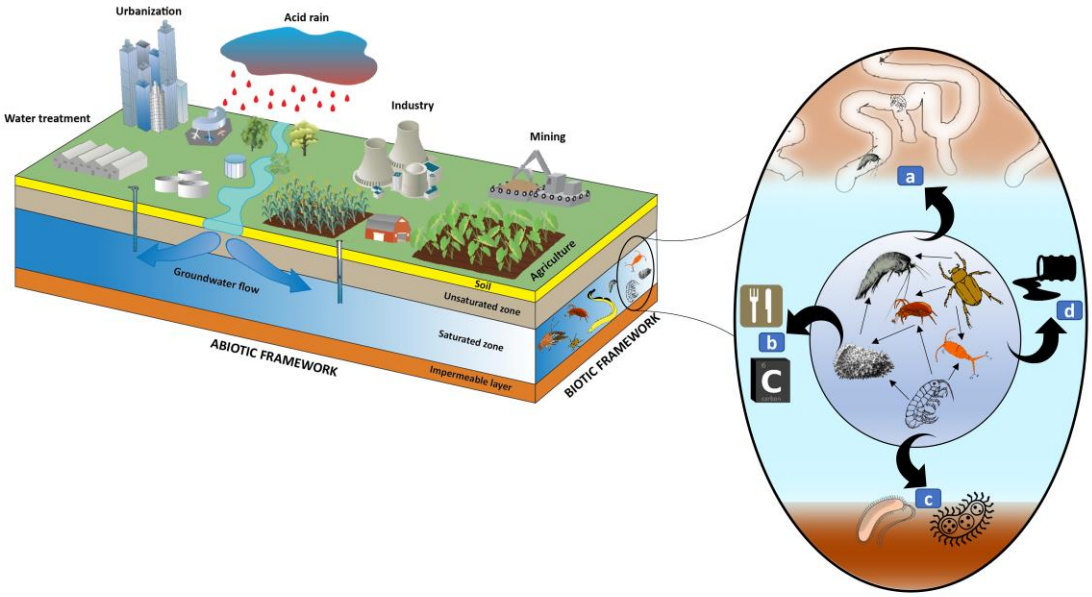


Figure 2.1. Groundwater impacts and stygofaunal functions in terms of maintenance of the environmental quality: a) preservation of groundwater hydraulic connectivity through grazing, burrowing and bioturbation; b) water purification from the excess of nutrients and organic matter; c) biofilm proliferation through the trophic regime and d) water depuration from contaminants.

As a result, groundwater functional integrity is dependent on the health of its stygofaunal community assemblage (Boulton *et al.,* 2008). Overuse or pollution of groundwaters risks altering this delicate ecological balance (Nevill *et al.,* 2010). The consequent loss of individual food web components – followed by ecological cascade effects – is likely to have a considerable negative impact on both biodiversity and the vital ecosystem services provided by groundwater systems (Boulton *et al.,* 2008; Chapelle, 2001; Griebler & Avramov, 2014; Miller & Boulton, 2005).

2.3 Beyond the (subterranean) big picture

Only latterly has the complexity of groundwater ecosystems been considered as comparable to surface ecosystems (Gibert *et al.,* 2009; Humphreys, 2008), although with a characteristic lack of primary producers resulting in a truncated trophic structure (Gibert & Deharveng, 2002). The existing literature mostly addresses the general dynamics of subterranean ecosystems and their interaction with the surface environments (e.g. Camacho, 1992; Detty & McGuire, 2010; Gibert *et al.,* 1994b; Maxwell & Kollet, 2008). Moreover, our knowledge related to biodiversity, endemism and phylogenetic patterns is,

although steadily growing, far from complete (Humphreys, 2006). There is limited research on groundwater faunal communities with respect to biotic/abiotic interactions, trophic studies, and energy and nutrient flows, especially that applying a biogeochemical multidisciplinary approach (Boulton *et al.,* 2008; Griebler & Lueders, 2009; Hancock, Boulton & Humphreys, 2005). In the following sections, we review the most relevant works focusing on each of these three priority areas.

2.3.1 *Stygofaunal diversity*

Stygofauna comprise a diverse array of lineages derived from various geological periods (Culver & White, 2005), and groundwater ecosystems can be seen as 'living museums' (Griebler, Malard & Lefebure, 2014). Stygofaunal diversity is dominated by crustaceans, accounting for about 40% of all freshwater crustacean species (Danielopol, Pospisil & Rouch, 2000). The groundwater fauna also includes nematodes, molluscs, mites, anellids and a few groups of insects (e.g. Deharveng *et al.,* 2009; Des Châtelliers *et al.,* 2009; Galassi, Huys & Reid, 2009; Gibert & Deharveng, 2002; Spangler & Botosaneanu, 1986) (Figure 2.2).

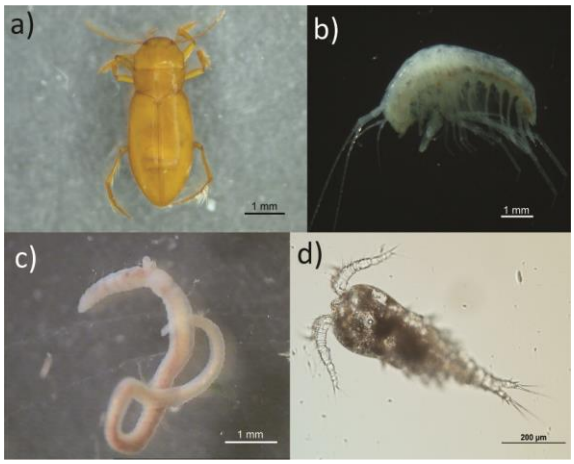


Figure 2.2. Examples of stygofaunal (including meiofauna) specimens from the community at Sturt Meadows calcrete aquifer (Leonora, Western Australia): a) *Paroster macrosturtensis* (Watts & Humphreys, 2014), b) *Scutachiltonia axfordi* (King, 2012), c) Tubificidae (Vejdovský, 1884) and d) Cyclopoida (Burmeister, 1834).

Stygofaunal biodiversity hotspots are mainly distributed within karst and pseudokarst settings along temperate gradients in the northern temperate hemisphere and the Australian continent (Culver & Sket, 2000; Guzik *et al.,* 2011) (Table 2.1). There have been a number of attempts to estimate global or regional stygofaunal diversity (Culver & Holsinger, 1992; Gibert *et al.,* 2009; Guzik *et al.,* 2011), despite the lack of reliable data

from Africa, Asia or South America and only sparse taxonomy from Australia. Culver and Holsinger (1992) proposed 50,000 to 100,000 species around the globe. Culver and White (2005) cited 7700 known stygofaunal species, of which 2000 were found in Europe.

Gibert *et al.* (2009) analysed the results from surveys carried out in six European regions (Wallonia (Belgium), Roussillon (France), French Jura (France), Cantabria (Spain), Lessinia (Italy) and Krim (Slovenia)) within the PASCALIS project, the biggest large-scale coordinated project on groundwater biodiversity. A total of 112 new species to science were reported, and 515 species were registered in the six areas, with Krim (151 species known after the survey) hosting the highest diversity.

Guzik *et al.* (2011) estimated 4140 species for subterranean systems in the western half of Australia, suggesting the Pilbara and Yilgarn regions are global diversity hotspots. Halse *et al.* (2014) collected 350 species in the Pilbara, citing similar approximations for the Yilgarn (Humphreys *et al.,* 2009). These areas harbour the highest stygofaunal diversity in the world, along with the Balkan Peninsula (330 species, excluding a large portion of Slovenia) (Sket, Paragamian & Trontelj, 2004).

However, most stygofaunal diversity is undescribed (Gibert & Deharveng, 2002; Humphreys, 2009; Maurice & Bloomfield, 2012), including 80% of the Western Australian stygofauna (Guzik *et al.,* 2011). Taking into account the high level of endemism and cryptic species, together with recent genetic technological progress, the number of recognised taxa is expected to considerably increase in the near future. Here, molecular methods are key in identifying species (Bickford *et al.,* 2007; Eme *et al.,* 2018), due to the lack of taxonomists and the morphological convergence typically associated with subterranean lineages.

Table 2.1. Major stygofaunal diversity hotspots around the world.

COUNTRY OR STATE	REGION	NO. OF STYGOBIONTS	KEY TAXA	REFERENCES
Italy	Lessinia	98	Copepoda, Amphipoda	Galassi *et al.,* 2009
Slovenia	Krim	151	Oligochaeta, Mollusca	Sket, 1999; Sket *et al.,* 2004
Spain	Cantabria	83	Oligochaeta, Amphipoda	Achurra *et al.,* 2015; Gibert *et al.,* 2009
France	Jura	62	Copepoda, Gastropoda	Dole-Olivier *et al.,* 2009
Belgium	Wallonia	32	Oligochaeta, Ostracoda	Martin *et al.,* 2009
Texas	Edwards/Balcones	27	Amphipoda, Mollusca	Gibert *et al.,* 2009; Longley, 1981
Tennessee	Appalachians	24	Isopoda, Amphipoda	Niemiller & Zigler, 2013
Kentucky	Interior low plateu	14	Copepoda, Amphipoda	Gibert *et al.,* 2009; Lewis & Reid, 2007
Western Australia	Kimberley, Pilbara, Yilgarn, Nullarbor	219	Coleoptera, Ostracoda	Guzik *et al.,* 2011
New South Wales	Peel valley	63	Syncarida, Acarina	Hancock & Boulton, 2008
Queensland	Pioneer valley	19	Copepoda, Syncarida	Hancock & Boulton, 2008
China	Yunnan, Guizhou, Guangxi	17	Decapoda	Pan *et al.,* 2010
C, L, B, T, V, M*	Southest Asia	122	Decapoda, Isopoda	Brancelj *et al.,* 2013

* refers to Cambodia, Laos, Burma, Thailand, Vietnam and Malaysia

2.3.2 *Pressures on groundwater ecosystems*

In spite of being one of the most valuable natural resources, groundwaters are subjected to increasing overexploitation pressures worldwide (e.g. Kapoor & Maheshwari: Reddy, 2005; Scheihing & Tröger, 2018). Although there is a controversy about the definition and quantification of aquifer overexploitation (Custodio, 2002), it is undeniable that the growing water demand from urban and agricultural developments is generating major threats to the conservation of biodiversity and environmental quality in subsurface ecosystems (e.g. Changming, Jingjie & Kendy, 2001; Humphreys, 2009; Liu *et al.,* 2018; Menció & Boix, 2018). Groundwater over-extraction is often linked with environmental processes like seawater intrusion and increased pollution vulnerability, amongst others, whose effect on the biota is likely to be enhanced under a global warming scenario (e.g. Esteller & Diaz-Delgado, 2002; Gejl *et al.,* 2018; Guermazi *et al.,* 2018;)

Nonetheless, it has been demonstrated only recently that artificial warming of groundwater - on a local to regional scale - coincides with a decrease in biodiversity (Brielmann *et al.,* 2009; Spengler & Hahn, 2018). As a result, a number of studies have started incorporating global warming into species distribution models (Kløve *et al.,* 2014; Mammola & Leroy, 2018; Mammola, 2018 and references therein). However, our understanding of climate change effects on subterranean habitats is still incomplete with the field under-researched.

Besides overexploitation and climate change, anthropogenic impacts on groundwater ecosystems include increasing contamination with chemicals and nutrients that respectively pose ecotoxicological risks and modulation of community composition and functioning (e.g. Appelo & Postma, 2004; Marmonier *et al.,* 2018; Mösslacher, 2000). Growing industrialization and agricultural production, waste deposition, and the exponentially increasing production and use of synthetic chemicals (currently over 80 million registered) have led to poor groundwater quality in many areas of the world (Griebler, Avramov & Hose, 2018). Prominent classes of pollutants involve organic chemicals such as petroleum hydrocarbons and halogenated solvents that typically distribute from point sources. From diffuse (non-point) sources, nutrients from fertilization (e.g. nitrate and ammonia), pesticides (e.g. triazines), as well as a multitude of ECCs (Emerging Contaminants of

Concern) such as pharmaceuticals, personal care products, artificial sweeteners, and nanoparticles, to name a few, are increasingly detected in aquifers (Lapworth *et al.,* 2012). This level of pollution implies a real or a potential consequence on the health of the ´ecological´ receptor, i.e. the communities in groundwater ecosystems.

However, the direct and indirect effects of these chemicals on groundwater biota and ecosystem services provision is hardly known. There is evidence that some groundwater species are more sensitive to individual pollutants than their close epigean relatives (Caschetto *et al.,* 2017; Di Lorenzo *et al.,* 2014; Di Lorenzo *et al.,* 2015a), likely due to their lower metabolic rates (Di Lorenzo *et al.,* 2015b). However, other studies have obtained different results indicating that sensitivity is pollutant specific (Avramov, Schmidt & Griebler, 2013; Reboleira *et al.,* 2013). Ecotoxicity data for groundwater taxa are almost exclusively derived from acute tests, and although chronic tests are more appropriate for stygobionts and groundwater ecosystems, such tests are rare (Di Marzio *et al.,* 2013). Currently, new concepts in ecotoxicology and biological risk assessment are being developed and tested (Cifoni *et al.,* 2017; Di Lorenzo *et al.,* 2018).

Although aquifers have the intrinsic capacity to purify incoming water to a high quality (Meckenstock *et al.,* 2015), this is based on a sensitive balance between the low microbial biomass and activity in aquifers and the flux of organic carbon and nutrients to the aquifer (Griebler *et al.,* 2018). Pollutant overload, however, soon overtaxes the ecosystem's capacity for "natural attenuation" (Rivett *et al.,* 2008). In this context, micropollutants are of particular importance. Since they occur at very low concentrations, generally they cannot be degraded efficiently by microbes, and hence persist in the environment for long periods (Wick & Chatzinotas, 2019). As a consequence, chronic toxic effects are an overseen threat for the higher organisms (i.e., invertebrates) and micropollutants have already initiated modulation of groundwater food webs (e.g. Arslan *et al.,* 2017; Ding *et al.,* 2015; Yan *et al.,* 2016).

2.3.3 *Biogeochemical patterns*

2.3.3.1 *Biotic/abiotic transitions*

To understand ecological dynamics in groundwater we need to characterise the processes shaping these ecosystems. Several reviews of groundwater ecosystems (e.g. Danielopol &

Griebler, 2008; Griebler *et al.,* 2010; Hancock, Boulton & Humphreys, 2005; Tomlinson & Boulton, 2010) are based on the analysis of the structural and functional relationships between abiotic (e.g. trace elements, physicochemical and environmental parameters) and biotic (community assemblage) components. Within groundwaters, the main controls on stygofaunal diversity are thought to be the depth of the water column (Datry, Malard & Gibert, 2005; Mauclaire & Gibert, 2001), pH (Galassi *et al.,* 2009; Hancock, Boulton & Humphreys, 2005) and dissolved oxygen levels (Dole-Olivier *et al.,* 2009; Tomlinson & Boulton, 2010).

These variables, and any variation in water chemistry leading to fluctuations in carbon and nutrient levels, are thought to influence biotic distribution patterns (Maurice & Bloomfield, 2012). Galassi *et al.* (2017) investigated the effect of geochemical conditions on the invertebrate community (mainly copepods) of sulfidic caves in central Italy (Frasassi). They showed that, while pH and oxygen shaped the non-sulfidic pool invertebrate assemblages (showing a positive correlation), sulfide concentration is the major variable controlling the community composition in sulfidic karst environments. Dumnicka, Galas and Krodkiewska (2017) compared stygofaunal distributions associated with two bedrocks (limestone and flysch) and found that neither geology nor water chemistry were major controls. This is in line with the lack of explanatory interactions between abiotic and biotic frameworks found in several other studies (Dumas, Bou & Gibert, 2001; Galassi *et al.,* 2009; Hahn & Fuchs, 2009; Halse *et al.,* 2014).

Besides inaccessibility, the difficulty in assessing ecological processes in groundwater resides in the complexity of integrating factors operating on different spatial and temporal scales (Hancock, Boulton & Humphreys, 2005). As a result, our understanding of the linkage between stygofauna, microbial communities and groundwater physical-chemical dynamics is still unclear (Griebler, Malard & Lefebure, 2014). However, the incorporation of biochemical approaches based on organic matter (OM) flows is providing new insights (e.g. Hutchins *et al.,* 2016). Carbon sources in freshwater environments are major drivers of biogeochemical processes, influencing the availability of nutrients and shaping food webs (Hancock, Boulton & Humphreys, 2005). These patterns have been the subject of several studies during the last decade (e.g. Datry, Malard & Gibert, 2005; Hancock & Boulton, 2008; Menció, Korbel & Hose, 2014). Griebler *et al.* (2010) found statistically significant positive correlations between stygofaunal α-diversity and abundance, and the dissolved organic

carbon (DOC) content in aquifers. This correlation must be mediated *via* microbial activity, as stygofauna do not live directly on DOC (Datry, Malard & Gibert, 2005). However, while many studies (e.g. Chapelle, 2001; Hofmann & Griebler, 2018; Mermillod-Blondin *et al.,* 2015; Pabich, Valiela & Hemond, 2001) show a positive correlation between microbial biomass and DOC, none also looked at fauna.

Brankovits *et al.* (2017) focused on the investigation of DOC and methane paths – derived from degraded terrestrial OM – as carbon sources for the microbiota in a Yucatan peninsula coastal karst aquifer. Their results underlined the importance of heterotrophy, methanotrophy and chemoautotrophy as basal, microbial trophic pillars of the food web. Other systems characterized by different biogechemical frame conditions are likely to be organized differently. However, our knowledge about how the flow of OM – and biochemical flows in general – influence stygofaunal communities remains incomplete. Here, advanced analytical and statistical techniques, leading to the study of groundwater processes through integrative and novel perspectives offer the opportunity to advance the field (Bradford *et al.,* 2014).

2.3.3.2 *Trophic dynamics*

During the past three decades, trophic studies have focused on marine (e.g. Bizzarro *et al.,* 2017; Edgar & Shaw, 1995; Hoekstra *et al.,* 2002) and terrestrial (e.g. Dorresteijn *et al.,* 2015; Elmhagen & Rushton, 2007; Schmitz, Hambäck & Beckerman, 2000) ecosystems. The field of trophic ecology is still in its infancy within groundwater research, with little known about ecosystem functioning in terms of food webs and energy sources (Humphreys, 2009). The current trophic ecology paradigm in groundwater is simplified trophic dynamics dominated by generalists and opportunistic consumers (Sherry, 1990). However, this archetype has hardly been tested (Francois *et al.,* 2016).

Trophic studies on stygofauna – often small in size with poor access to gut or faecal contents – are subject to substantial technical and methodological challenges. As a consequence, the field of groundwater trophic ecology requires a multidisciplinary approach. Bishop, Humphreys and Longley (2014) investigated the metabolism and energy sources of epigean and hypogean *Palaemonetes* sp. (Decapoda, Palaemonidae) from the Edward aquifer (Texas USA) by applying stable C ($\delta^{13}C$), N ($\delta^{15}N$) and S ($\delta^{34}S$) isotope

analyses (SIA) to individual stygofauna. They revealed a composite geochemical system with organic and inorganic energy sources from methane and sulfates respectively, suggesting metabolic adaptation to the environmental conditions shaping the community. Hutchins *et al.* (2016) combined isotopic and biological approaches to assess the current subterranean ecology paradigm of subsurface obligate invertebrate communities characterized by simplified food web assemblages dominated by generalist feeding behaviours (e.g. Gibert & Deharveng, 2002; Poulson & White, 1969). They linked the microbial community with the stygofaunal one, depicting a complex food web based on chemolithoautotrophic production. Francois *et al.* (2016) used C and N SIA to delineate the degree of trophic specialization in two species of isopods - *Proasellus valdensis* (Chappius, 1948) and *P. cavaticus* (Leydig, 1871). In agreement with Hutchins *et al.* (2016), their findings oppose the classic archetype of simplified generalist feeding niches.

2.4 Filling (groundwater) voids: CSIA, 14C and DNA analysis

Compared to surface systems there is a paucity of research into subterranean ecosystems, particularly with respect to functioning and response to environmental perturbation. To resolve this research gap, further studies linking ecology and the geochemical component are needed (Maurice & Bloomfield, 2012). To accomplish this, we propose complementing classic groundwater ecology research with three technical approaches (Figure 2.3): (1) Compound Specific Stable Isotope Analysis (CSIA), (2) Radiocarbon analysis (^{14}C), and (3) molecular analysis including DNA metabarcoding and eDNA analysis.

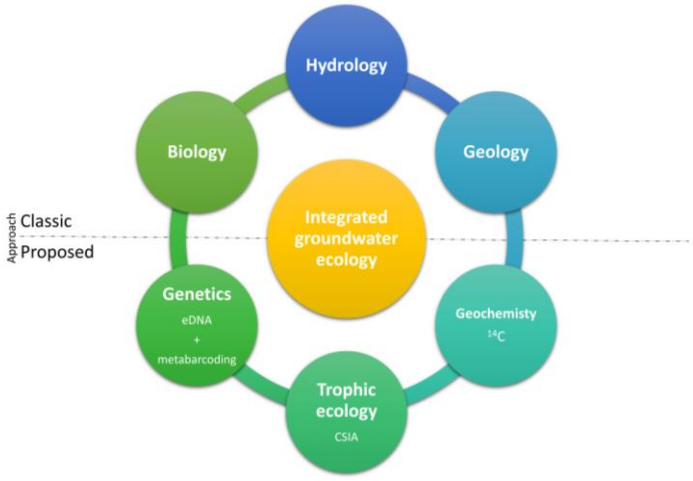


Figure 2.3. Diagram representing the integrative groundwater ecology design as a combination between the classic and the proposed approaches.

2.4.1 *CSIA: deciphering food webs*

Carbon and nitrogen are the most important elements in biomass besides water (hydrogen and oxygen) (Eswaran, Van Den Berg & Reich, 1993; Hecky, Campbell & Hendzel, 1993; Meybeck, 1982). In pristine groundwater systems, the lack of photosynthesis leads to low abundances of organic carbon (Gibert *et al.,* 1994b) - the concentration of DOC is in the range of a few mg/L with the fraction readily available to microbes being 10-100 times lower (Aiken, 2002; Gooddy & Hinsby, 2008; Shen *et al.,* 2015). Concurrently, nitrogen content in groundwater varies in concentration and forms (Kreitler, 1974). While nitrate is the most abundant species and is usually transported through the groundwater flow (Spalding & Exner, 1993), dissolved organic nitrogen (DON) and ammonia are less concentrated and less mobile. The biological and geochemical processes involving these two essential elements play a key role in shaping the aquatic biota found in groundwater environments (Hancock, Boulton & Humphreys, 2005). Figure 2.4 illustrates a simplified model for the biological carbon and nitrogen paths in groundwaters.

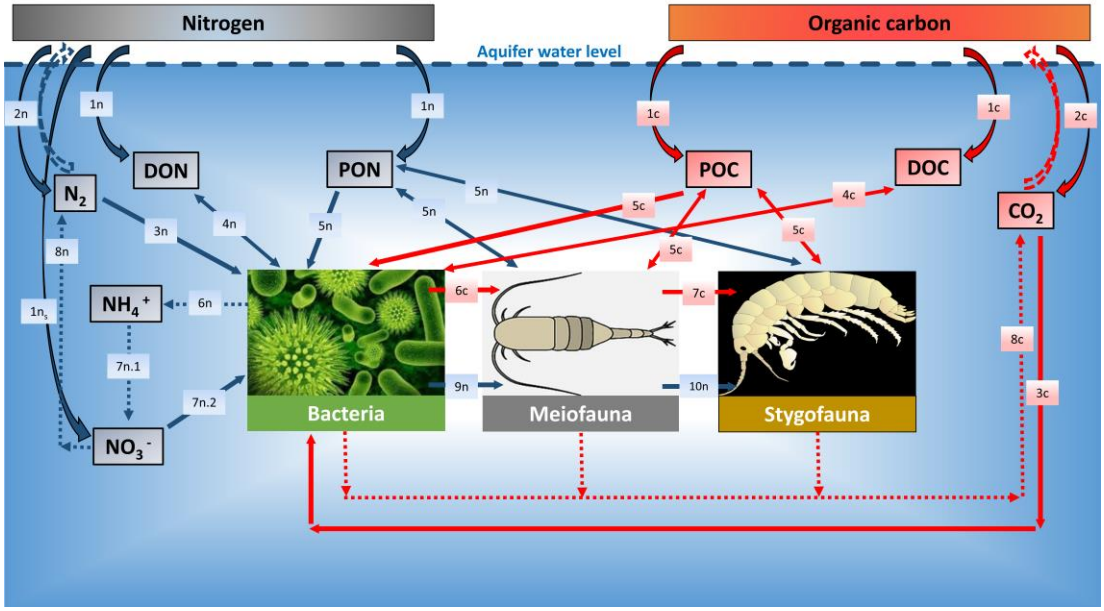


Figure 2.4. Combined biochemical cycles for carbon and nitrogen in aquifers. Carbon and nitrogen share similar first phases within their biological cycles in groundwaters. They both enter in the subsurface biological cycle either *via* the atmosphere (N_2 and CO_2 biological fixation carried out by bacteria, pathway 2n and 3n, and 2c and 3c, respectively) or *via* bores, subterranean streams to the hyporheic zone and soil percolation (pathway 1n, $1n_s$ (seepage of nitrate)) and once subsurface (1c), both the particulate (PON and POC, both in minor concentrations) and dissolved organic phases (DON and DOC) are present. As per the carbon cycle, nitrogen can be either directly assimilated by microorganisms in its dissolved status (DON, pathway 4n; DOC, pathway 4c) or consumed/decomposed by bacteria or higher trophic levels (meiofauna and stygofauna) if particulate (PON, pathway 5n; POC,

pathway 5c). Nitrogen and carbon are then transferred along the trophic pathways in the food chain (from bacteria to meiofauna, pathways 9n and 6c), up to the top consumers (stygofauna, pathway 10n and 7c). Both bacterial and faunal respiration generate carbon dioxide (CO2, pathway 8c), which is fixed by the former or released to the atmosphere where groundwater exfiltrates, closing the cycle. The death of these organisms triggers the mineralization process, where microbes decompose organic N from the organic phase to NH_4^+ (pathway 6n). Ammonium can then follow a double path: nitrification (microorganism convert ammonium to nitrate to obtain energy, pathway 7n.1) or N assimilation (pathway 7n.2). The further chemical pathway can either lead to denitrification (N_2 is released to the atmosphere, pathway 8n) or, once again, N assimilation (pathway 3n). POC: Particulate Organic Carbon; DOC: Dissolved Organic Carbon; PON: Particulate Organic Nitrogen; DON: Dissolved Organic Nitrogen. Dashed lines illustrate respiration (for carbon) or denitrification (for nitrogen). Information composed from Hancock, Boulton and Humphreys (2005) and Beaumont and Robert (1999).

Only three carbon isotopes occur naturally: ^{12}C, ^{13}C and ^{14}C. While ^{12}C and ^{13}C are stable, ^{14}C exhibits radioactive decay. Nitrogen has two stable (^{14}N and ^{15}N) and several radioactive isotopes. Slight differences arise in the chemical and physical properties of stable isotopes from a quantum mechanical effect depending on dissimilar zero-point energies of the heavy and light isotopes. The higher zero-point energy of the lighter isotope means that a chemical bond formed by a lighter isotope is weaker than one by the heavier isotope (Bigeleisen *et al.,* 1959). This principle controls the reactivity of the individual stable isotopes in biochemical reactions that may be accompanied by isotope fractionation (Bigeleisen & Wolfsberg, 1957), and thus isotope ratios in natural compounds and biomass vary due to kinetic effects during their production and consumption (transformation, mineralization). Commonly denoted by δ, isotope fractionation refers to the stable isotopic ratio between the heavy and the light isotopes, relative to a standard. Data are reported as per mil (‰):

$$\delta = [(R_{sample}/R_{standard}) -1)] \times 1{,}000\ ‰$$

R_{sample}: the isotopic ratio of a sample (e.g., ^{13}C $R_{sample} = {}^{13}C_{sample}/{}^{12}C_{sample}$)
$R_{standard}$: the isotopic ratio of a standard or reference material

The analysis of biochemical patterns of isotopic fractionation allows trophic pathways to be tracked through food webs (Du *et al.,* 2015; Finlay & Kendall, 2007; Herman *et al.,* 2000; Hondula *et al.,* 2014; Middelburg, 2014). Isotope ratios measured per each individual/group of individuals can provide detailed isotopic fingerprints of the feeding interactions within specific trophic levels. This refined approach, once integrated within an ecological design

(isotopic ecology), allows tracking of biochemical energy flows and specific trophic assemblages to be deciphered.

The $\delta^{13}C$ signature of a heterotrophic organism is derived from the carbon signature of the primary food source, i.e. primary producers, at the base of the food web, and can for example be used to distinguish between consumers of C3 and C4 plants, heterotrophic microbial biomass and chemoautotrophs (Layman *et al.,* 2007). The $\delta^{15}N$ signature of biomass varies with both primary producers and specific food web positions, because animals preferentially incorporate ^{15}N from their diet (Post, 2002). As a result, combined $\delta^{13}C$ and $\delta^{15}N$ analyses allow a detailed elucidation of system food sources and trophic dynamics (Arcagni *et al.,* 2013; Finlay & Kendall, 2007; Huang *et al.,* 2007; Middelburg, 2014).

Ecological SIA has mostly focused on 'bulk' analysis of whole tissues or organisms (Fry, 2006). In groundwaters, trophic SIA has been focused on stygofaunal web interactions and biogeochemical processes (see section '2.3.3.2 *Trophic dynamics'*). However, this approach has also several shortcomings (Boecklen *et al.,* 2011), including the conflation of separate biochemical pathways and signals, leading to difficult and sometimes conflicting data interpretation (Steffan *et al.,* 2013). A more sophisticated approach involves the tracking of isotopes through detailed empirical fractionation rules for individual compounds known as compound specific SIA (CSIA – see Hayes (2001)). It targets major biochemical constituents of OM such as amino acids (e.g. Popp *et al.,* 2007), sterols (e.g. Grice *et al.,* 1998) and fatty acids (e.g. Collister *et al.,* 1994), and serves to identify pathways of carbon and nitrogen flow through food webs.

^{13}C fractionation effects in herbivory experiments have been performed in a series of mesocosm experiments with copepods (common components of stygofaunal assemblages) and flagellates as food sources (Breteler *et al.,* 2002). Faecal pellets were depleted in ^{13}C by up to 11.3‰, but in mass balance with the ^{13}C enrichment of both the copepod biomass and the carbon dioxide through respiration. The $\delta^{13}C$ of faecal pellets adds a variable to the $\delta^{13}C$ of particulate organic carbon (POC) in the environment, and DIC can be influenced by seasonal changes and availability of food. As a result, when food is scarce, proteins can be fractionated by copepods and lead to ^{13}C-depleted POC (Breteler *et al.,* 2002). $\delta^{13}C$ CSIA studies of sterols in feeding experiments show that the dominant sterol in copepods retains

the $\delta^{13}C$ "signature" of its dietary algal precursor. Thus, $\delta^{13}C$ of sterols prove conservative tracers for herbivory regimes; $\delta^{13}C$ of cholesterol in the faecal material released from copepods was the same as the $\delta^{13}C$ of the sterols in their diet (Grice *et al.,* 1998).

Individual amino acids are typically divided into two categories: essential and non-essential. While plants, algae, and bacteria can synthesise essential amino acids *de novo* from a bulk carbon pool, animals have lost the enzymatic capability to do so at a rate sufficient for growth. As such, animals must acquire essential amino acids directly from their diet, resulting in little to no trophic fractionation (Du *et al.,* 2015). Consequently, essential amino acid carbon isotope patterns provide an isotopic fingerprint of the food sources down to the bottom of the trophic pyramid (Fantle *et al.,* 1999).

With respect to nitrogen, two amino acids have been shown to discriminate between the source signal and trophic enrichment (Steffan *et al.,* 2013). These are phenylalanine, an essential amino acid which experiences little enrichment with each trophic transfer, and glutamic acid, a non-essential amino acid (i.e. one which is synthesised by the animals, rather than being obtained directly from the food source), which experiences substantial enrichment because the carbon-nitrogen bonds are commonly cleaved during the metabolic processes (Macko *et al.,* 1987). Based on these attributes, Chikaraishi *et al.* (2007) provided a concept for the characterization of the specific trophic position (TP) of an organism in a food web. The TP is calculated according to the following equation:

$$TP_{Glu/Phe} = [(\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + \beta)/TDF] + 1$$

β: Isotopic difference between glutamic acid ($\delta^{15}N_{Glu}$) and phenylalanine ($\delta^{15}N_{Phe}$) in primary producers; TDF: Trophic Discrimination Factor (7.6 ± 1.2‰ = $\delta^{15}N_{Glu}$ - $\delta^{15}N_{Phe}$) at each shift of trophic level.

CSIA in consequence permits a more refined analysis than the bulk approach alone (Ishikawa *et al.,* 2014) (Table 2.2). By confining the isotopic analysis to selected amino acid molecules, it is further possible to define the food web structure based on the metabolic pathway of specific amino groups, removing complicating influences from other biochemical fractionation (Chikaraishi *et al.,* 2007; Chikaraishi *et al.,* 2011; Steffan *et al.,* 2013). To date, the $\delta^{13}C$ and $\delta^{15}N$ CSIA approach has been used to unravel the trophic position of consumer species in aquatic (marine and freshwater) and terrestrial ecosystems (e.g. Chikaraishi *et al.,* 2007; Chikaraishi *et al.,* 2011; Lorrain *et al.,* 2009; Popp *et al.,* 2007;

Steffan *et al.,* 2013). However, the study of trophic patterns *via* $\delta^{13}C$ and $\delta^{15}N$ CSIA is still in its infancy when it comes to groundwater research (Steffan *et al.,* 2013). The majority of CSIA studies for aquifers have focused on contaminants and biomarkers (e.g. Amaral *et al.,* 2011; Bashir *et al.,* 2015; Segal, Kuder & Kolhatkar, 2018). To the best of our knowledge all the SIA trophic studies in groundwater ecosystems so far employed bulk analysis (e.g. Bradford *et al.,* 2014; Francois *et al.,* 2016; Humphreys, 1999; Hutchins *et al.,* 2016). Isotope ecology based on $\delta^{13}C$ and $\delta^{15}N$ CSIA therefore represents a further step towards a comprehensive understanding of groundwater ecosystems food web interactions.

Table 2.2. Comparison of the main features of CSIA and bulk (SIA) analysis for the study of trophic relationships. Advantages (+) and disadvantages (-). [1] Takizawa *et al.,* 2017; [2] Steffan *et al.,* 2013; [3] Sauheitl, Glaser & Weigelt, 2009; [4] Chikaraishi *et al.,* 2007; [5] Chikaraishi *et al.,* 2011; [6] Fry *et al.,* 2008; [7] Thorp & Bowes, 2017; [8] Hannides *et al.,* 2009; [9] Fry, 2006.

CSIA		BULK ANALYSIS	
Novel approach [1,4,5]	-	Long used [6,9]	+
$\delta^{15}N_{Glu/Phe}$: centred ^{15}N- enrichment values (+7.6‰) [2,5]	+	$\delta^{15}N_{bulk}$: broad ^{15}N - enrich. values (-2.1 to +9.2 ‰) [4,1]	-
$\delta^{13}C_{CSIA}$: not affected by the uptake of tracer C-fragm. [3,7]	+	$\delta^{13}C_{bulk}$: overestimation of direct amino acid uptake [3]	-
Detailed trophic discrimination [4,5,8]	+	Imprecise/Inaccurate trophic discrimination [3,4]	-

2.4.2 *Radiocarbon dating (14C): digging into the path of carbon*

Radiocarbon (or ^{14}C), a radioactive isotope of carbon, is produced continuously in the upper atmosphere by the interaction of neutrons generated from cosmic radiation with atmospheric ^{14}N (Gäggeler, 1995). The resulting ^{14}C reacts with atmospheric oxygen to form $^{14}CO_2$. In this form ^{14}C is quickly distributed throughout the Earth's atmosphere, transferred to other carbon reservoirs and included in the global carbon cycle. Radiocarbon enters the biosphere and living organisms *via* fixation in autotrophic production (e.g. photosynthesis). Once an organism dies, the ^{14}C uptake ceases and the ^{14}C content of the organism starts to decrease at a rate governed by its half-life of 5730 years (Godwin, 1962). Since its discovery (Libby, 1946), radiocarbon has been a reliable dating tool for the past 50,000 years in archaeological, geochemical and climate change research (e.g. Taylor, 1978; Walker & Walker, 2005). Radiocarbon is also produced artificially. Aboveground nuclear testing in the late 1950s and early 1960s produced high fluxes of thermal neutrons, which reacted with atmospheric nitrogen to form ^{14}C. This caused a dramatic increase in the ^{14}C content of the atmosphere, known as "Bomb-pulse radiocarbon" (Hua & Barbetti, 2004; Levin &

Hesshaimer, 2000). The excess atmospheric ^{14}C level peaked in the mid-1960s and has since been decreasing due to rapid exchange between the atmosphere and other carbon reservoirs and injections of ^{14}C-free fossil fuel into the atmosphere. Currently, the atmospheric ^{14}C level is only slightly higher than its pre-bomb value (Graven *et al.,* 2017; Hua, Barbetti & Rakowski, 2013).

Conventionally, the atmospheric ^{14}C level in 1950 (or year zero before present [BP]) is defined as 0‰ in $\Delta^{14}C$ unit, which is the per mil deviation from the absolute radiocarbon standard (Stuiver & Polach, 1977). Natural carbon-bearing materials have a large range of $\Delta^{14}C$, spanning from -1000‰ for geological materials such as limestone and carbonates containing no ^{14}C, to ~1000‰ for terrestrial plants growing in the Northern Hemisphere during the bomb peak period. This enables ^{14}C to be employed as a powerful tracer for ecological research not only to distinguish between contributions of recent and old carbon sources to an ecosystem but also to provide the time frame of its carbon cycling (Ishikawa, Hyodo & Tayasu, 2013; Larsen, Yokoyama & Fernandes, 2018). For example, a high negative $\Delta^{14}C$ value of samples indicates that they contain subfossil or aged carbon, while a small negative value suggests that their carbon is formed before 1950. A positive $\Delta^{14}C$ value reveals that they contain modern carbon produced during the bomb period after 1955 (Ishikawa, Hyodo & Tayasu, 2013; Keaveney, Reimer & Foy, 2015a).

For groundwater, ^{14}C mainly enters the system through water recharge. The main forms of carbon in groundwater are dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC). Groundwater DIC mainly consists of CO_2 and bicarbonate, which are generated from different steps of chemical reactions in unsaturated and saturated zones (Geyh, 2000; Han & Plummer, 2016). In the unsaturated zone, soil CO_2 gas is dissolved in infiltrating water. The resulting dissolved CO_2 reacts with possible soil carbonate minerals to form bicarbonate, and the DIC is then in exchange with soil CO_2. When the infiltrating water reaches the saturated zone, the DIC reacts with aquifer carbonate minerals. The initial $\Delta^{14}C$ value of groundwater DIC is usually lower than the $\Delta^{14}C$ value of contemporaneous atmospheric (or terrestrial organic) samples, due to groundwater DIC comprising a mixture of modern carbon (soil CO_2 partly derived from root respiration), aged carbon (contributed to soil CO_2 from biological decomposition of dead OM) and ^{14}C-free carbon (derived from carbonate minerals in soil and aquifer matrix). Radiocarbon in groundwater DIC has been long used to estimate the groundwater residence time for hydrogeological studies (Geyh,

2000; Han & Plummer, 2016 and references therein). Groundwater DOC consists of a diverse mixture of organic compounds, i.e. lipids, hydrocarbons, methane and humic components (Geyh, 2000). It is produced in soil, unsaturated and saturated zones by microbial degradation of organic detritus and *via* the oxidation of lignite or kerogen (Bryan *et al.,* 2017; Geyh, 2000). Similar to groundwater DIC, groundwater DOC contains a mixture of young and old carbon derived from various pedogenic and/or geogenic sources (Wassenaar *et al.,* 1990), and the initial $\Delta^{14}C$ value of groundwater DOC is, therefore, usually lower than that of contemporaneous terrestrial organic samples. According to Geyh (2000) and references therein, organic compounds of fulvic acids rather than abundant humic substances are more suitable for radiocarbon dating of groundwater DOC. Although there is not much research on ^{14}C in groundwater DOC (e.g. Aravena & Wassenaar, 1993), the combined use of DIC and DOC and their isotopes can improve our understanding of the evolution of groundwater, and better age determination based on ^{14}C dating of groundwater DIC (Bryan *et al.,* 2017).

Atmospheric CO_2, dissolved CO_2 (a portion of groundwater DIC), particulate organic carbon (POC) and groundwater DOC are assimilated by microorganisms (archaea, bacteria and fungi), as indicated in the hypothetical groundwater food web shown in Figure 2.4. The resulting biofilms and POC (in minor concentration) are the basal food source for meiofauna and stygofauna. There is a large range of $\Delta^{14}C$ values in the food web components, spanning from aged carbon (for POC), aged-to-recent carbon (for DIC and DOC), to modern carbon (atmospheric CO_2).

Similar approaches based on the large range of $\Delta^{14}C$ of different carbon sources in ecosystems have been applied for ecological studies in freshwater, marine and terrestrial environments. Fernandes *et al.* (2013) investigated the trophic ecology of Lake Schwerin, a freshwater lake in Germany, by analysing ^{14}C in flora and fauna collected from the lake as well as in DIC, POC and DOC. The results showed similar ^{14}C content for an aquatic plant (primary producers), a zebra mussel (filter feeders), fishes (including a bream, a pike and a large eel; higher trophic species), and water DIC, indicating one important grazing food chain in the lake. Similarly, ^{14}C content of a smaller eel and that of water POC were similar, but were much lower than water DIC, suggesting the smaller eel was a part of a detritus food chain. The ^{14}C content of DOC was substantially lower than DIC and POC, suggesting DOC might not constitute a major nutrient contributor to Lake Schwerin's food web. A

similar study was carried out in Lower Lough Erne, a humic, alkaline lake in northwest Ireland (Keaveney, Reimer & Foy, 2015a; Keaveney, Reimer & Foy, 2015b). The authors analysed zooplankton (calanoid copepod and *Daphnia* spp.), phytoplankton (algae), fishes (roach, perch and pollan), and water DIC, DOC and POC for ^{14}C. They reported similarly depleted $\Delta^{14}C$ values for Daphnia and water POC of ca. -100‰, indicating that Daphnia consumed some detrital fossil carbon. In contrast, much higher values for calanoid $\Delta^{14}C$ were observed, suggesting modern carbon inputs within the food chain. These values varied between seasons with higher values in winter (similar to DOC $\Delta^{14}C$ of ~0‰) and lower values in summer (within the range of algae $\Delta^{14}C$ of ca. -50 ‰), suggesting a seasonal shift between allochthonous (terrestrial) and autochthonous carbon sources for calanoid copepods. For most of the cases, measured fish $\Delta^{14}C$ values were found in the range between winter and summer calanoid $\Delta^{14}C$ values. These results indicated that terrestrial organic carbon was evident in all trophic levels of Lower Lough Erne.

In marine environments, ^{14}C has been used for distinguishing the food sources between surface and bottom waters given that fresh algae usually have $\Delta^{14}C$ higher than that of detritus (Larsen, Yokoyama & Fernandes, 2018 and references therein). Radiocarbon has also been applied in migration ecology (e.g. Eisenmann *et al.,* 2017; Levin & Hesshaimer, 2000).

In terrestrial ecology, Briones, Garnett and Piearce (2005) employed radiocarbon to estimate the diet ages for earthworms. ^{14}C ages of epigeic earthworms were younger than those of anecic or endogeic earthworms, indicating that the former species assimilated more recently fixed carbon, while older mineralised organic carbon formed a portion of the diet of the latter earthworms. A similar investigation on termites (primary consumers in a detrital food web) and bees (primary consumers in a grazing food web) was reported by Hyodo, Tayasu and Wada (2006). ^{14}C has also been used to trace the fate of newly fixed carbon and to improve our knowledge on the turnover times of terrestrial ecosystem carbon pools (Pataki *et al.,* 2003 and references therein). This becomes important under the current global change scenario as elevated atmospheric CO_2 levels can result in increased rate of fixation of carbon in photosynthesis, which may change ecosystem function, affecting populations at different trophic levels.

The application of ^{14}C analyses in groundwater ecosystems (as opposed to the waters themselves) is largely unexplored, but offers a powerful tool to decipher vital ecological dynamics. By analysing ^{14}C in stygofauna and comparing them with $\Delta^{14}C$ values of groundwater DIC, DOC and POC, the feeding ecology of subsurface faunal communities can be characterised. With recent technical advances in accelerator mass spectrometry (AMS), ^{14}C measurement of sub-milligram/microgram carbon (e.g. Hua *et al.,* 2004; Santos *et al.,* 2007; Smith *et al.,* 2010) and therefore analysis of small sample sizes of stygofauna and meiofauna can be achieved.

2.4.3 *eDNA and DNA metabarcoding: the new biomonitoring frontiers*

During the last 30 years, metagenomics (DNA sequencing of multiple genomes) and metabarcoding (DNA sequencing for genetic identification) have gained relevance within molecular ecology (Reusch & Wood, 2007). Originally mainly focused on microorganisms (Bass & Cavalier-Smith, 2004; Henne *et al.,* 1999), these techniques involve the extraction and identification of genetic material directly from environmental samples (Bohmann *et al.,* 2014). As predicted by Shokralla *et al.* (2012), the latest advances in sequencing technology are providing new perspectives in ecological and environmental research. In this context, also analysis of free, environmental DNA (eDNA) is gaining prominence as a powerful tool for biological surveys and species monitoring.

eDNA is total genomic DNA (nuclear or mitochondrial) that is shed or deposited from a biological organism (e.g. faeces, hair, saliva, chitin, mucus, etc.) into their environment. Sources of eDNA include soil, water and air. Once shed, DNA is subject to decay and degradation as a result of physical (e.g. UV light), chemical and/or biological processes (e. g. enzymatic digestions, microrganisms, etc.). eDNA can be detected in cellular or extracellular forms and allow a researcher to obtain a biological sample that is non-invasive to the target organism(s). As a result, individual species from a wide range of environments can be determined without the need for capture or trapping. This has the potential to avoid the often difficult sampling that is applied during traditional biological surveys, specifically in groundwater environments.

Due to its typically low environmental concentration, eDNA is usually amplified *via* PCR and longer sequences able to specifically depict species diversities are characterized. Recent 2nd

generation sequencing techniques allow the generation of thousands of sequences at once, improving the reliability and repeatability of this analytical approach (Bohmann *et al.,* 2014). One of the most common techniques consists of targeting specific short fragments of mitochondrial DNA (mtDNA) and coupling them with other analyses such as DNA metabarcoding.

eDNA offers a wide variety of applications, ranging from the detection and monitoring of common, invasive and endangered species to the study of intra- and interspecific evolutionary processes in ecosystems. As per Bohmann *et al.* (2014) three main eDNA application areas can be identified: 1) conservation biology/policy making decisions, 2) ecology, and 3) the understanding of ecosystems processes. The majority of eDNA to date studies focus on marine (e.g. Minamoto *et al.,* 2012; Stoeck *et al.,* 2010; Thomsen *et al.,* 2012) and surface freshwater (e.g. Ficetola *et al.,* 2008; Goldberg *et al.,* 2013; Pilliod *et al.,* 2013) environments. However, this new approach still suffers from methodological uncertainties such as the quantitative relationship and time frame behind the continuous eDNA production and degradation in the environment. False positives can occur as a result of false eDNA detection from external sources (e.g. water discharge, excrements, etc.). Given the hydraulic continuum between groundwater habitats and freshwater environments, both quality and reliability of the eDNA analyses can be affected by external factors such as sewage or waste water runoff. Nonetheless, as suggested by Bohmann *et al.* (2014), quality controls and optimized protocols can be implemented at each stage (e.g. samples collection, primer amplifications, sequence comparing) to improve the reliability of the analyses.

Despite the aforementioned constraints, eDNA constitutes a powerful tool for the study of biological dynamics in aquatic ecosystems (Thomsen *et al.,* 2012). Individuals from specific species can be detected anywhere - in free water or sediments - and not just at their source, providing reliable insights into the study of cryptic and vast environments such as rivers, lakes and oceans. As a result, the biological monitoring of endangered, cryptic and invasive species – usually difficult to detect with the classic sampling protocols – can be optimized.

However, published eDNA studies from groundwater environments are sparse. The work of Gorički *et al*. (2017) focused on the detection of the rare cave-dwelling amphibian *Proteus*

anguinus (Laurenti, 1768). Their findings confirmed that a combination of eDNA and qPCR can be used as a species-specific monitoring tool in ecology, evolutionary history and taxonomy. Niemiller *et al.* (2018) collected and extracted eDNA directly from groundwater water samples to monitor the rare, autochthonous and endangered amphipod *Stygobromus hayi* (Hubricht and Mackin, 1940) and the widespread congener *Stygobromus tenuis potomacus* (Holsinger, 1967). They were able to detect the presence of the former species in springs where traditional sampling methodologies were not successful.

The eDNA approach is often coupled with DNA metabarcoding, a technique based on the extensive parallel sequencing of entire communities (Cristescu, 2014). This latter approach refers to sequencing of individuals from different groups of species and ecological functions within a system. Metabarcoding is gaining prominence in the field of trophic ecology due to its accuracy and reliability (De Barba *et al.,* 2014; Nilsson *et al.,* 2006), and application to faecal analysis (Deagle, Kirkwood & Jarman, 2009; Zeale *et al.,* 2011), gut content extraction (Krehenwinkel *et al.,* 2017; Valdez-Moreno *et al.,* 2012) or whole organisms (e.g. Deagle *et al.,* 2017; Fonseca *et al.,* 2017). While most of the studies targeted marine (e.g. Lakra *et al.,* 2011; Leray & Knowlton, 2015; Wangensteen *et al.,* 2018) and surface freshwater environments (e.g. Civade *et al.,* 2016; Evans *et al.,* 2016), a few studies have focused on subsurface ecosystems (e.g. Asmyhr *et al.,* 2014; Bradford *et al.,* 2010; Meleg *et al.,* 2013).

Stygofaunal crypticism and complexity of site-specific adaptations are the major obstacles in groundwater trophic ecology (Bradford *et al.,* 2010; Hancock, Boulton & Humphreys, 2005). The barcode analysis of faecal contents (e.g. amphipods) or entire individuals (e.g. copepods) has the potential to unravel stygofaunal trophic patterns with a species-specific design (Taberlet *et al.,* 2012; Yoccoz, 2012). As a result, once coupled with eDNA, metabarcoding studies can provide extraordinary powerful biomonitoring tools in groundwater studies. The integration of this qualitative design (eDNA and metabarcoding, diet digested) with biogeochemical approaches (CSIA and ^{14}C, diet assimilated) will lead to a broader perspective in groundwater ecology investigations.

2.5 The statistical linkage: Bayesian mixing models

One of the biggest challenges in ecology, and scientific quantitative multidisciplinary studies in general, involves establishing statistical analyses able to generate robust results to

support or invalidate hypotheses. The wider the scope of a specific investigation, the more complex are the statistical approaches needed to unravel those trends. Therefore, the analytical design ideally aims to include all factors potentially shaping the specific patterns. Diverse spatial and temporal scales can make all analytical processes more difficult.

Bayesian Mixing Models (BMM) represent a powerful tool able to overcome this intrinsic complexity. Based on the principle of Bayes' theorem, these models express statistical evidences in terms of 'degrees of belief' known as Bayesian probabilities. The study of trophic ecology has been boosted by the incorporation of BMM applied to the study of food webs *via* analysis of stable isotopes (Phillips *et al.,* 2014). Isotopic BMM aims to address fundamental questions such as how trophic habits (and therefore food web assemblages and energy flows) link to population dynamics in a much broader ecological focus. As a result, since its first incorporation (Haines, 1976), isotopic BMM have gained prominence and applicability in a wide range of research fields having been incorporated into a number of analytical packages (e.g. SIAR (Parnell *et al.,* 2010), MixSIAR (Stock & Semmens, 2013) and FRUITS (Fernandes *et al.,* 2014)).

Recently, as BMM have improved in sophistication and accuracy (Phillips *et al.,* 2014), many studies have focused on their application beyond SIA, including radiocarbon dating (for a thorough review see Bronk Ramsey, 2009) and genetic studies (e.g. MrBayes (Ronquist *et al.,* 2012), BAMBE (Simon & Larget, 1998) or PHASE (Jow *et al.,* 2002)). When compared to geometric approaches and linear mixing models, BMM allow incorporation of uncertainty and variation in input parameters and the inclusion of prior information into the model (Layman *et al.,* 2011). To take full advantage of the Bayesian approach any useful prior information can be provided to the modelling process. As a result, BMM provide a crucial statistical framework for the integration of data coming from different scientific disciplines. To date, few examples in the literature apply such an approach and all involve fish dietary investigations. However, given the crucial insights provided by the application of these designs, we believe that the field of groundwater ecology can deeply benefit from the advances brought by other research areas.

Chiaradia *et al.* (2014) showed how the inclusion of prior information gathered from DNA analysis can improve the estimates of stable isotope BMM. They performed a captive feeding experiment with the aim of reconstructing the diet of Little Penguins (*Eudyptula*

minor) on the basis of $\delta^{15}N$ and $\delta^{13}C$. To accomplish this goal, they fed animals with known percentages of food type (i.e. pilchard, tuna, etc.) and then measured stable isotopes values of both food and penguins. Concurrently, they collected scats to perform DNA analysis to quantify the proportion of prey eaten by penguins. Their outcomes showed how BMM based only on stable isotope values failed to pinpoint the real diet of penguins, while the inclusion of prior information gathered from DNA analysis improved the accuracy of the analyses.

Radiocarbon has proved to be a powerful tool to complement the results of stable isotopes in food webs analysis. According to Keaveney, Reimer and Foy (2015a), the use of radiocarbon allows a more refined separation of individual carbon sources when compared with $\delta^{13}C$. They used ^{14}C to determine the carbon sources in a humic lake, and demonstrated that carbon pools and invertebrate communities were supported by terrestrial carbon sources. The same authors used radiocarbon together with stable isotope values to infer the percentage contribution sources of fish diets in the same lake (Keaveney, Reimer & Foy, 2015b). They showed that the combination of stable isotopes and radiocarbon data gave more detailed insights into the feeding behaviours of the three investigated fish species. Similarly, Larsen, Yokoyama and Fernandes (2018) combined data from $\delta^{15}N$ and $\delta^{13}C$ with and without prior information derived from fish food preferences and radiocarbon data to reconstruct the diet of a freshwater fish (*Coregonus pollan* (Thompson)). The inclusion of this prior information in the BMM provided a more specific characterization of diet compared with a BMM performed on stable isotope values alone. These investigations highlight the usefulness of incorporating prior information to improve BMM estimates both in term of precision and interpretability.

BMM have the ability to ease the complex transition from theory to practice, allowing an effective, novel and practical application of multidisciplinary approaches. We therefore consider that the combined application of ecological, geochemical and genetic data *via* a bespoke BMM is the key to successful characterisations of complex groundwater ecosystems. By using the food web characterization *via* CSIA, prior information from radiocarbon eDNA data can be incorporated into the modelling and unravel - together with carbon flows - the relationships between predators and consumers. As a result, within a conceptual framework defined by both quantitative (CSIA and ^{14}C) and qualitative (eDNA and DNA metabarcoding) approaches, the accuracy in the interpretation of trophic

interactions is enhanced, providing outputs in form of true probability distribution, and not just a summary of all the potential solutions (Layman *et al.,* 2011).

2.6 Conclusions

This review provides an innovative and multidisciplinary framework for the study of the stygofaunal ecological dynamics in groundwater environments. Given the intrinsic crypticism and scarce accessibility of subsurface environments, it is crucial to bring new conceptual designs and techniques to the field. The integration of CSIA, DNA and ^{14}C analyses can elucidate the linkage between the biotic and abiotic frameworks, as well as contribute to widening the knowledge of stygofaunal trophic assemblages and genetic diversity. This will help to facilitate management decision processes within ecosystems deeply affected by the pressure of climate change and anthropogenic overexploitation. Improved ecologically focused studies will provide insights into the biochemical dynamics in groundwater fauna and open new perspectives into the subsurface environmental conservation. In summary, the advances generated by the incorporation of isotopic chemistry, radiocarbon analysis and molecular genetics into the field of groundwater ecology represent a crucial step towards the holistic scenario suggested by Gibert and collaborators (2009) almost a decade ago.

Acknowledgements

The authors thank all the people (colleagues, friends, partners and family members) involved in the vital and continuous brainstorming process that allowed the development of this literary review.

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Chapter 3 | Stygofaunal community trends along varied rainfall conditions: deciphering ecological niche dynamics of a shallow calcrete in Western Australia

Published as a research article in *Ecohydrology* 13(1), e2150 (2019).

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Abstract

Groundwaters host highly adapted fauna, known as stygofauna, which play a key role in maintaining the functional integrity of subterranean ecosystems. Stygofaunal niche studies provide insights into the ecological dynamics shaping the delicate balance between the hydrological conditions and community diversity patterns. This work aims to unravel the ecological trends of a calcrete stygofaunal community, with special focus on niche dynamics through the Outlying Mean Index analysis (OMI) and additional calculation of Within Outlying Mean Indexes (WitOMI), under three rainfall regimes. Temperature and pH changed significantly among different rainfall conditions ($P < 0.001$), and together with salinity were the most influential drivers in shaping stygofaunal assemblages. These environmental conditions, linked with nutrient fluctuations in the groundwater, constrained changes in niche occupation for water mites, two species of beetles and juvenile amphipods (OMI analysis, $P < 0.05$). The WitOMI analysis revealed differential subniche breadths linked with taxa-specific adaptations after different rainfall conditions. Our results indicate that stygofaunal niches are closely linked to the hydrodynamic conditions influenced by different rainfall regimes. Further long-term investigations, incorporating broader ecological perspectives, will help to understand the impacts associated with climate change and anthropogenic pressures on one of the most threatened ecosystems in the world.

Key-words: stygofauna, ecological niche, groundwater, calcrete, rainfall, WitOMI.

3.1 Introduction

The study of ecological dynamics in groundwaters is notoriously complex (Gibert, Stanford, Dole-Olivier, & Ward, 1994; Dole-Olivier, Malard, Martin, Lefébure, & Gibert, 2009; Steube, Richter, & Griebler, 2009). This is primarily because the geological and physical conditions (occurrence of interstitial spaces, voids) of an aquifer, where the groundwater resides, create a complex abiotic structure (Danielopol, Griebler, Gunatilaka, & Notenboom, & 2003). Additionally, hydrological processes, together with chemical mechanisms, can have significant impacts on the environmental conditions shaping the subsurface faunal community (Galassi et al., 2017).

Aquifer recharge, or replenishment, plays a key role in maintaining ecological balance in groundwaters (Humphreys, 2008). As the absence of light limits autochthonous carbon production (Humphreys, 2006), groundwater recharge provides an essential input of resources needed to support the stygofaunal community (Meyer, Meyer, & Meyer, 2003; Konrad, Brasher, & May, 2008). Recharge-related changes in water quality, such as increased oxygen levels (e.g. Hakenkamp & Palmer, 2000) and nutrient availability (Datry, Malard & Gibert, 2005) constitute vital drivers in shaping biotic assemblages in groundwaters (Hahn, 2006; Reiss et al., 2019).

Generally, rainfall patterns and aquifer characteristics such as permeability and porosity control groundwater recharge processes (Berkowitz & Balberg, 1993). Recharge dynamics are particularly influenced by climate variability (Scanlon, Healy, & Cook, 2002), land use/land cover (e.g. Niemiller & Taylor, 2019) and thickness of the vadose zone (e.g. Manna et al., 2019). These factors, together with the position of the groundwater in the landscape, influence the hydrogeochemical mechanisms defining the interactions with the aquifer (e.g. Arnold, Allen, & Bernhardt, 1993; Rau et al., 2017).

Worldwide, climate change is predicted to result in profound shifts in weather patterns (Stocker & Raible, 2005), and several studies over the last two decades have predicted negative impacts on groundwater (e.g. Eckhardt & Ulbrich, 2003; Holman, 2006; Green et al., 2011). In Australia, where climate is strongly influenced by the El Niño-Southern Oscillation (ENSO) and Indian Ocean Dipole (IOD), global warming is predicted to trigger high rates of biodiversity loss and habitat fragmentation (Hughes, 2003; Horwitz et al.,

2008). The vast array of Australian groundwater habitats host vulnerable aquatic fauna, termed stygofauna (Humphreys, 2006; Humphreys, 2019), which are expected to face major threats in response to rapidly changing regional climates (Davis, Pavlova, Thompson, & Sunnucks, 2013). Recent investigations have linked artificial warming to increased biodiversity loss rates (e.g. Brielmann, Griebler, Schmidt, Michel, & Lueders, 2009), while increased rainfall variability will affect recharge patterns (e.g. Rosenberger al., 1999; Hendrickx & Walker, 2017). A comprehensive understanding of the functional ecology and ecosystem dynamics in groundwater systems is therefore urgently needed in order to understand the threat posed by climatic change (Mammola et al., 2019).

Over the past two decades, the incorporation of multidisciplinary approaches, such as biogeochemistry, microbiology and genetics, into groundwater ecology has helped define the vital role played by stygofauna in the conservation of subsurface environmental conditions (Hancock, Boulton, & Humphreys, 2005). Stygofaunal activities such as grazing, burrowing or bioturbating help maintain the hydraulic connectivity between aquifers and surface environments (Murray, Zeppel, Hose, & Eamus, 2008). Stygobionts also interact with microbes, vital actors in shaping groundwater biogeochemical cycling of nutrients (Griebler & Lueders, 2009), and their consumption and excretion of organic matter regulates the proliferation of microbial biofilms (Mermillod-Blondin & Rosenberg 2006; Schmidt, Cuthbert, & Schwientek, 2017). As a result, groundwater functional integrity is dependent on the health of its stygofaunal community assemblages (Boulton, Fenwick, Hancock, & Harvey, 2008).

The ecological niche of a species reflects the set of required resources, encountered abiotic conditions and biotic interactions that enable its persistence through time and space (Chase & Leibold, 2003). During the last century, many definitions of this central concept in ecology have been proposed, with the Hutchinsonian niche (Hutchinson, 1957) being one of the most widely employed. A Hutchinsonian niche is a multi-dimensional volume (Levin et al., 2009) where habitat conditions and resources influence population dynamics, namely birth and death rates (Holt, 2009). This definition led to the formulation of vital concepts such as niche breadth (the conceptual inverse of niche specialization) and niche partitioning (coexisting species occupying different ecological niches) (Colwell & Futuyma, 1971). Due to the urgent need to predict ecological patterns under rapidly changing environmental conditions such as climate change (Soberón, 2007), re-evaluation of Hutchinson's approach

has led to recent upsurges in ecological niche research (e.g. Chase & Leibold, 2003; Holt, 2009; Pironon et al., 2017).

The measurement of ecological niches faces two major challenges (Peterson, Papeş, & Soberón, 2008). On one side, the characterization of the abiotic conditions is strictly dependent on the accuracy of sampling procedures (Karasiewicz, Dolédec, & Lefebvre, 2017). On the other, the understanding of the role of biotic interactions still remains poor in many ecosystems (Soberón & Nakamura, 2009). These obstacles grow exponentially when groundwater environments are considered, due to the poor accessibility of these systems (Halse et al., 2014), and the sparse knowledge of stygofaunal population dynamics (Guzik et al., 2011) and biotic interactions between stygofauna and microbial communities (Schmidt et a., 2017). As a result, studies of ecological niches in groundwater environments are scant, with the majority being surveys at a regional scale carried out in Europe (Dole-Olivier et al., 2009; Galassi, Stoch, Fiasca, Di Lorenzo, & Gattone, 2009; Martin et al., 2009).

In Australia, the calcrete aquifers of the Yilgarn region (WA) harbour a myriad of short-range endemic invertebrate species (Humphreys, 2001). This area, together with the Pilbara, is a global diversity hotspot that hosts one of the highest stygofaunal densities in the world (Humphreys, Watts, Cooper, & Leijs, 2009; Guzik et al., 2011). However, this diversity is largely undescribed (80% Western Australia stygofauna are unidentified taxa, see Guzik et al., 2011), which creates a major obstacle for the investigation of the biological dynamics shaping stygofaunal communities. Consequently, despite the urgent need for a detailed understanding of the ecological patterns in the region (Humphreys, 2006), research is still in its infancy.

The Sturt Meadows calcrete aquifer in Western Australia provides a unique opportunity to investigate the linkage between hydrogeochemical conditions and taxa-specific ecological niche dynamics. The fauna of this relatively pristine shallow groundwater has been the subject of genetic and taxonomic studies during the last 15 years (e.g. Leys, Watts, Cooper, & Humphreys, 2003; Allford, Cooper, Humphreys, & Austin, 2008; Cooper, Saint, Taiti, Austin, & Humphreys, 2008; Guzik, Cooper, Humphreys, & Austin, 2009; Bradford, Adams, Humphreys, Austin, & Cooper, 2010; Bradford et al., 2013). It therefore provides a well-defined stygofaunal community on which to base studies linking the fauna to broader biotic

and abiotic dynamics. Hyde, Cooper, Humphreys, Austin, and Munguia (2018) investigated diversity patterns within the invertebrate assemblages using data from a six years collection period. Here we extend this research by comparing the environmental parameters and the stygofaunal distributions from three sampling campaigns undertaken during two contrasting rainfall periods (defined as low rainfall and high rainfall, see Hyde et al., 2018). We aim to: 1) test the importance of physicochemical and environmental parameters as species distributions' descriptors in calcrete aquifers, 2) determine the linkage between the changes in potential aquifer recharge events and stygofaunal assemblages and 3) identify potential shifts in ecological niche occupation within the invertebrate community in the calcrete aquifer.

3.2 Methods

3.2.1 *Study site*

The field work was carried out at the Sturt Meadows calcrete aquifer (28˚41'S 120˚ 58'E) located on Sturt Meadows pastoral station, Western Australia, ~42 km from the settlement of Leonora (833 km northeast of Perth, Figure 3.1a). The surface vegetation is dominated by open Acacia woodlands, primarily *Acacia aneura* (F. Muell. ex Benth.). The understorey is herbaceous mixed with saltbush shrubs and grasses. The area experiences combined grazing pressure from domestic stock, feral animals and macropods. The average monthly rainfall of the area ranges from 6.9 mm in September to 30.6 mm in March (data from the Australian Bureau of Meteorology (BoM)), and the daily average temperature varies between 37°C (January) and 18.4°C (July). The average pan evaporation of 2400 mm per year exceeds the average rainfall (200 mm per annum, BoM).

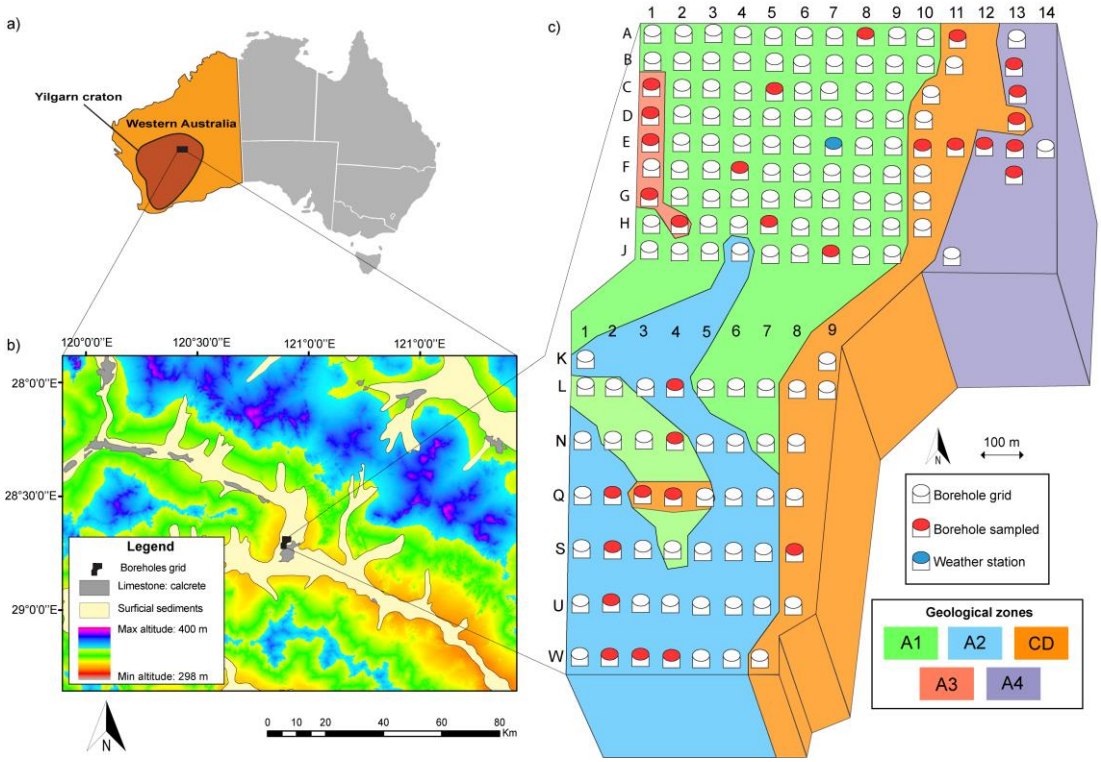

Figure 3.1. Sturt Meadow calcrete: a) location within the Yilgarn craton region, b) elevation map showing the surficial sediments and the calcretes nearby the borehole grid and c) grid map showing the location of the boreholes sampled, the weather station at bore E7 and the five geological zones (A1, A2, A3, A4 and CD).

The Sturt Meadows aquifer occurs in a calcrete deposit formed in a palaeodrainage channel located in the northeast Yilgarn, the largest Archean craton in Australia (Figure 3.1a). The surrounding area is dominated by Quaternary surficial unconsolidated deposits (Figure 3.1b). The calcrete systems of this region formed as secondary sedimentary deposits *via* precipitation of calcium carbonate in the groundwater flowpath of palaeodrainage channels during the Late Eocene to Early Oligocene (37–30 Mya) (Morgan, 1993). The groundwater within the Sturt Meadows calcrete has a strong northeast to southwest biogeochemical gradient that is comparable to estuarine habitats (Humphreys et al., 2009).

Groundwater was accessed through a grid of shallow bores, initially drilled for mineral exploration, which comprises 115 bore holes of between 5-11 m in depth forming a 1.4 km X 2.5 km (3.5 km^2) area (Figure 3.1c). These bores are unlined, except within about 0.5 m from the surface, where they are lined with PVC pipe, to stabilise the surface, and capped (Allford et al., 2008). Two sub-grids can be identified: the northern area (bores separated by 100 m in each direction) and the southern portion (bores separated by 100 m EW and 200 m NS). According to previous investigations of the depth and lithography of the calcrete

(Bradford et al., 2013), the study area can be divided into two major geological zones (A1 and A2, the deepest calcrete intersected with clayey material), a clay bar (CD) and two minor zones (A3 and A4 (together with A5), the shallowest calcretes overlying clay) (Figure 3.1c). Previous studies at the Sturt Meadows aquifer have found a multitude of endemic invertebrate species, including both stygobitic (e.g. Bradford et al., 2010) and troglobitic (e.g. Barranco & Harvey 2008; Javidkar, Cooper, King, Humphreys, & Austin, 2015) taxa.

3.2.2 *Samples and data collection*

Preliminary analysis of historical data (eleven sampling campaigns, see Hyde et al., 2018) was undertaken on the hydrological (i.e water level fluctuations, water physicochemical parameters) and stygofaunal distribution patterns throughout the bore grid. Thirty boreholes (six samples from A1, seven from A2, seven from CD, five from A3 and five from A4, Figure 3.1c) were then selected by simple random sampling among the most reliable (i.e. lowest risk of drying, broadest ranges of stygofaunal abundances) and most representative (i.e. widest ranges of stygofaunal diversity and water level changes) bores. The *in situ* sampling campaign involved collecting stygofauna and water physicochemical parameters from each bore, while samples for nutrient analysis were collected from bores J7 (zone A1), W4 (zone A2) and D13 (ZONE CD).

A weather station with rain gauge was installed near bore E7 (Figure 3.1c, in blue) to monitor rainfall events and changes in the groundwater water level *via* a differential pressure water level sensor. Daily data of both parameters were recorded for the period ranging from 18/06/2017 to 17/06/2018 (Figure 3.2a). By following the Sturt Meadows' rainfall periods categorization proposed by Hyde et al. (2018), three sampling campaigns were carried out. Two were associated with low rainfall periods (LR: <10 mm of rain during the 30 days prior to sampling, Figure 3.2b and c): LR1 on the 26/07/2017 (4.4 mm of cumulative rainfall) and LR2 on the 7/11/2017 (0.8 mm of cumulative rainfall). A final sampling trip associated with a higher rainfall event (HR: >30 mm of rain in the previous 30 days, Figure 3.2d) was carried out on the 17/03/2018 (37.8 mm of cumulative rainfall).

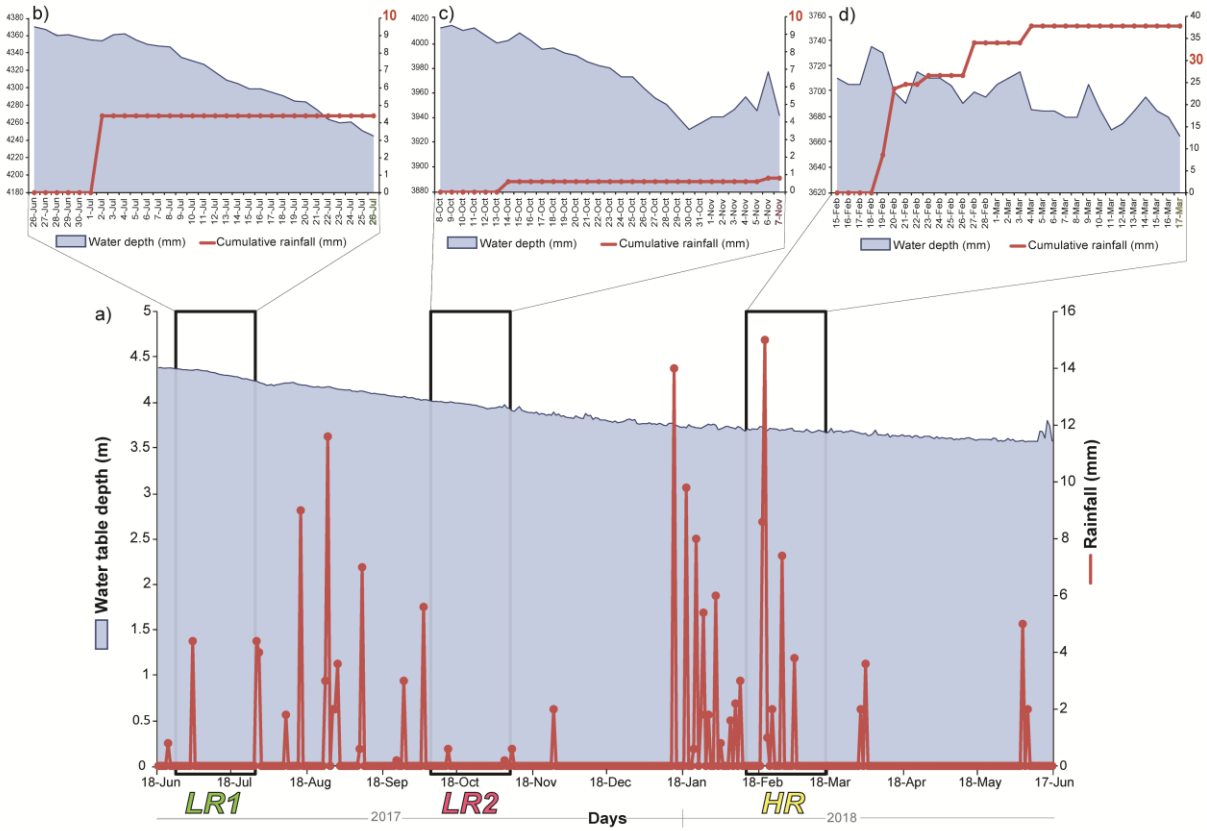

Figure 3.2. Weather station data from Sturt Meadows aquifer (bore E7) water level (in mm, in light blue) and the rainfall events (in mm, in red): a) annual evolution (1 year: from 18/06/2017 to 17/06/2018), b) cumulative rainfall and water depth trend for the 30 days before sampling campaign LR1 (sampling date: 26th of July 2017), c) cumulative rainfall and water depth trend for the 30 days before sampling campaign LR2 (sampling date: 7th of November 2017) and d) cumulative rainfall and water depth trend for the 30 days before sampling campaign HR (sampling date 17th of March 2018). Red numbers in b), c) and d) refer to the category thresholds established by Hyde et al. (2018).

Temperature, pH, ORP, salinity, DO, and depth were measured *in situ*, using portable field measurement equipment (Hydrolab Quanta Multi-Probe Meter®), for all the bores sampled in this study. Water samples for nutrient analysis from the bores J7 (zone A1), W4 (zone A2) and D13 (ZONE CD) were collected by using a 2 L bailer during the sampling campaigns corresponding to LR2 and HR. Bailers were washed with bleach and rinsed with distilled water. Standard pre-purging of the bores was not conducted to preserve in-site specific hydrogeological dynamics and avoid increased sample turbidity, as recommended by Gray et al. (2016) for shallow aquifers in this arid region.

Groundwater samples were stored in 1 L high density polyethylene (HDPE) bottles, immediately frozen and kept at –20°C until further analyses. In the laboratory, nitrites (HACH kit, Method 10207), nitrates (HACH kit, Method 10020), ammonia (HACH kit, Method 8038), phosphates (HACH kit, Method 8048), sulphates (HACH kit, Method 8051) and total alkalinity (CaCO3 mg/L, titration method) were analysed. These parameters were measured to test nutrient concentrations in the system, and detect fluctuations linked with potential recharge processes.

Adult and larval stygofaunal specimens were collected by haul netting, with five hauls of a weighted plankton net with a mesh size of 100 µm (Allford et al., 2008) through the water column in each of the thirty bores sampled. All biological samples were kept frozen (–20°C) in darkness until further processing in the laboratory where individual organisms were counted and identified to the lowest taxonomic level *via* optical microscopy and reference to specific taxonomic keys (e.g. Alarie, Michat, & Watts, 2009; Watts & Humphreys, 2006; King, Bradford, Austin, Cooper, & Humphreys, 2012).

3.2.3 *Statistical analysis*

Physicochemical water parameters - 90 samples in total - were compared across the three rainfall events and the geological zones using a factorial ANOVA (outliers were identified using box plot methods (package 'rstatix' in R software version 3.5.1), homogeneity of variances was tested through the Levene's test (function *leveneTest()* in R 3.5.1) and normality was tested through the Shapiro-Wilk test (function *shapiro.test()* in R 3.5.1)). Tukey's *post hoc* comparisons were used to test pairwise interactions. Nutrients (nitrites, nitrates, ammonia, phosphate and sulphates) and alkalinity trends between LR2 and HR

were tested *via* ANOVAs. Kruskal-Wallis with Dunn's *post hoc* analyses were run to test the same patterns for the number of individuals, α-diversity, Shannon diversity index (H) and Buzas and Gibson's evenness (E).

To investigate niche overlap and co-occurrence, we calculated Pianka's index of niche overlap and the species segregation (co-occurrence) by mean of the Stone and Robert's C-scores (package 'EcoSimR' in R software version 3.5.1). Pianka's index (Levins, 1968) calculates the mean overlap of all possible species pairs. It ranges from 0 (no overlap) to 1 (complete overlap) and allows detection of niche overlaps at basic community-levels in groundwaters (Fattorini et al., 2017). C-scores enable comparison of randomness across sampling events, with lower average index values indicating higher probability that the presence of one species might be affected by the distribution of the others (Ulrich & Gotelli, 2007). While abundance data is employed for the analysis of niche overlapping (*via* Pianka's index), C-scores (randomness of the species assemblages) are calculated by using a presence/absence data matrix. Overall, we employed both indices as descriptive tools to compare potential changes in the community niche occupations across the rainfall regimes (LR1, LR2 and HR).

We investigated the ecological niches by means of Outlying Mean Index (OMI) analysis (Dolédec, Chessel, & Gimaret-Carpentier, 2000), package 'ade4' in R software version 3.5.1. The OMI analysis required the stygofaunal abundance table and the environmental matrix containing the values of the hydrological parameters. Details about the mathematical insights associated with the analysis can be found in Dolédec et al. (2000).

OMI analyses provide the position of each taxonomic group in a two-dimensional Euclidean space by decomposing species distribution patterns into marginality, tolerance and residual tolerance. The marginality of a species (*G*) is defined as the squared Euclidean distance between the mean conditions used by the species and the average environmental conditions (Dolédec et al., 2000). Species are positioned according to their respective deviation to a theoretical ubiquitous species. Species with high marginality values indicate that they occur in less common habitats compared to the rest of the community. Contrarily, low marginality values express a common occurrence of the species within the environment studied. The tolerance, or niche breadth, represents the variance of the environmental condition used by the species. A species can be considered generalist or specialist to the

environment studied by having a high or low tolerance value respectively. The residual tolerance is the quantification of the species niche variability not incorporated in the marginality axes. The niche statistical significance is tested using Monte Carlo permutations by comparing the observed marginality with 1000 simulated marginalities.

Specific seasonal shifts linked with the rainfall patterns were detected by calculating Within Outlying Mean Indexes (WitOMI, 'subniche' package in R version 3.5.1, Karasiewicz et al., 2017). The WitOMI indexes use the environmental space created by the OMI analysis and integrates the characteristics of the K-select analysis (Calenge, Dufour, & Maillard, 2005), enabling niche breakdown into subniches linked with temporal subsets. As a result, interactions such as competition or predation are deciphered at both population and individual scales, and can be further linked with community responses to changes in environmental conditions (Karasiewicz et al., 2017). The subniche parameters are similar to the niche equivalents calculated in the OMI analysis, with the difference that they consider one subset at a time. The environmental subsets of this study were defined by the three rainfall conditions LR1, LR2 and HR. Similar to the OMI analysis, subniche parameters for each species (compared to *G*) were calculated (WitOMI*G*), and additionally compared to the mean environmental condition within a specific subset (G_K) (WitOMIG_K). OMI and WitOMI analytical details for the present study are illustrated in Figure 3.3.

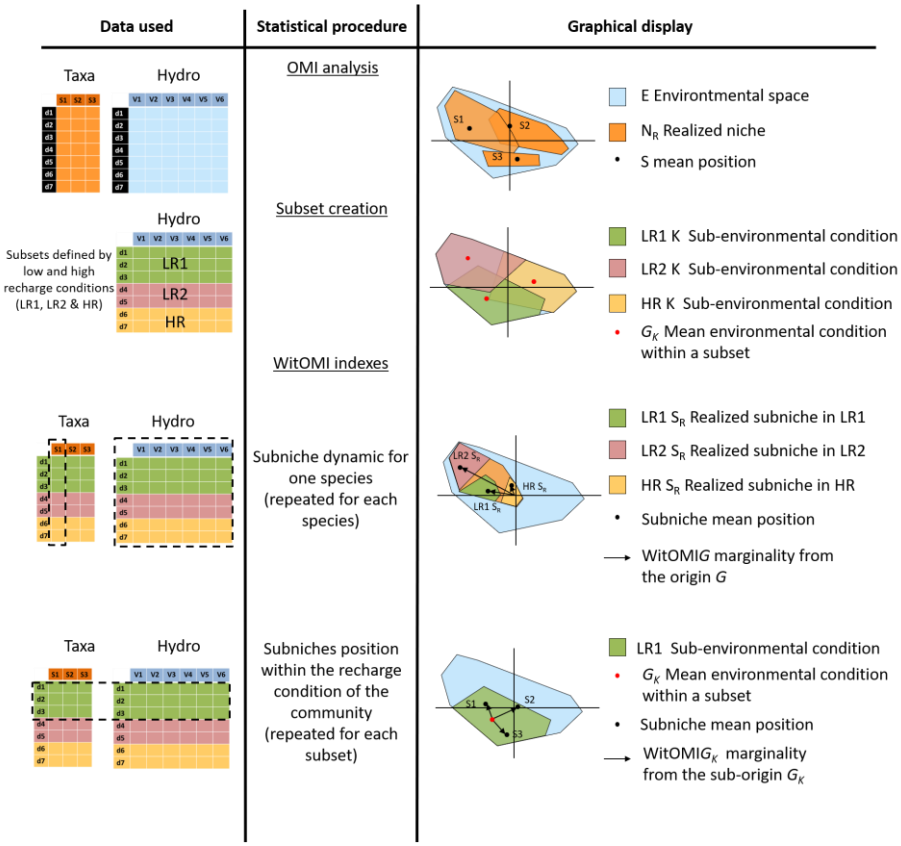

Figure 3.3. Data sets, statistical procedures and graphical displays of the OMI and WitOMI analyses carried out in this study. Taxa: table containing stygofaunal abundance data; Hydro: table containing data from environmental parameters.

3.3 Results

3.3.1 *Environmental gradients*

Figure 3.2 illustrates the annual water level and rainfall trends at Sturt Meadows. Over one year, the water table dropped from 4.4 to 3.6 m. A previous survey in 2006 from the same bore (Hyde et al., 2018) indicated initial and final water levels of 5.26 m and 5.12 m, respectively. Rainfall during our study (171.8 mm) was below the mean annual rainfall for Sturt Meadows (222.7 mm; 100 years of data, Australian Bureau of Metereology). Together, this suggests that Sturt Meadows calcrete groundwater level is in a recessional state (Figure 3.2a).

Table 3.1 summarises the mean values of the hydrochemical parameters across the three rainfall events. Temperature of the groundwater was significantly higher during the HR period (pairwise comparisons: LR1 vs HR: $P < 0.001$, LR2 vs HR: $P < 0.001$). pH was

significantly higher during LR2 when compared with LR1 and HR ($P < 0.001$), and this pattern is linked with interactions between space (geological zones) and time (rainfall regimes) ($P < 0.001$, Table 3.2).

Salinity ($P < 0.05$), DO ($P < 0.005$) and depth ($P < 0.05$) changed significantly between geological zones. Salinity (Tukey's test, *A2 vs A4*, $P < 0.05$) was significantly higher downstream (zone A2) than upstream (zone A4), and deeper calcretes (zone A1) had higher oxygen concentrations when compared with the clay bar area (zone CD) (Tukey's test, *A1 vs CD*, $P < 0.05$) (Table 3.2). These patterns are in accordance with Humphreys et al. (2009) and confirm the influence of a northeast to southwest biogeochemical gradient along the borehole grid. Total alkalinity increased in groundwater after rainfall (*LR2 vs HR,* $P < 0.001$), ammonia increased ($P < 0.001$) and phosphates decreased ($P < 0.001$). All the other nutrients (sulphates, nitrites and nitrates) revealed steady, non-significant trends in time and space (geological zones) (Table 3.1).

Table 3.1. Mean values of the hydrochemical parameters. PSS, Practical Salinity Scale; DO, Dissolved Oxygen; ORP, oxidation reduction potential; *, parameters showing significant trends within rainfall periods (LR1/LR2 and HR; results of the physicochemical pairwise comparisons are illustrated as 'a' and 'b').

Rainfall period	Temperature* (°C)	pH*	Salinity (PSS)	DO (mg L^{-1})	ORP	Depth (m)	NO_2^- (mg L^{-1})	NO^{3-} (mg L^{-1})	NH_3* (mg L^{-1})	PO_4^{3-}* (mg L^{-1})	SO_4^{2-} (mg L^{-1})	Alkalinity* (mg $CaCO_3$ L^{-1})
LR1	22.9 ± 1.7[a]	7.3 ± 0.3[a]	14.4 ± 4.9	3.7 ± 2.2	85.1 ± 126.6	3.6 ± 2.2	n/a	n/a	n/a	n/a	n/a	n/a
LR2	23.3 ± 0.7[a]	7.7 ± 0.5[b]	15.9 ± 3.2	3.7 ± 1.8	74.2 ± 91.6	3.5 ± 2.3	0.01 ± 0	0.81 ± 0.17	1.74 ± 1.10	0.14 ± 0.05	102.56 ± 4.56	205.11 ± 36.61
HR	26.2 ± 0.5[b]	6.7 ± 0.2[a]	16.6 ± 2	4.1 ± 1.5	88.3 ± 33.3	3.3 ± 2.3	0.01 ± 0	0.78 ± 0.11	3.44 ± 0.83	0.05 ± 0.02	103.33 ± 6.48	291.33 ± 28.93

Table 3.2. Results of ANOVA using rainfall periods (LR1, LR2 and HR), geological zone (A1, A2, A3, A4, CD) and their interaction as factors. Significant results are highlighted in bold. d.f. degrees of freedom; PSS, Practical Salinity Scale; DO, Dissolved Oxygen.

	RAINFALL PERIOD			ZONE			ZONE*RAINFALL PERIOD		
	d.f.	F	*P*	d.f.	F	*P*	d.f.	F	*P*
Temperature (°C)	2	82.94	**< 0.001**	4	2.19	0.08	8	1.19	0.32
pH	2	89.24	**< 0.001**	4	1.72	0.16	8	3.06	**< 0.005**
Salinity (PSS)	2	2.94	0.06	4	2.73	**< 0.05**	8	0.27	0.97
DO (mg L^{-1})	2	0.64	0.53	4	4.94	**< 0.005**	8	0.63	0.75
ORP (mV)	2	0.18	0.83	4	1.08	0.37	8	0.40	0.92
Depth (m)	2	0.12	0.88	4	3.20	**< 0.05**	8	0.06	1.00

3.3.2 *Stygofaunal trends*

The collected stygofauna comprised 7424 individuals from 11 taxa belonging to five different Classes: Oligochaeta (Family Tubificidae (Vejdovský 1884)), Subcohort Hydrachnidia, Maxillopoda (Order Harpacticoida (G. O. Sars, 1903) and Cyclopoida (Burmeister, 1834)), Malacostraca (Family Chiltoniidae (Barnard, 1972) (juveniles) and species *Scutachiltonia axfordi* (King, 2012)*, Yilgarniella sturtensis* (King, 2012) and *Stygochiltonia bradfordae* (King, 2012)) and Insecta (species *Paroster macrosturtensis* (Watts & Humphreys, 2006), *Paroster mesosturtens*is (Watts & Humphreys, 2006) and *Paroster microsturtensis* (Watts & Humphreys, 2006) and respective larvae). Table 3.3 illustrates taxa codes and abundances for each bore and geological zone within the three sampling campaigns.

Table 3.3. Species matrix of Sturt Meadows stygofaunal taxa. Abbreviations: ID: Taxonomic Code, LR1: 1st low rainfall period, LR2: 2nd low rainfall period, HR: high rainfall period. Results from 30 bores are displayed (A8 to F13).

RAINFALL PERIOD		LR1																																		
GEOLOGICAL ZONES		A1						A2							A3					CD								A4								
TAXON	ID	A8	C5	F4	H5	J7	N4	L4	S2	U2	W2	Q2	W3	W4	C1	D1	E1	G1	H2	A11	D13	E10	E11	Q3	Q4	S8	B13	C13	E12	E13	F13					
Tubificidae	TU														2																					
Oribatida	OR			2			2					1	1									1		1		3		1	1		2					
Harpacticoida	H	1	3	9	17	18	5	7	13	17	12		8	27	5	15	12	3	72	21	8	19		2	8	27	7	1		6	11					
Cyclopoida	C	18	6	1	11	16	36	8	9	14	2	5	3	3	2	6	1	5	131	92	1	33	11	19	14	55	2	269	3	26	1					
Chiltoniidae (juveniles)	AMJ				15	1		3	15	4	5			1					4								3			6	11					
Scutachiltonia axfordi	AM1		19	1	14		13		1	7		1		1		1		1	9	4	5		2					6		7					
Yilgarniella sturtensis	AM2		1	1	23		13		21		2		2	2				3	2	2	7	2			2			3							
Stygochiltonia bradfordae	AM3			1	3				6	3			1					1	4			2							2						
Paroster macrosturtensis	B		1		1	1							1				1		2								1		11	1	6				
Paroster mesosturtensis	M			3	1		3		9	1	1		1	1	4	1	4	1	3	9	1				1				1	9					
Paroster microsturtensis	S		1		2		1		6							3	2	1	1		6			1					1	5	1				
Paroster macrosturtensis larvae	Blv		1										1				1	1	2	1	1									1					
Paroster mesosturtensis larvae	Mlv								1																		1									
Paroster microsturtensis larvae	Slv		1						1	1	1		3			1	1	1	1		2		2	1	1				1	2					
		LR2																																		
Tubificidae	TU																								1										
Oribatida	OR			1			2		1	1	8	123		3		1		1		1		2			2		1		1	1					
Harpacticoida	H	82	26	5	12	2	18	2	8	2	17	7	4	29	259	7	22		11	6	1	2	3	34	3	3	12		1	2	1					
Cyclopoida	C	86	29	22	24	4	16	2	16	129	7	97	87	19	68	16	31	4	22	35	31	9	29	66	1		6	33	7	81	38					
Chiltoniidae (juvelines)	AMJ	32	6		1	1													6	1		1							2	5					
Scutachiltonia axfordi	AM1	3	1		3	1	1		1	6	1		12	4			2	2		2				1			8	1	9	1	1				
Yilgarniella sturtensis	AM2	28	11		1	2				6		2	11	2		2	6		4	6	1	5		1	1		4		6	4	2				
Stygochiltonia bradfordae	AM3	2	2		1	1	3			1			1				1	1	2	2	1	5	2	1				2	2		1				
Paroster macrosturtensis	B	1				2			2	1	2		7					3		3	1						4		7	3	9				
Paroster mesosturtensis	M	3				1	1			7		3	6	2	2		3			5	22						1			2					
Paroster microsturtensis	S	1				1	3	1			1		7	1		1				1	17						7		2	3					
Paroster macrosturtensis larvae	Blv		1				1						3							6							1								
Paroster mesosturtensis larvae	Mlv																				2														
Paroster microsturtensis larvae	Slv	1	2			3						2	1			2	1			5	6			4			2			1					
		HR																																		
Tubificidae	TU	14	1			1													3			1									1				
Oribatida	OR											29						2	3											3					
Harpacticoida	H	64	44	13	1	32	17	8	4	83	6		5	4	3	7	154	11	19	13	12	2	26	9	2	13	8	3	7	14	4				
Cyclopoida	C	54	91	23	17	22	12	4	8	19	4	2	23	13	15	2	87	1	29	148	77	66	76	15	11	16	137	41	39	36	47				
Chiltoniidae (juveniles)	AMJ	7	8		14	2				3	1			1			8		2	6	2		1			3	2		2	7	4				
Scutachiltonia axfordi	AM1				2		4			13			2	2			2	1	1	1			1				4	2	8		4				
Yilgarniella sturtensis	AM2	2	3		9	4	2			12			7	1			13		2	4		3	4				2	8		5	7				
Stygochiltonia bradfordae	AM3		1							1										1		8						7		1				
Paroster macrosturtensis	B	4			3					3	9		28			3				6			1	3			1		2	5	8				
Paroster mesosturtensis	M	7	2		6	1	3	1	1	1		6	4	2	1		1		1	11		1	6	1	1		4	1		11					
Paroster microsturtensis	S	22	3	3	6	1	3			14	1	3	9	7				1		8			2				7		2	6	5				
Paroster macrosturtensis larvae	Blv									1				1			1		1		2									1					
Paroster mesosturtensis larvae	Mlv																			2		1						1							
Paroster microsturtensis larvae	Slv		1																	2	1						2			4				

Overall, the most abundant taxa were cyclopoids (C) and harpacticoids (H), accounting for 49.99% and 28.27% of the total respectively, followed by the amphipod *Y. sturtensis* (AM2, 4.36%), water mites (OR, 2.95%), the amphipod *S. axfordi* (AM1, 2.8%), juvenile amphipods Chiltoniidae (AMJ, 2.76%) and the beetles *P. mesosturtens*is (M, 2.61%), *P. microsturtensis* (S, 2.42%) and *P. macrosturtensis* (B, 2.22%). The remaining taxa (AM3, Slv, Blv, Tu and Mlv) accounted for 2.6% of the total. The abundance of copepods (H and C), tubificids (TU), juvenile amphipods (AMJ) and beetles (B, M and S) was higher during HR compared with LR1 and LR2, whilst adult amphipods (AM1, AM2 and AM3) were more abundant during LRs (LR1 and LR2) compared to HR. None of these differences were statistically significant. Statistical analysis confirmed that abundances of tubificids (TU, Kruskal-Wallis test, χ^2= 6.7698, $P < 0.05$), water mites (OR, Kruskal-Wallis test, χ^2= 7.8973, $P < 0.05$) and *S. bradfordae* amphipods (AM3, Kruskal-Wallis test, χ^2= 9.2196, $P < 0.05$) changed significantly among rainfall regimes. However, pairwise comparisons between rainfall events showed significant (decreasing) patterns only for the latter two (OR: Dunn's test, *LR2 vs HR, Z* = -2.802977, $P < 0.05$; AM3: Dunn's test, *LR2 vs HR, Z* = -2.974035, $P < 0.05$).

The number of individuals was higher, but not statistically significant, during the HR period (2875) when compared with LR1 (2152) and LR2 (2397), while the average number of taxa per bore was similar, ranging from 6.17 ± 2.35 (LR1) to 6.53 ± 2.71 (LR2). The Shannon diversity index calculated per each bore was higher on average within low rainfall campaigns (S_{LR1} = 1.20 ± 0.48 and S_{LR2}= 1.22 ± 0.48 vs S_{HR} = 1.13 ± 0.33) and average evenness remained steady – with a slight but not statistically significant decrease from LR1 to HR – across the three sampling events (E_{LR1} = 0.61 ± 0.15; E_{LR2} = 0.60 ± 0.18; E_{HR} = 0.57 ± 0.19). None of the diversity patterns commented above revealed significant results. The community was not distributed differently across the five geological areas and the number of individuals, taxa, Shannon and Evenness indexes did not change significantly according to the geological zones across the different rainfall periods.3.3.3 *Ecological niche analysis*

3.3.3.1 *Overlap, co-occurrence and realized niches*

Overall observed niche overlap was 28% for LR1, 27% for LR2 and 32% for HR. This increase was paralleled by a decrease in co-occurrence (C-scores): from C= 22.74 for LR1, to C= 16.71 for LR2 and C= 15.36 under HR conditions (Table S3.5).

The first two OMI axes accounted for 76.30% of the explained variability, with OMI axis one representing 56.55% and OMI axis two 19.74% of the variance. The average marginality of the theoretical ubiquitous species was significant ($P < 0.05$; 1000 Monte Carlo permutations), suggesting an influence of the environmental conditions on the stygofaunal community assemblages (Table 3.4). Temperature ($P < 0.001$) and pH ($P < 0.001$) for LR2 and HR, and temperature ($P < 0.001$) and salinity ($P < 0.05$) for LR1 were the most influential environmental parameters on the taxa's realized niches (Figure 3.4).

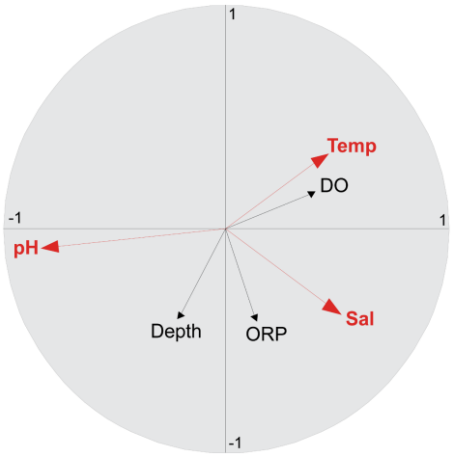

Figure 3.4. Results of the canonical weights of six environmental variables extracted by OMI analysis (axis 1 and axis 2, 76.30% of the variability in the data set). The significant environmental variables are in red. Temp, Temperature; DO, Dissolved Oxygen; Sal, Salinity; ORP, Oxidation Reduction Potential.

Table 3.4. Result of OMI and WitOMI analyses. OMI, Outlying Mean Index; WitOMI*G*, marginalities from the average habitat condition *G*, Tol, tolerance; Rtol, residual tolerance; $\bar{x}$, average marginality.

Rainfall period	All				LR1			LR2			HR		
ID	OMI	Tol	Rtol	*P*	WitOMI*G*	Tol	Rtol	WitOMI*G*	Tol	Rtol	WitOMI*G*	Tol	Rtol
TU	2.00	0.86	7.12	0.16									
OR	1.09	3.06	5.07	**< 0.05**	2.00	3.73	5.81	2.69	1.61	5.37	3.42	0.20	1.46
H	0.04	2.25	5.35	0.13									
C	0.02	1.92	6.07	0.12									
AMJ	0.51	1.13	3.79	**< 0.05**	0.70	0.43	6.04	0.85	0.76	1.82	2.77	0.13	2.38
AM1	0.13	1.03	4.89	0.34									
AM2	0.16	0.92	4.95	0.12									
AM3	0.24	1.47	5.01	0.27									
B	0.46	1.20	3.49	**< 0.05**	1.32	1.70	2.35	1.15	0.63	2.94	3.11	0.38	1.88
M	0.11	1.18	6.94	0.32									
S	0.34	1.10	5.06	**< 0.05**	1.85	1.41	6.62	1.54	0.75	3.31	2.60	0.18	2.59
Blv	0.30	0.58	4.84	0.55									
Mlv	1.96	0.77	5.19	0.17									
Slv	0.20	1.14	5.68	0.37									
$\bar{x}$ (*P*)				**< 0.05**	**< 0.001**			**< 0.001**			**< 0.001**		

The subsets of the environmental conditions associated with each rainfall regime were statistically different from the origin (the realized environmental space) (LR1: $P < 0.001$; LR2: $P < 0.001$; HR: $P < 0.001$; 1000 Monte Carlo permutations, Table 3.4), confirming that the three regimes are associated with differential habitat settings. Overall, stygofauna showed less scattered distributions along the environmental gradients during HR conditions (Figure 3.5). All the taxa showed low OMI values indicating a common use of the habitat (OMI<2). Four taxa (28.5%) out of 14 had a significant niche (OMI, $P < 0.05$, Table 3.4): water mites (OR), juvenile amphipods (AMJ*), P. macrosturtensis* (B) and *P. microsturtensis* (S). Water mites had the highest marginality, (OMI=1.09), and tolerance (TOL= 3.06), while AMJ and B had similar marginality (OMI=0.51 and OMI=0.46) and tolerance (TOL=1.13 and TOL=1.20) values. S had very low marginality (OMI=0.34), indicating a use of the available habitat which is ubiquitous to the community object of study. Overall, during low rainfall conditions (LR1 and LR2) the sub-environmental conditions revealed fewer constraints on the species realized niches than for HR (Figure 3.5).

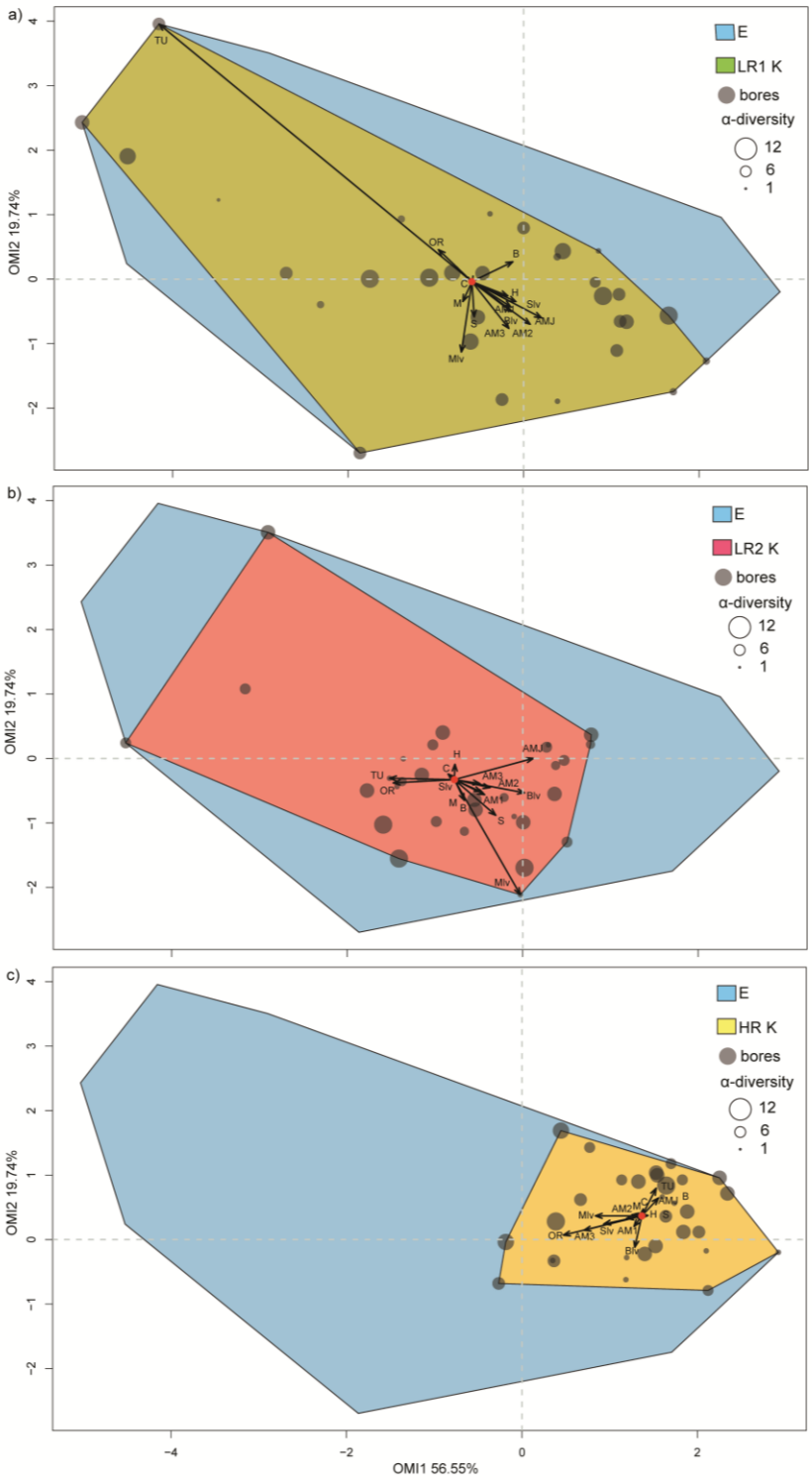

Figure 3.5. Rainfall periods (LR1, LR2 and HR) subset's constraints of habitat conditions (green, red and yellow polygons respectively), found within the overall available habitat conditions (light blue polygons). Bore distribution is depicted with the respective number of taxa (α-diversity) and species subniche positions per each sampling event are also displayed. Red dots represent the suborigins. E, realized environmental space; K, subset realized environmental space. Refer to Table 3.3 for the stygofaunal codes and to Karasiewicz et al. (2017) for further details about the indexes.

3.3.3.2 *Subniche trends*

All the WitOMI were significant (Table S3.6) and the observed changes in realized subniche occupations are linked with the differences between rainfall regimes' subset average environmental conditions (red dots in Figure 3.5). The significant taxa (B, AMJ, OR and S) occupied smaller ecological niches during HR relative to the other rainfall regimes (see Table 3.4 for taxa tolerance (Tol) values).

Apart from water mites (Figure 3.6a), realized subniches were wider for HR than during LR2, and showed the biggest departures from the average conditions (Figure 3.6b, c and d). Water mites (OR) and *P. microsturtensis* (S) occupied wider realized subniches than *P. macrosturtensis* (B) and juvenile amphipods (AMJ), and during the HR regime the latter three species occupied more similar niches than that used by the stygofaunal assemblage if compared with the two low rainfall periods (Figure 3.5 and Figure 3.6). Aside from water mites, the other taxa occupied common environments (OMI values below one, Table 3.4) and they used more marginal habitats (higher WitOMI*G* values) under HR conditions than during LR regimes (lower WitOMI*G* values, Table 3.4). Concurrently, all the significant taxa decreased their tolerance under HR conditions (Table 3.4).

In contrast, the average positions of the two most widely distributed taxa, the cyclopoid and harpacticoid copepods, along the first two axes of OMI analysis (Figure S3.7) were closest to the origin of the axis, indicating wide ecological tolerance to environmental variation. Considering each rainfall category separately, water mites were the only taxon group that revealed higher marginality under HR (WitOMIG_K=2.36) when compared with LR1 (WitOMIG_K=0.59) and LR2 (WitOMIG_K=0.67). In contrast, marginalities of amphipod juveniles (AMJ) and *P. microsturtensis* (S) plummeted under HR conditions, and marginality values for *P. macrosturtensis* (B) were 1.05 (LR1), 0.23 (LR2) and 0.54 (HR) (Supplementary Table 3.2).

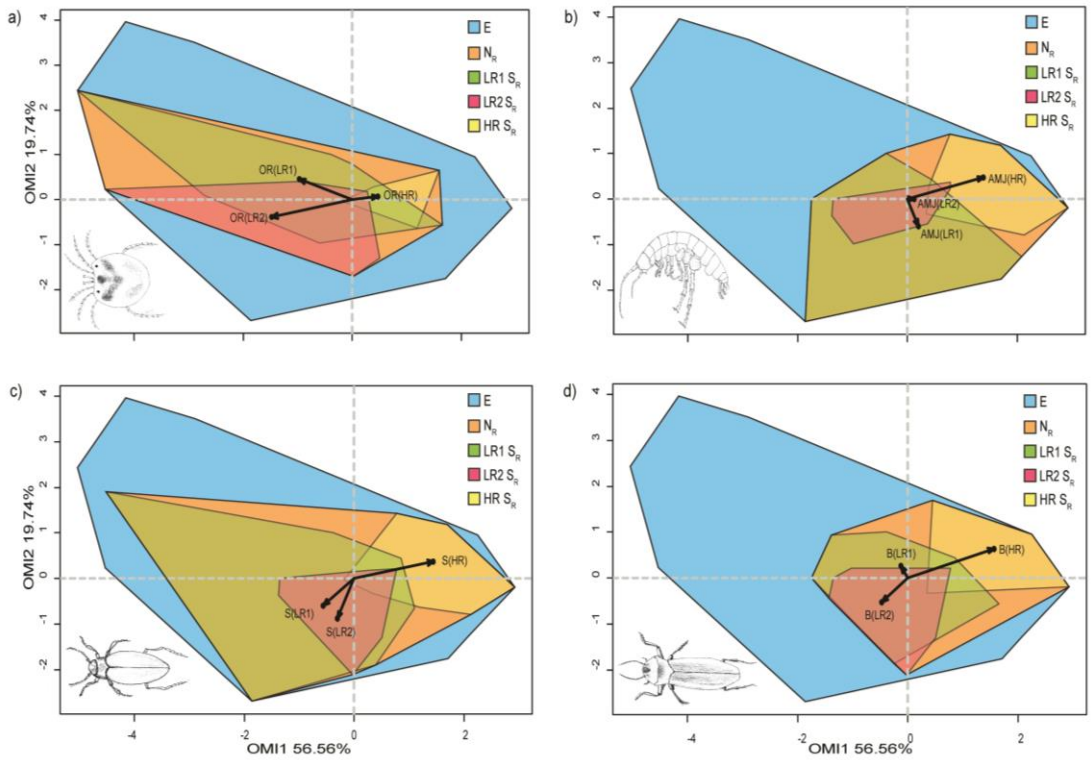

Figure 3.6. Species realized subniches positions of the statistically significant taxa for the OMI and WitOMI analyses: a) water mites (OR), b) amphipods juveniles (AMJ), c) *P. microsturtensis* (S) and d) *P. macrosturtensis* (B). The arrows represent the marginality vectors from the average habitat conditions. E, Environmental space; N_R realized niche; S_R, realized subniche. Refer to Karasiewicz et al. (2017) for further details about the ecological niche indexes.

3.4 Discussion

3.4.1 *Environmental dynamics*

Water level monitoring of the groundwater at Sturt Meadows was conducted under dry conditions when compared with previous years (see Hyde et al., 2018). Such conditions are not unexpected, given that the Australian climate regimes, the driest inhabited continent on earth, have erratic and largely unpredictable patterns (Buys, Miller, & van Megen, 2012). Groundwater recharge is controlled by climatic conditions such as rainfall intensity and frequency, together with water exchange between aquifers and catchments (Datry et al., 2005). With this in mind, the sampling design for the present study not only focused on specific rainfall periods that maybe linked to groundwater recharge into the aquifer, but also incorporated a broad approach involving the yearly long term cyclic alternation between the wettest (January (26.3), February (29.2) and March (30.6), average monthly

rainfall (mm) within parentheses; from BoM) and driest period (from July (17) to December (15.6)) (Figure 3.2a).

Soil conditions such as temperature and moisture are strongly linked with rainfall events (Maxwell, Chow & Kollet, 2007), and play a key role in aquifer recharge (e.g. Edmunds & Wright, 1979; Wooding, Tyler, & White, 1997; Nasta, Adane, Lock, Houston, & Gates, 2018). Although water levels did not respond to the low rainfall amounts, we did see a change in groundwater chemistry. While pH significantly decreased, temperature increased after rainfall (i.e. under HR conditions). A possible explanation for these trends is that at Sturt Meadows the surface soil layers can reach temperatures of up to 70 °C (Saccò's unpublished data). Therefore, when the limited rainfall does occur at the site, it infiltrates the soil zone and the water becomes warmer and more acidic suggesting a recharge pulse has indeed migrated to the water table through either diffuse or point sources.

Rainfall inputs also linked with changes in nutrient concentrations. The significant increase in ammonia is most likely derived from the dissolution and overland transport of animal waste (Bradford et al., 2013; over 1000 bovines feed on the surficial saltbush) across the study site after increased rainfall, and subsequent migration to the groundwater (Kendall, 1998; Mallin & Cahoon, 2003). However, nitrate concentration showed no significant change, suggesting that microbial processing may play an important role in the biogeochemical patterns of the system. Further investigations involving isotopic fingerprinting would allow more refined elucidation of nutrients flows at Sturt Meadows.

Concurrently, the inflow of acidic water under HR conditions (pH = 6.7) has the potential to access phosphorus from its insoluble forms (Cook & Heizer, 1965) and as a result, phosphates significantly decrease in concentration relative to LR2. The groundwater level remained steady across all rainfall periods, and together with ORP, was not a significant driver in determining the stygobiotic dynamics at Sturt Meadows. The DO concentration was higher under HR conditions, suggesting that dissolved oxygen is transported through the aquifer, most likely *via* advection (Malard & Hervant, 1999). Overall, these shifts in water chemistry and nutrient availability might be responsible for driving ecological changes in the stygofaunal community (Humphreys, 2008; Datry et al., 2005).

Local geology (denoted as the five different geological zones) was not a significant driver shaping the stygofaunal assemblages and it was finally discarded for our OMI analysis. This

does not rule out geology being significant in notably different contexts (e.g. karst vs alluvial sediments, or areas with hydrocarbons). It is important to emphasise that our study focused on a considerably smaller scale compared with those more extensive investigations of marginality at regional scales (Dole-Olivier et al., 2009; Galassi et al., 2009).

3.4.2 *Stygofaunal patterns*

Although stygofaunal diversity did not change significantly after the three rainfall periods, some interesting distribution patterns can be unveiled. Copepods (C and H) were the most abundant taxa, and increased their number under HR. Hyde et al. (2018) showed similar patterns for the same system, suggesting that HR events might increase the hydrological connectivity, together with increased nutrient availability, throughout the calcrete at Sturt Meadows and trigger an overall increase in copepods' population sizes. Galassi et al. (2009) reported colonization processes by free-swimming cyclopoids in low water velocity karst systems coupled with harpacticoid dominance in interstitial voids within the sediment (also in Galassi & Laurentiis, 2004; Di Lorenzo et al*.,* 2005). However, our results indicated that among the copepods, cyclopoid populations increased more markedly with increasing rainfall than the harpacticoids, although the differences within these two groups were not significant. This suggests that species-specific and scale-specific patterns may play a role in the observed patterns.

Copepods, together with amphipods, graze biofilms and assimilate microbially-derived DOC and POM, fuelling carbon transfers along the trophic chain and maintaining the biochemical flows in groundwater (Humphreys, 2006; Tomlinson & Boulton, 2010; Galassi et al., 2017). Subterranean amphipods display opportunistic trophic habits characterized by a wide range of feeding modes (filter feeders, scrapers, scavengers, predators) (Hutchins, Schwartz, & Nowlin, 2014). Hartland, Fenwick, and Bury (2011) suggest that switches of feeding modes according to environmental conditions could explain the dominance of amphipods in oligotrophic groundwaters. At Sturt Meadows, abundances of adult amphipods (AM1, AM2 and AM3) decreased through the three rainfall regimes (from LR1 to HR).

The changes in distribution patterns we observed are likely to be linked with differential resource availability after rainfall periods, together with predatory pressures from higher trophic levels. Previous DNA analyses on beetles (B, M and S) at Sturt Meadows provided

evidence of amphipods and copepods as prey items (Bradford, 2010). Our results show that adult beetles increased their abundances through the rainfall regimes (from LR1 to HR), indicating similar potential predator-prey interactions. Abundances of beetle larvae (Blv, Mlv and Slv) were very low, indicating they may occupy different trophic niches to the adults, as suggested by Bradford et al. (2013).

Water mites (OR) did not follow a specific trend, and were most widely distributed (15 bores out of 30) and abundant (149 in total) in the LR2 rainfall period, suggesting that limited rainfall, or stable groundwater conditions, provide the most favourable environmental conditions for this taxonomic group (Irmler, 2004). In contrast, oligochaete tubificids (TU) were most abundant after rainfall, i.e. under the HR regime. As reported by Learner, Lochhead, and Hughes (1978), high water temperatures stimulate rates of growth and asexual reproductions in oligochaetes, and these conditions are likely to be responsible for the population increases observed at Sturt Meadows.

3.4.3 *Ecological niche interactions*

The environmental settings resulting from different rainfall events revealed diverse ecological niche patterns, suggesting specific biotic adaptations to the changes in hydrogeochemistry in the aquifer. Overall, species increased their niche overlaps under HR conditions. Increased rainfall recharge over the site was linked with more suitable environmental conditions and increased overlaps in the use of resources. Concurrently, lower species segregations (C-scores) also indicated that existing interspecific interactions were strengthened under HR conditions. In line with the dynamic reported by Fattorini et al. (2017) in a different context, this result indicates species 'displacements' linked with differential subsurface conditions. Further, WitOMI analysis confirmed a shift in ecological niche occupation (between low rainfall (LR1 and LR2) compared to higher rainfall conditions (HR)) for OR, AMJ, B, and S. Overall, the patterns revealed by AMJ, B and S seem to be driven by dissolved oxygen flux after rainfall (from LR1 and LR2, to HR).

Several studies (e.g. Hakenkamp & Palmer 2000; Dumas, Bou, & Gibert, 2001; Hahn, 2006) have shown the importance of oxygen gradients in shaping stygofaunal assemblages. Dole-Olivier et al. (2009) concluded that the main drivers shaping stygofaunal biodiversity patterns in the French Jura region were salinity and high dissolved oxygen in waters. This is

consistent with our results, where oxygenated conditions and higher resource availability (higher marginality values, Table 3.4) were key factors in shaping shifts in niche occupation. Simultaneously, the four taxa reduced their niche breadths (tolerance) under HR conditions and this, associated with increased levels of specialization (Karasiewicz et al., 2017), seems to indicate biotic strategies driven by higher levels of adaptations to the environmental conditions.

Amphipod juveniles (AMJ) revealed coupled ecological patterns with *P. macrosturtensis* (B) and *P. microsturtensis* (S) (Figure 3.6b, d and c). Our results suggest that the beetles' predatory pressures, reported at Sturt Meadows by Bradford et al. (2013), are predominantly exerted on adult amphipods. In line with this axiom, Sudo and Azeta (1992) suggest that amphipod juveniles in benthic and pelagic environments might be protected from predation because of their small size. In groundwaters, where interstitial voids shape stygofaunal distributions within the aquifer matrix (Hose & Stumpp, 2019), small bodies might represent a practical advantage in avoiding predation. Concurrently, adults usually display greater activity than juveniles, including mate searching behaviour, and this may expose them to more frequent interception by active predators like *P. microsturtensis* or *P. macrosturtensis* (Strong, 1972; Peer, Linkletter, & Hicklin, 1986; Conlan, 1994). However, while reported for surface freshwater environments (e.g. McGrath, Peeters, Beijer, & Scheffer, 2007), there is a lack of evidence for these patterns in groundwaters and they need to be empirically tested.

In another study, Bradford (2010) found discordant diversity patterns among the two sympatric sister species *P. microsturtensis* and *P. macrosturtensis*, and suggested an ecological niche partitioning process occurs within the calcrete environment. Our results illustrate a substantial overlap between *P. macrosturtensis* (B) and *P. microsturtensis* (S) in ecological niche occupation through the three rainfall periods (Figure 3.6d and c). These patterns suggest that the shifts in environmental conditions, and therefore resource availability, provided by rainfall (HR) do not shape differential inter-specific competition. As suggested by Allford et al. (2008), vertical partitioning down the water column, as a result of contrasting oxygen requirements (Jones, Cooper, & Seymour, 2019), is likely to play an important role in beetles' interactions at Sturt Meadows, and further investigations will help unravel these ecological dynamics.

Unlike the other significant taxa, water mites showed contrasting ecological shifts within LR regimes (LR1 and L2), and depth was an influential environmental factor for their biotic assemblages during LR2 (Figure 3.6a). In shallow aquifers, deeper and more oxygenated water columns can provide mites with more accessible and heterogeneous habitats (i.e. voids) that might contain adult beetles to parasitize. Dystiscids host suitable sites such as thorax or abdomen or wings for acari larval attachments (e.g. Smith & Oliver, 1986; Mortazavi, Hajiqanbar, & Lindquist, 2018). However, given the low and scattered abundances of this group, conclusions about specific population dynamics at Sturt Meadows are at risk of bias. Moreover, despite their morphological and physiological adaptations to the aquatic environment (Schatz & Behan-Pelletier, 2008) and their occurrence in the hyporheic zone (Williams, 1993; Irmler, 2004), oribatid mites are unstudied in the region and their adscription to stygofauna is still uncertain. Further species-specific investigations involving different habitats and locations from expanded research areas will allow a better comprehension of the ecological dynamics of this group in calcrete systems (i.e. Sabatino, Cicolani, & Gerecke, 2003).

Overall, residual tolerance values for all the taxa (Table 3.4) indicate that there are environmental drivers present that are not being captured in our data. These results are in line with those presented by other groundwater 'OMI analysis' investigations in Europe (PASCALIS project, Dole-Olivier et al., 2009; Galassi et al., 2009; Martin et al., 2009). Indeed, the quantification of the ecological niches represents one of the major keystones in ecology (Miklos, 1959; Larson, Holden, & Usio, 2010). Several models have attempted to unravel ecological niche determinations *via* different approaches, namely, experimental (e.g. Moore, 2009), mechanistic (e.g. Kearney et al., 2008) and statistical (e.g. Peterson, 2001), and all of them require the definition of 'environmental' conditions (Holt, 2009).

In groundwaters, the habitat characterisation usually includes hydrological and geological parameters coupled with water chemistry parameters (e.g. Datry et al., 2005; Martin et al., 2009; Galassi et al., 2017). Dole-Olivier et al. (2009) suggest that the incorporation of factors like food availability, habitat fragmentation and biotic interactions would considerably improve the analysis of niche dynamics. However, the reduced accessibility of the majority of subsurface ecosystems is a crucial limiting factor for the integration of broader sampling techniques (Allford et al., 2008). Therefore, additional perspectives, such as the integration of genetic and geochemical information, need to be brought to the field.

Metagenomics (branded environmental DNA, eDNA) is an emerging and extremely powerful biomonitoring tool able to unravel stygofaunal functions and biotic community structures (Niemiller et al., 2018). Providing the crucial linkage between the stygofaunal and microbial communities, this technique has the potential to unravel crucial biochemical mechanisms that will provide novel insights into groundwater ecology investigations. Given the recent advances towards a multidisciplinary focus (e.g. Hancock et al., 2005; Danielopol & Griebler, 2008; Humphreys, 2008; Murray et al., 2008; Steube et al., 2009; Fleckenstein, Krause, Hannah, & Boano, 2010), the field of groundwater ecology can benefit from the incorporation of designs from different disciplines such as hydrology, isotope geochemistry and genetics (Saccò et al., 2019).

Overall, the stygofaunal community at Sturt Meadows aquifer displayed broad ecological tolerances, tendencies in line with several other investigations (e.g. Martin et al., 2009; Schulz, Steward & Prior, 2013). Our results show that ecological shifts are driven by the influx of dissolved oxygen from rainfall. However, climate change, linked with anthropogenic pressures such as overexploitation and contamination, puts at risk the maintenance of the delicate ecological balance sustaining these communities (Spengler & Hahn, 2018). Further investigations involving whole-system approaches, considering spatiotemporal ecological dynamics of the aquatic fauna and their linkage to microbial assemblages (e.g. Žutinić et al., 2018), are needed (Datry et al., 2005) to address how our indisputably changing climate will affect the aquatic biota in groundwaters, one of the most distinct and understudied ecosystems on Earth.

Acknowledgements

We wish to acknowledge the traditional custodians of the land at Sturt Meadows, the Wongai people. We pay respect to their Elders past, present and emerging, and their continuing connection to land, waters and culture in the Yilgarn region. The authors thank Dr Elizabeth Ooi, Bernardo Dewey and Lucila López Gibson for their amazing attitude and crucial assistance with field collections. Flora, Peter and Paul Axford of the Sturt Meadows pastoral station are thanked for their kindness and generosity in providing both accommodation and access to their property.

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Supplementary material

Table S3.5. Overlap (Pianka' index) and co-occurrence (Stone and Robert's C-scores) observed and expected overlap for null assemblages. RA2 (relaxed niche breadth) and RA3 (retained niche breadth) for the overlap and sim9 for the co-occurrence are the algorithms used for the null assemblages.

Rainfall period	Observed overlap	RA2 Expected overlap	RA3 Expected overlap	Observed co-occurrence	sim9 Expected co-occurrence
LR1	0.284	0.319	0.214	22.736	22.560±0.065
LR2	0.269	0.354	0.191	16.714	16.629±0.058
HR	0.317	0.350	0.212	15.363	15.014±0.094

Table S3.6. Environmental preferences parameters of the 14 invertebrate taxa sampled during LR1, LR2 and HR. *P* and *SubniP* number of 1000 Monte-Carlo random permutations that yielded a higher value than the observed marginality (OMI, WitOMI*G* or WitOMIG_K) (in bold significant values). OMI, Outlying Mean Index (marginality); WitOMI*G*, marginalities from the average habitat condition *G*; WitOMIG_K, subset marginality; Tol = tolerance, Rtol = residual tolerance; $\bar{x}$, average marginality.

Rainfall period		All		LR1			LR2			HR		
	OMI	WitOMI*G*	WitOMIG_K									
ID	*P*	*SubniP*	*SubniP*	WitOMIG_K	Tol	Rtol	WitOMIG_K	Tol	Rtol	WitOMIG_K	Tol	Rtol
TU	0.16	**< 0.001**	**< 0.001**									
OR	**< 0.05**	**< 0.001**	**< 0.001**	0.59	3.00	6.54	0.67	3.46	3.51	2.36	0.76	0.90
H	0.13	**< 0.001**	**< 0.001**									
C	0.12	**< 0.001**	**< 0.001**									
AMJ	**< 0.05**	**< 0.001**	**< 0.001**	1.05	1.40	5.07	1.54	0.97	1.61	0.04	0.19	2.32
AM1	0.34	**< 0.001**	**< 0.001**									
AM2	0.12	**< 0.001**	**< 0.001**									
AM3	0.27	**< 0.001**	**< 0.001**									
B	**< 0.05**	**< 0.001**	**< 0.001**	1.05	0.46	3.59	0.23	0.47	3.11	0.54	0.73	1.53
M	0.32	**< 0.001**	**< 0.001**									
S	**< 0.05**	**< 0.001**	**< 0.001**	0.70	0.55	7.48	0.65	0.66	3.39	0.02	0.49	2.29
Blv	0.55	**< 0.001**	**< 0.001**									
Mlv	0.17	**< 0.001**	**< 0.001**									
Slv	0.37	**< 0.001**	**< 0.001**									
$\bar{x}$	**< 0.05**	**< 0.001**	**< 0.001**									

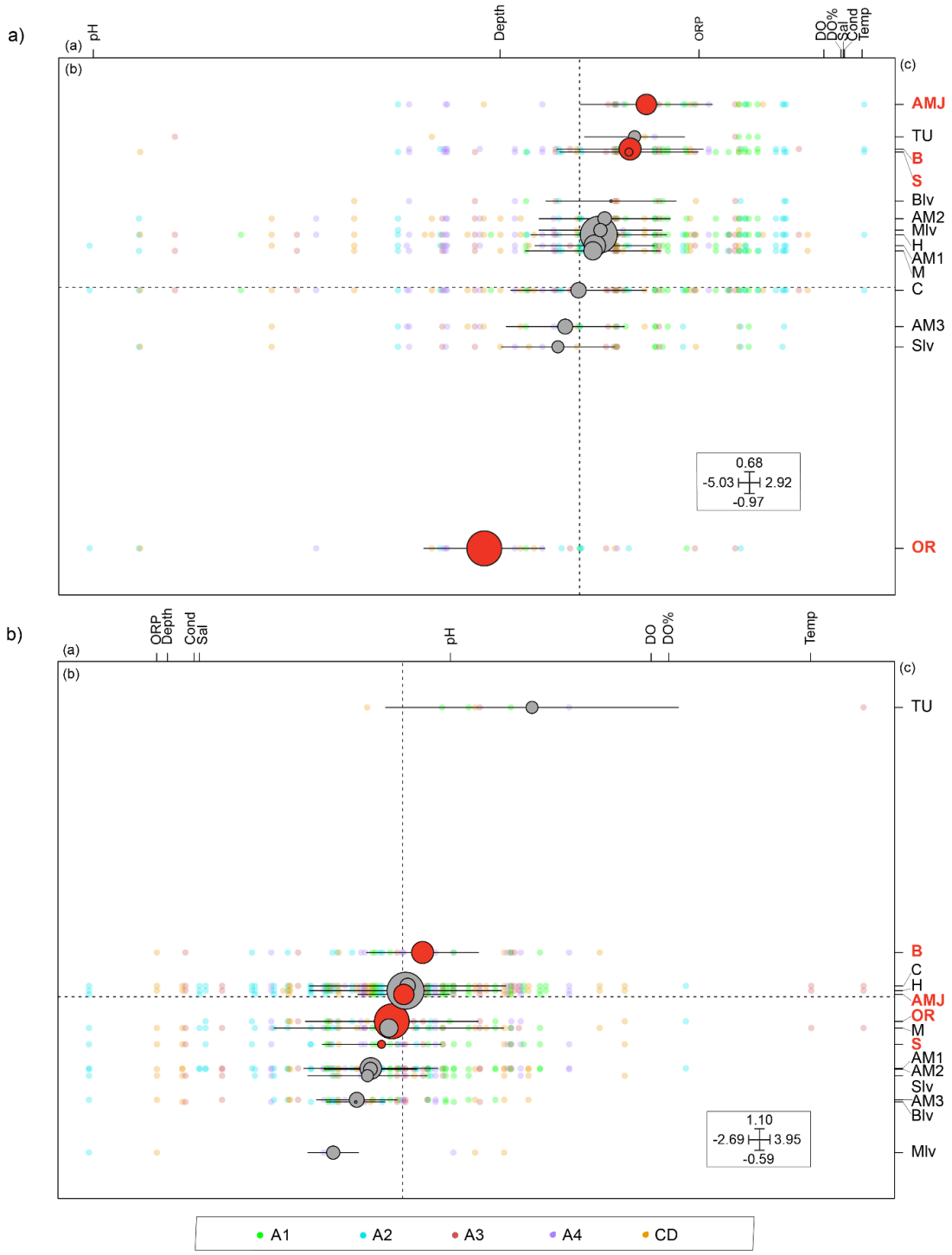

Figure S3.7. First (a)) and second (b)) axes extracted by the OMI analysis, which respectively explain 56.55% and 19.74% of the variability in the stygofaunal dataset: (a) Environmental gradients (8 variables) along the first axis; (b) taxa distribution along the environmental gradient. Small coloured circles represent bores where species occurred (separated by geological areas), while large grey circles illustrate the occurrence of a given taxa (proportional to the size of the circle) at its centroid (mean position) along the environmental gradient. Species with a statistically significant marginality are shown in red. Horizontal lines shows the standard deviation; (c) ordination of the 14 stygofaunal taxa along the first axis using their factorial scores.

Chapter 4 | Elucidating stygofaunal trophic web interactions *via* isotopic ecology

Published as a research article in *PLOS ONE* 14(10) (2019).

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Abstract

Subterranean ecosystems host highly adapted aquatic invertebrate biota which play a key role in sustaining groundwater ecological functioning and hydrological dynamics. However, functional biodiversity studies in groundwater environments, the main source of unfrozen freshwater on Earth, are scarce, probably due to the cryptic nature of the systems. To address this, we investigate groundwater trophic ecology *via* stable isotope analysis, employing $\delta^{13}C$ and $\delta^{15}N$ in bulk tissues, and amino acids. Specimens were collected from a shallow calcrete aquifer in the arid Yilgarn region of Western Australia: a well-known hot-spot for stygofaunal biodiversity. Sampling campaigns were carried out during dry (low rainfall: LR) and the wet (high rainfall: HR) periods. $\delta^{13}C$ values indicate that most of the stygofauna shifted towards more ^{13}C-depleted carbon sources under HR, suggesting a preference for fresher organic matter. Conversion of $\delta^{15}N$ values in glutamic acid and phenylalanine to a trophic index showed broadly stable trophic levels with organisms clustering as low-level secondary consumers. However, mixing models indicate that HR conditions trigger changes in dietary preferences, with increasing predation of amphipods by beetle larvae. Overall, stygofauna showed a tendency towards opportunistic and omnivorous habits - typical of an ecologically tolerant community - shaped by bottom-up controls linked with changes in carbon flows. This study provides baseline biochemical and ecological data for stygofaunal trophic interactions in calcretes. Further studies on the carbon inputs and taxa-specific physiology will help refine the interpretation of the energy flows shaping biodiversity in groundwaters. This will aid understanding of groundwater ecosystem functioning and allow modelling of the impact of future climate change factors such as aridification.

Key-words: stygofauna, $\delta^{13}C$ and $\delta^{15}N$ CSIA, SIA, food web, rainfall, groundwater, calcrete aquifer.

4.1 Introduction

During recent decades, investigations of trophic webs have become a cornerstone for the interpretation of functional biodiversity in freshwater ecosystems. Within both lentic and lotic environments, macroinvertebrate food web dynamics play a key role in shaping process-level aquatic ecosystem attributes [1]. Aquatic faunal trophic characterization is usually conducted by employing the morpho-behavioural based concept of functional feeding groups (FFGs) [2]. Since its inception, FFGs have been extensively used in ecological assessments and biomonitoring studies, and have allowed detailed assessment of ecological patterns in both natural and disturbed environments [3,4,5].

However, despite the hydraulic and ecological continuum in groundwater dependent ecosystems, the subsurface ecosystem and the study of its food chain interactions have suffered from a conceptual disconnection from surficial aquatic habitats. The main reasons are attributable to methodological limitations [6,7], scarce aquifer accessibility [8] and the lack of interdisciplinary approaches [9]. Moreover, compared to surface freshwater ecosystems, groundwaters are subjected to relatively extreme environmental conditions: sparse organic inputs, lack of light and primary production, and truncated trophic webs [10,11,12,13]. Altogether, these unique conditions shape obligate subterranean aquatic communities (stygofauna) dominated by plastic and opportunistic trophic behaviours [14,15], whose categorization *via* feeding modes such as FFGs is constantly at risk of misinterpretation. As a result, our knowledge about how food web interactions shape groundwater ecological functioning and community patterns is fragmented [16].

Stygofauna - when present - play a key role in regulating both ecological and hydrological dynamics in aquifers [17,18]: they actively bioturbate the sediment, facilitate nutrient recycling and, in combination with microbial communities, degrade/retain contaminants. In groundwaters, carbon inputs (allochthonous dissolved organic carbon (DOC) and chemoautotrophic production) are mediated by microbes which are then grazed by stygofauna at the base of the food chain [19]. Organic matter (OM) is transferred along the trophic chain *via* prey-predator interactions. Therefore, OM inputs, microbial communities, and the stygofaunal trophic web, all shape the energy flows sustaining the subterranean biodiversity [20].

The incorporation of biogeochemical approaches (i.e. stable isotopes composition, fatty acids content, radiocarbon analysis) has recently led to re-evaluation of the archetype of poorly structured – and generalist-dominated – trophic dynamics in groundwaters [21]. These designs are leading a vital transition from purely descriptive to functionally-based investigations, providing wider perspectives to the field [22].

Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope analysis (SIA) is a well-established approach enabling quantitative investigation of food webs [23,24]. Since its initial application in groundwater trophic ecology, several studies have benefited from the insights provided by the study of naturally-occurring stable isotopes [25,26]. However, $\delta^{13}C$ and $\delta^{15}N$ SIA investigations on bulk material are limited by the mixing of tissues and different biochemical pathways [27]. These limitations can be addressed by the complementary or alternative use of compound-specific approaches.

$\delta^{13}C$ and $\delta^{15}N$ Compound Specific Isotope Analysis (CSIA) on amino acids (AAs) allows detailed characterization of food web interactions [28], by focusing on compounds created by definable biosynthetic pathways. Single amino acids can be divided into essential (EAA) and non-essential (NEAA). Whilst primary producers (plants, algae and bacteria) biosynthetise *de novo* EAA from a bulk carbon pool, animals lack these enzymatic pathways and acquire EAA from their diet [29]. As a result, tracking of EAA allows carbon fingerprinting of food sources down to the base of food webs [30]. Concurrently, $\delta^{15}N$ CSIA can distinguish between compounds reflecting the source isotopic signal, and that enriched with each trophic step, thus providing crucial information on prey-predator interactions [31]. The application of CSIA in amino acids has allowed a much more thorough understanding of food web dynamics in freshwater [32], marine [33] and terrestrial environments [34], but despite the greater potential than bulk analysis [35], this technique has yet to be applied to food web studies of groundwater environments.

This study is, to our best knowledge, the first based on the combination of carbon and nitrogen CSIA in groundwaters, and focuses on a calcrete stygofaunal community under two contrasting environmental conditions: low rainfall (LR, dry season) and high rainfall (HR, wet season). We hypothesise that different environmental conditions trigger species-specific adaptations that are ultimately responsible for distinct food web interactions. The specific objectives of this work are: 1) unravel OM incorporation trends across the

stygofaunal community, 2) decipher the trophic habits of the species and elucidate prey-predator interactions and 3) provide biochemically-based knowledge about trophic web interactions in arid zone calcrete aquifers.

4.2 Methodology

4.2.1 *Study area and field work*

The field work was carried out at a calcrete aquifer (28˚41'S 120˚ 58'E) located on Sturt Meadows pastoral station, Western Australia, ~42 km from the settlement of Leonora (833 km northeast of Perth, Figure 4.1a).

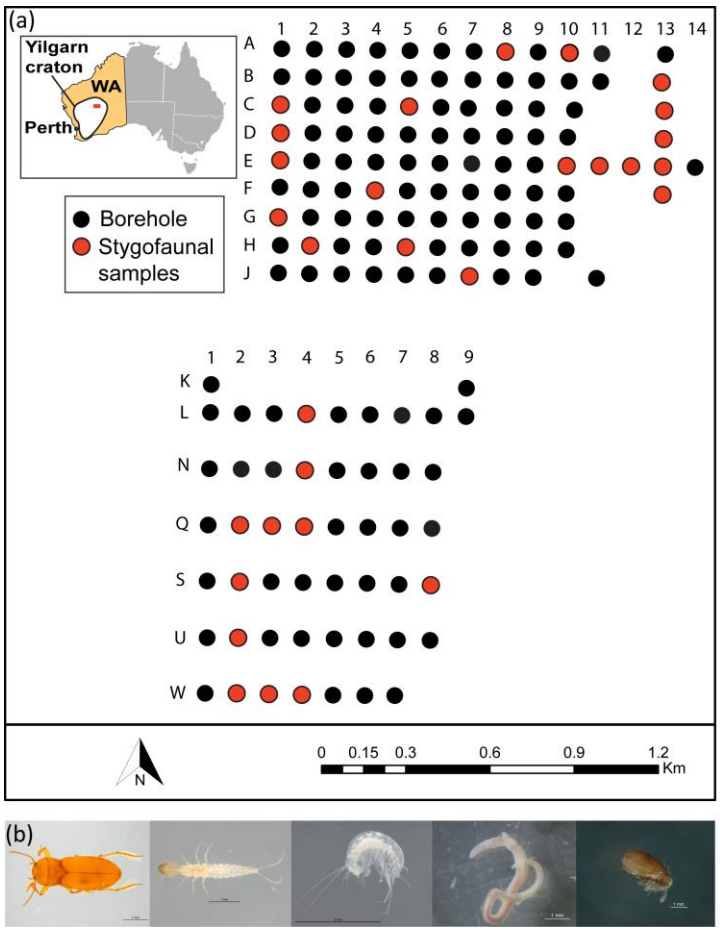

Figure 4.1. a) Borehole grid and its location in the Yilgarn region, Western Australia. b) Photos of some specimens from the bore samples (from left to right *Paroster macrosurtensis* adult, *Paroster microsturtensis* larvae, *Scutachiltoni axfordi*, Oligochaeta sp. and Oribatida sp.).

The Sturt Meadows calcrete hosts a very shallow aquifer, located two to four metres below the surface, and accessible through bores characterised by water depths ranging from a few centimetres to ten metres. The bore grid was initially drilled for mineral exploration and comprises 115 bore holes of between 5-11 m in depth forming a 1.4 km by 2.5 km (3.5 km^2) area (Figure 4.1a). The bores are unlined, except for about the upper 0.5 m which are lined with 10 cm diameter PVC pipe for stability, and capped [36]. Three sampling campaigns – two of them corresponding to low rainfall periods (LR) and one during the wet season (high rainfall, HR) [37] – were carried out in July and November 2017, and March 2018. More details about the sampling design, monitoring of water depth and hydrogeological background at Sturt Meadows can be found in Saccò et al. [38].

The high morphologically (Figure 4.1b) and taxonomically diverse stygofaunal community at Sturt Meadows comprises three sister species of subterranean beetles (*Paroster macrosturtensis* (Watts & Humphreys 2006), *Paroster mesosturtensis* (Watts & Humphreys 2006) and *Paroster microsturtensis* (Watts & Humphreys 2006) and respective larvae)), three species of amphipods (*Scutachiltonia axfordi* (King, 2012), *Yilgarniella sturtensis* (King, 2012) and *Stygochiltonia bradfordae* (King, 2012)), aquatic worms (family Tubificidae (Vejdovský, 1884)) and water mites (order Oribatida; Dugès, 1834). Within the stygobiotic meiofaunal community, two species of harpacticoids (*Novanitocrella cf. aboriginesi* (Karanovic, 2004), *Schizopera cf. austindownsi* (Karanovic, 2004) and four species of cyclopoids (*Halicyclops kieferi* (Karanovic, 2004), *Halicyclops cf. ambiguous* (Kiefer, 1967), *Schizopera slenderfurca* (Karanovic & Cooper, 2012) and *Fierscyclops fiersi* (De Laurentiis et al., 2001)) can be found.

Adult and larval stygofaunal specimens were collected by hauling a small weighted plankton net (mesh 100 µm, [36]) five times from the bottom through the water column of 30 boreholes (Figure 4.1a) selected by simple random sampling [38]. Stygofaunal abundance data across the boreholes are reported in Table S4.4.

All biological samples were kept frozen (–20°C) in darkness until further processing in the laboratory where individual organisms were counted and identified (and consequently separated) to the lowest taxonomic level *via* optical microscopy and reference to specific taxonomic keys. Roots and sediment samples from the bottom of the aquifer were obtained through the stygofaunal haul netting procedure, and were separated by using

sterile glass pipettes during the sorting in the laboratory according to the sampling campaign (LR or HR). Sediment samples were soaked in acid (0.1 N HCl) to remove inorganic carbon and dried at 60 °C for 24 hours.

Given the delicacy of the hydrological dynamics in shallow calcretes [39], extensive water extractions spread along the bores were avoided and preliminary tests were carried out to quantify the potential risk of dewatering the calcrete. Bores D13 and W4 host groundwater systems which are representative of the geological conformations of the area - phreatic and vadose calcretes interspersed with clay material - and were finally selected because of their hydrological and biotic stability (lowest risk of drying and representative ranges of Sturt Meadows stygofaunal diversity) [38]. Water samples for POC (particulate organic carbon) analysis were collected using a submersible centrifugal pump (GEOSub 12V Purging Pump) after wells were purged of three well-volumes and stabilisation of in-field parameters was observed. POC samples were obtained by filtering water from the bores D13 and W4 through GF/F filters (pre-combusted for 12 hours at 450 °C), washed with 1.2 N HCl to remove any inorganic carbon, and subsequently dried at 60 °C for 24 hours. The field site was accessed and samples were collected with permit approval (permit number 08-003150-1) from the Department of Parks and Wildlife of Western Australia.

4.2.2 *Sample preparation and study design*

Prior to sample preparation for analytical tests, images of adults and larvae of beetles *P. macrosturtensis*, *P. mesosturtensis and P. microsturtensis* were prepared at the Western Australian Museum (Perth) using the Leica Application Suite version 4.6 utilizing multiple images taken with a Leica DFC 500 digital camera, attached to the Leica MZ16A microscope. All individuals from a single taxon were then pooled for each sampling campaign (LR1, LR2 or HR) and subsequently washed with MilliQ water to remove external contaminants. Subsequently, samples were oven dried at 60 °C overnight and crushed to a fine powder which was stored at –20 °C until further analysis (Figure 4.2).

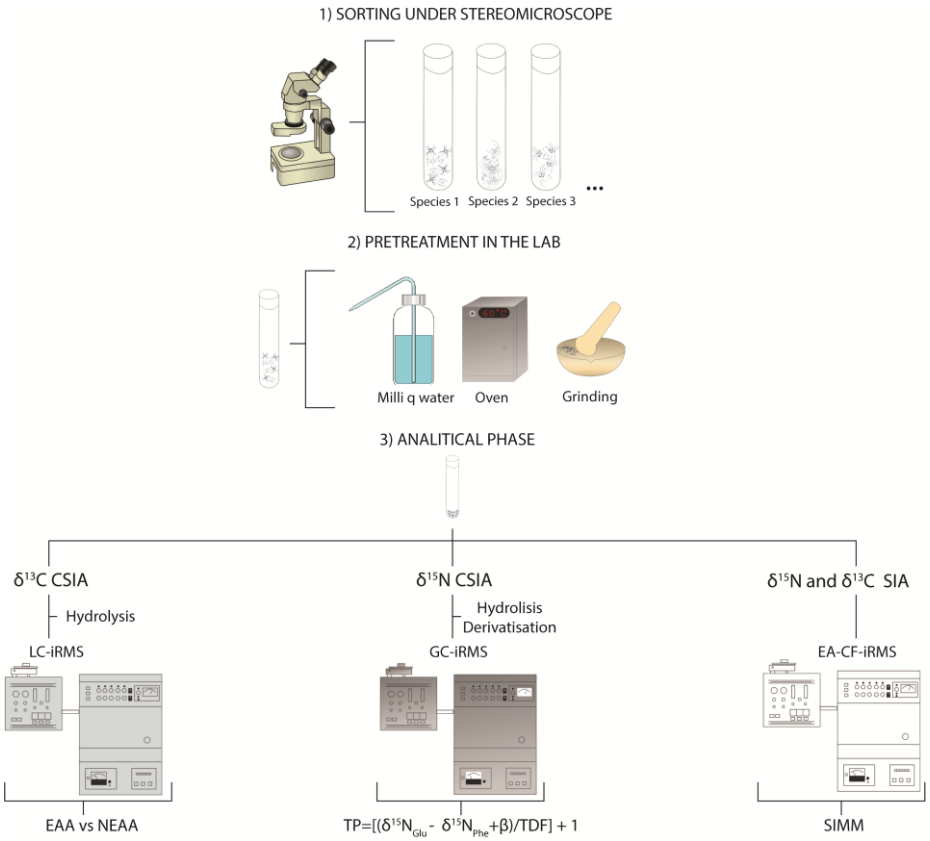

Figure 4.2. Methodological scheme of the study for stygofaunal samples (including copepods for bulk SIA). EAA: essential amino acids; NEAA: non-essential amino acids; TP: trophic position; TDF: trophic discrimination Factor; β = ratio between $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values in primary producers; SIMM: stable isotopes mixing models; LC-iRMS: Liquid Chromatography-isotope Ratio Mass Spectrometry; GC-iRMS: Gas Chromatography-isotope Ratio Mass Spectrometry; EA-CF-iRMS: Elemental Analyser-Continuous Flow-isotope Ratio Mass Spectrometry.

Due to sample size constraints, the samples for each taxon from the two low rainfall periods were further combined. Previous metagenomics investigations, together with mesocosm experiments and field observations at Sturt Meadows provided some information about the trophic habits of beetles and amphipods [40]. Adult subterranean beetles had active predatory feeding on epigean amphipods and copepods (including group feeder behaviours) together with scavenger habits (and potential active predatory pressures) on sister species. Beetle larvae (third and last instar) showed opportunistic predatory habits with a range of prey from copepods and amphipods to adult beetles from the three species (inter and intraspecific cannibalism), while amphipods displayed predation of copepods, epilithic biofilm grazing, root shredding and sediment filter feeding.

4.2.3 *Bulk stable isotope analysis*

$\delta^{13}C$ and $\delta^{15}N$ SIA on bulk homogenised samples of sediment, roots and stygofauna (respectively 1.28 mg, 0.08-0.14 mg and 0.63-2.79 mg per samples, Table S4.5) were performed at the Australian Nuclear Science and Technology Organisation (ANSTO, Sydney). Samples were loaded into tin capsules and analysed with a continuous flow isotope ratio mass spectrometer (CF-IRMS, Delta V Plus, Thermo Scientific Corporation, U.S.A.), interfaced with an elemental analyser (Thermo Fisher Flash 2000 HT EA, Thermo Electron Corporation, U.S.A.) following the procedure of Mazumder et al. [41]. $\delta^{13}C$ values are reported in per mil (‰) relative to the Vienna Peedee Belemnite (VPDB), while $\delta^{15}N$ values are reported relative to reference N_2 of known nitrogen isotopic composition (in ‰), previously calibrated against the AIR international isotope standard. $\delta^{13}C$ POC (0.6 mg, Table S4.5) was analysed at the Western Australian Biogeochemistry Centre at The University of Western Australia using a GasBench II coupled with a Delta XL Mass Spectrometer (Thermo-Fisher Scientific). Results have a precision of ± 0.10 ‰, and are reported relative to the NBS19 and NSB18 international carbonate standard [42].

4.2.4 *Single amino acid carbon and nitrogen isotope analysis*

4.2.4.1 *$\delta^{13}C$ CSIA*

Stygofaunal samples (0.16-2.89 mg per sample, Table S4.5) were hydrolysed under vacuum with 0.5 to 1 mL of amino acid-free 6 M HCl (Sigma-Aldrich) at 110 ˚C for 24 h. The protein hydrolysates were dried overnight in a rotary vacuum concentrator and stored in a freezer. Prior to analysis, the samples were resolved in Milli-Q water and 10 µL of 1-mmol solution of 2-aminoisobutyric acid (Sigma-Aldrich) was added as internal standard. The sample stock had a concentration of approximately 8 to 10 mg/mL, which was further diluted as needed. Single amino acid carbon isotope analysis was carried out at the La Trobe Institute for Molecular Sciences (LIMS, La Trobe University, Melbourne, Australia) using an Accela 600 pump connected to a Delta V Plus Isotope Ratio Mass Spectrometer *via* a Thermo Scientific LC Isolink (Thermo Scientific).

The amino acids were separated using a mixed mode (reverse phase/ion exchange) Primesep A column (2.1 x 250 mm, 100 °C, 5 µm, SIELC Technologies) following the chromatographic method described in Mora et al. [43], after Smith et al. [44]. Mobile

phases are those described in Mora et al. [45]. Percentage of Phases B and C in the conditioning run, as well as flow rate of the analytical run and timing of onset of 100% Phase C were adjusted as needed. Samples were injected onto the column in the 15 µL - partial loop or no waste - injection mode, and measured in duplicate or triplicate.

4.2.4.2 *$\delta^{15}N$ CSIA*

CSIA nitrogen analyses were undertaken at the Organic Geochemistry Unit of the University of Bristol, UK. To extract the AAs, crushed samples (2.47-5.19 mg per sample, Table S4.5) were hydrolysed in culture tubes (6 M HCl, 2 mL, 100 °C, 24 h). A known quantity of norleucine (1 mg mL^{-1} in 0.1 M HCl) was added to each sample as an internal standard prior to hydrolysis. After heating, the tubes were allowed to cool then after centrifugation (3000 rpm, 5 min) the supernatant containing the hydrolysate from each tube was transferred to a clean culture tube and dried under N_2 whilst being heated to 70°C. Once dry, each sample was re-dissolved in 0.1 M HCl and stored in the dark at -18°C until required for analysis.

The derivatisation procedure followed Styring et al. [46] and included isopropylation, with a 4:1 mixture of 2-propanol and acetyl chloride heating to 100 °C for 1 hour, the reaction was quenched by rapidly cooling in a freezer. After removing the residual solvents under N_2, acetylation of the amino group was achieved by adding a 5:2:1 mixture of acetone, triethylamine and acetic anhydride then heating to 60°C for 10 minutes before being allowed to cool. The derivatised AAs were isolated *via* liquid-liquid separation, residual solvent being removed by evaporating under N_2. Samples were again stored at -18°C until required for analysis.

A Thermo Finnigan Delta Plus XP isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) was used to determine the $\delta^{15}N$ values of derivatised AAs. The mass spectrometer (EI, 100 eV, three Faraday cup collectors for m/z 28, 29 and 30) was interfaced to a Trace 2000 gas chromatograph *via* a Combustion III interface (CuO/NiO/Pt oxidation reactor maintained at 980°C and reduction reactor of Cu wire maintained at 650°C), both from Thermo Scientific.

Samples were dissolved in ethyl acetate and 1 µL of solution was injected *via* a PTV injector. Helium at a flow of 1.4 mL min^{-1} was used as the carrier gas and the mass spectrometer source pressure was maintained at 9 X 10^{-4} Pa. The separation of the AAs was accomplished

using a DB-35 capillary column (30 m X 0.32 mm i.d., 0.5 mm film thickness; Agilent Technologies, Winnersh, UK). The oven temperature of the GC started at 40°C where it was held for 5 min before heating at 15 °C min^{-1} to 120 °C, at 3 °C min^{-1} to 180 °C, at 1.5 °C min^{-1} to 210 °C and finally at 5 °C min^{-1} to 270 °C and held for 1 min. A Nafion dryer removed water and a cryogenic trap was employed to remove CO_2 from the oxidised and reduced analyte.

All the $\delta^{15}N$ values are reported relative to reference N_2 of known nitrogen isotopic composition, previously calibrated against the AIR international isotope standard, introduced directly into the ion source in four pulses at the beginning and end of each run. Each reported value is a mean of duplicate $\delta^{15}N$ determinations. A standard mixture of derivatised AAs of known $\delta^{15}N$ values was analysed every three runs in order to monitor instrument performance.

4.2.5 *Data treatment and statistical analysis*

Only AAs that returned results for each taxon were considered. EAA and NEAA were separated according to the classification provided by Boudko [47]. EAAs were used in the interpretation of carbon flows - and potential shifts in OM incorporations - because they persist through the trophic chain [48] due to the little fractionation they undergo when incorporated into consumer's tissue [49]. NEAA, which are subjected to much greater fractionation because of their *de novo* biosynthesis mainly from intermediates of the Krebs cycle (serine (Ser), glycine (Gly) and alanine (Ala)) and glycolysis (glutamic acid (Glx), aspartic acid (Asx) and proline (Pro)) [50], were compared to EAA to investigate taxa-specific carbon isotopic trends (biosynthesis vs assimilation through diet) across the two rainfall periods (LR and HR).

All the statistical analyses were performed in R software version 3.6.0 (Development-Core-Team, 2016). Analysis of variance (ANOVA; outliers were identified using box plot methods (package 'rstatix'), homogeneity of variances was tested through the Levene's test (function *leveneTest()*) and normality was tested through the Shapiro-Wilk test (function *shapiro.test()*)) coupled with Tukey's HSD pairwise comparisons (R-package 'stats') was employed to inspect significant differences between $\delta^{13}C_{EAA}$ (Val, Phe and Arg) and $\delta^{13}C_{NEAA}$ (Krebs (Ser, Gly and Ala) and glycolysis (Asx, Glx and Pro) cycles) within the different rainfall conditions (LR and HR). Principal component analyses (PCA, R-package 'vegan') and Linear

Discriminant Analysis (LDA, R-package 'vegan') among EAA were performed to explore sample distribution in the multi-dimensional space. Determination of EAA driving sample variability in the PCA was carried out *via* function *fviz_contrib* (R-package 'factoextra').

Trophic positions (TP) were calculated using the methodology reported by Chikaraishi et al. [33]:

$$TP = [(\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + \beta)/TDF] + 1$$

where $\delta^{15}N_{Glu}$ = $\delta^{15}N$ of glutamic acid, $\delta^{15}N_{Phe}$ = $\delta^{15}N$ of phenylalanine, β = ratio between $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values in primary producers, and TDF = the trophic discrimination factor at each shift of trophic position.

Incorporation of source carbon from terrestrial vegetation has previously been reported at Sturt Meadows, with roots from surficial saltbush vegetation (C3 metabolism) frequently found in the groundwater [40]. β was accordingly assigned the value of +8.4 ± 1.6 ‰, which is the established value for aquatic food webs involving C3 plants [31]. Although other carbon sources are possible in groundwaters, as they are not established in this system, a conservative approach has been taken in using the value of an evidenced source. TDF was assigned the value of 7.6 ± 1.2‰, based on Steffan et al. [51] who showed it did not vary across trophic levels one to four in multiple controlled-feeding experiments, and for trophic levels one to five in a natural food chain, using terrestrial arthropod species [28].

Pairwise comparisons for $\delta^{15}N$ were carried out with the same approach as for the carbon CSIA data. Robustness and consistency between CSIA and SIA data from beetles and amphipods were inspected using Pearson correlations (function *rcorr* in R-package 'Hmisc'). SIMM (Stable Isotope Mixing Models, R-package 'simmr') were then applied to establish dietary proportions of the key ecological taxa (Figure 4.2). Since a specific trophic discrimination factor has not been calculated for stygofauna, we used the widely accepted values of 3.4 ± 2 ‰ for nitrogen and 0.5 ± 1 ‰ for carbon [52]. Markov chain Monte Carlo (MCMC) algorithms were used for simulating posterior distributions in SIMM, and MCMC convergence was evaluated using the Gelman-Rubin diagnostic by using 1.1 as a threshold value for analysis validation.

4.3 Results

4.3.1 *Stygofaunal carbon fluxes*

During LR, $\delta^{13}C$ average values of AAs ($\delta^{13}C_{NEAA[LR]}$ and $\delta^{13}C_{EAA[LR]}$) spanned from -31.52 ‰ (Phe) to -5.72 ‰ (Gly). Similar values were found under HR conditions ($\delta^{13}C_{NEAA[HR]}$ and $\delta^{13}C_{EAA[HR]}$), ranging from -31.55 ‰ (Phe) to -4.92 ‰ (Ser) (Table 4.1).

Table 4.1. Low (LR) and high rainfall (HR) carbon amino acids spectrum ($\delta^{13}C$ values) for stygofaunal specimens separated by non-essentials (NEAA: aspartic acid (Asx), serine (Ser), glutamic acid (Glx), glycine (Gly), alanine (ala), and proline (Pro)), and essentials (EAA: valine (Val), phenylalanine (Phe), and arginine (Arg)). Average values (and standard deviation) for the analytical replicates are shown. *P* values for ANOVA Tukey's HSD pairwise comparisons between NEAA and EAA are also illustrated.

Taxon	ID	NEAA						EAA			NEAA[2] vs EAA[3]
		Asx	Ser	Glx	Gly	Ala	Pro	Val	Phe	Arg	
LR											
Paroster macrosturtensis	B	-17.64±0.59	-10.43±0.1	-18.74±0.53	-10.01±0.12	-20.01±0.12	-17.85±0.53	-24.39±0.55	-24.44±0.13	-19.56±0.55	*P* <0.05
Paroster mesosturtensis	M	-18.85±0.6	-11.32±0.16	-20.3[1]	-12.22±0.6	-21.23±0.52	-15.10±0.63	-24.55±0.27	-26.24±0.23	-20.42±0.07	*P* <0.05
Paroster microsturtensis	S	-22.42±0.18	-12.39±0.62	-22.42[1]	-14.84±0.64	-24.24±0.35	-18.67±0.62	-27.73±0.64	-28.98±0.07	-23.19±0.54	*P* <0.05
Paroster macrosturtensis larvae	Blv	-20.56±0.11	-9.22±0.18	-20.20[1]	-14.17±0.6	-22.01±0.58	-19.31±0.6	-25.97±0.17	-26.48±0.12	-20.59±0.24	*P* <0.05
Paroster mesosturtensis larvae	Mlv	-19.09±0.04	-6.70±0.18	-20.05±0.43	-16.12±0.65	-21.12±0.37	-16.38±0.3	-24.92±0.09	-27.08±0.1	-19.59±0.3	*P* <0.05
Paroster microsturtensis larvae	Slv	-19.1±0.5	-8.41±0.05	-17.75±0.03	-14.20±0.63	-21.57±0.15	-20.38±0.2	-25.34±0.07	-27.02±0.38	-18.59±0.21	*P* <0.05
Scutachiltonia axfordi	AM1	-16.1±1.3	-5.87±0.8	-14.09±1.4	-5.72±1.01	-20.42±2.88	-16.68±0.2	-24.55±2.01	-21.94±0.28	-15.31±0.74	*P* <0.05
Yilgarniella sturtensis	AM2	-19.09±0.08	-8.31±0.41	-21.35±3.77	-6.81±0.42	-20.29±0.09	-16.63±0.12	-25.48±0.17	-25.6±1.29	-19.56±1.39	*P* <0.05
Stygochiltonia bradfordae	AM3	-21.7±0.1	-9.15±0.64	-24.65±4.38	-9.00±0.33	-24.08±0.19	-22.76±0.64	-28.84±0.25	-28.27±0.1	-23.54±0.38	*P* <0.05
Tubificidae sp.	OL	-21.7±0.24	-16.01±0.44	-24.33±0.31	-20.93[1]	-26.36±0.1	-25.78±0.36	-31.46±0.1	-31.52±0.23	-27.58±0.04	*P* <0.05
Oribatida sp.	OR	-20.44±0.63	-11.99±0.23	-17.77[1]	-14.12±0.47	-21.18±0.07	-19.31±0.61	-26.36±0.03	-24.33±0.55	-18.85±0.2	0.0955
HR											
Paroster macrosturtensis	B	-18.67±0.45	-11.62±0.29	-18.19±0.59	-11.31±0.11	-19.648±0.45	-16.83±0.56	-25.44±0.64	-26.3±0.61	-20.40±0.6	*P* <0.05
Paroster mesosturtensis	M	-23.88[1]	-18.06[1]	-23.56[1]	-16.64[1]	-25.96[1]	-21.99[1]	-29.768[1]	-31.08[1]	-26.05[1]	*P* <0.05
Paroster microsturtensis	S	-20.87[1]	-11.46[1]	-20.8[1]	-13.94[1]	-22.62[1]	-17.82[1]	-26.773[1]	-29.04[1]	-22.63[1]	*P* <0.05
Paroster macrosturtensis larvae	Blv	-21.64±0.55	-12.58[1]	-20.63[1]	-13.56[1]	-24.222±0.1	-19.43[1]	-28.12±0.51	-28.22±0.57	-22.35±0.58	*P* <0.05
Paroster mesosturtensis larvae	Mlv	-23.88[1]	-18.06[1]	-23.56[1]	-16.94±0.42	-25.97[1]	-21.99[1]	-29.38±0.55	-31.08[1]	-26.05[1]	*P* <0.05
Paroster microsturtensis larvae	Slv	-24.15±0.35	-18.03±0.59	-24.24±0.01	-18.74[1]	-26.1115±0.11	-22.19[1]	-30.074[1]	-31.55[1]	-26.59±0.2	*P* <0.05
Scutachiltonia axfordi	AM1	-23.73±0.6	-12.36±0.21	-23.55±0.35	-12.89±0.26	-24.128±0.47	-21.68±0.46	-29.52±0.52	-29.56±0.43	-23.80±0.54	*P* <0.05
Yilgarniella sturtensis	AM2	-23±0.01	-13.67±0.25	-23.60±0.42	-14.92±0.32	-26.2775±0.65	-24.03±0.45	-30.87±0.13	-30.31±0.63	-24.99±0.58	*P* <0.05
Stygochiltonia bradfordae	AM3	-23.39±0.2	-12.37±0.62	-22.68±0.01	-13.19±0.01	-23.8365±0.14	-21.41±0.51	-28.7±0.22	-28.28±0.52	-22.94±0.11	*P* <0.05
Tubificidae sp.	OL	-20.25[1]	-14.11[1]	-20.25[1]	-15.6[1]	-23.94[1]	-20.47[1]	-27.93[1]	-28.79[1]	-21.62[1]	*P* <0.05
Oribatida sp.	OR	-20.42±0.64	-4.921	-22.11±0.61	-5.9±0.11	-22.191±0.09	-19.69±0.01	-27.52±0.11	-27.41	-21.02±0.39	*P* <0.005

[1] Unique run

[2] Calculated as average value of Ser, Gly and Ala

[3] Calculated as average value of Val, Phe and Arg

$\delta^{13}C_{NEAA[LR]}$ average values varied from -26.36 ‰ (Ala) to -5.72 ‰ (Gly), similar values to $\delta^{13}C_{NEAA[HR]}$ spanning from -26.28 ‰ (Ala) to -4.92 ‰ (Ser). Overall, $\delta^{13}C_{EAA}$ showed trends towards more negative values than $\delta^{13}C_{NEAA,}$ which is involved in both Krebs and glycolytic cycles (ANOVA, $P < 0.005$). This is consistent with the enrichment of NEAA during biosynthesis in the organism. With the exception of water mites (OR), *P. macrosturtensis* larvae (Blv), *Y. sturtensis* (AM2), *S. bradfordae* (AM3) under LR, and oligochaetes (OL) under HR, pairwise comparisons between $\delta^{13}C_{EAA}$ and $\delta^{13}C_{NEAA}$ confirmed a shift towards more negative values across the stygofaunal community (Table 4.1).

Neither PCAs nor LDAs on EAA distinguished different clusters within taxa or main groups (adult and larval beetles, and amphipods) nor among different rainfall periods (LR and HR). All three EAA correlated positively and significantly ($P < 0.005$), with phenylalanine and valine being the most informative AAs explaining the isotopic variability across stygofauna (~70%). $\delta^{13}C$ values of valine ($\delta^{13}C_{Val}$) and phenylalanine ($\delta^{13}C_{Phe}$) show that, with the exception of *P. microsturtensis* (S) and *S. bradfordae* (AM3), the entire stygofaunal community experienced a significant shift towards more ^{13}C-depleted values under HR (ANOVA, $P < 0.005$) (Figure 4.3, Table 4.2). Within the significant trends, *P. macrosturtensis* adults and larvae (B and Blv) showed the smallest change in carbon values (B: $\delta^{13}C_{Val+Phe}$= -2.91; Blv: $\delta^{13}C_{Val+Phe}$ = -3.89) between rainfall regimes, while amphipods *S. axfordi* and *Y. sturtensis* (AM1 and AM2) showed the largest depletion (AM1: $\delta^{13}C_{Val+Phe}$= -12.59 ‰; AM2: $\delta^{13}C_{Val+Phe}$= -10.10 ‰), suggesting differential carbon incorporations under HR conditions.

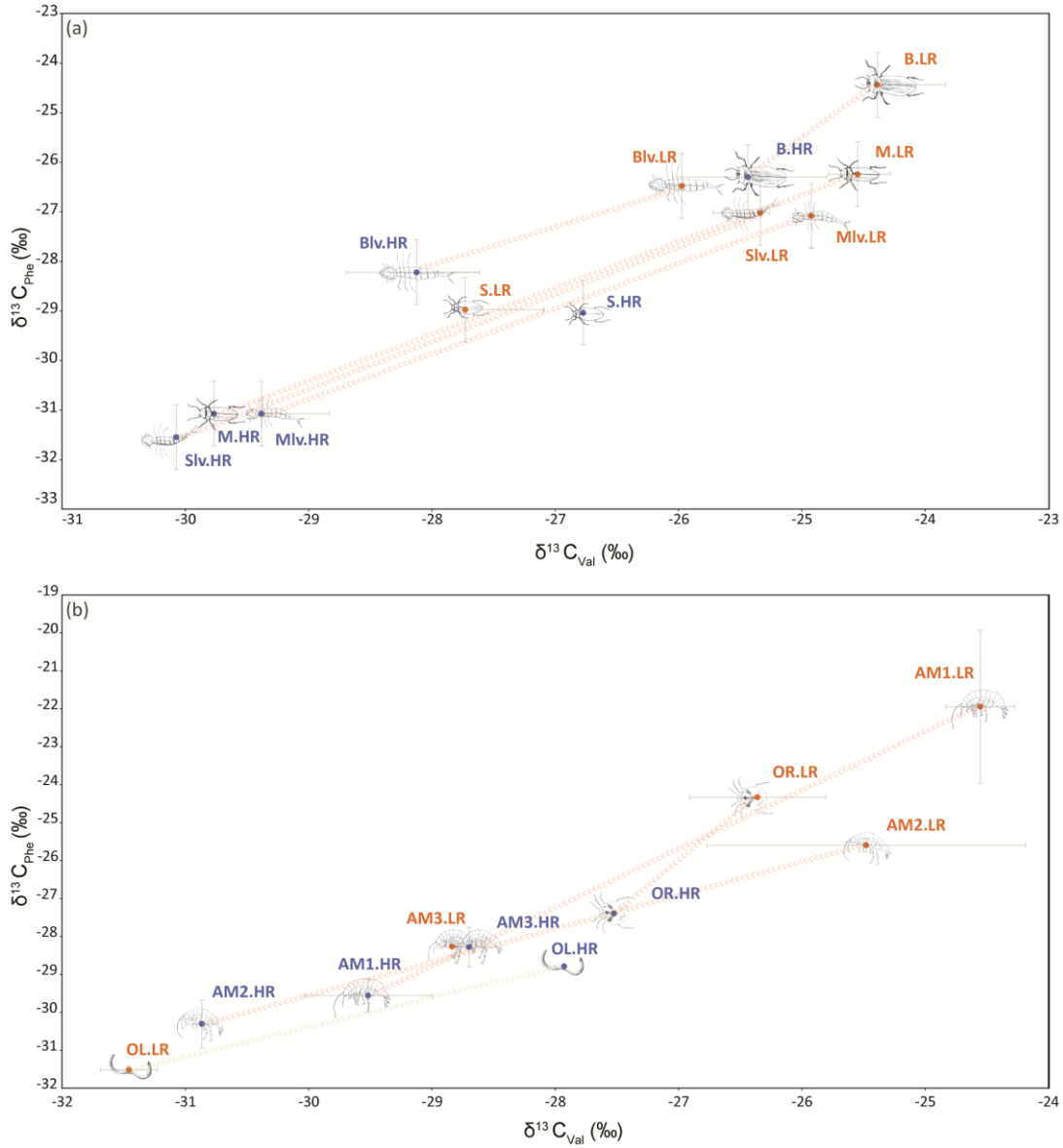

Figure 4.3. Biplot of $\delta^{13}C_{Phe}$ values vs. $\delta^{13}C_{Val}$ values for a) beetles (B, M, S, Blv, Mlv and Slv) and b) amphipods (AM1, AM2 and AM3), water mites (OR) and aquatic worms (OL). Red arrows indicate significant decreasing trends between LR and HR, while green arrows indicate increasing trends within rainfall periods. Refer to Table 4.1 for taxa IDs.

Table 4.2. Tuckey's post hoc pairwise comparisons between phenylalanine and valine values under low (LR) and high (HR) rainfall conditions. In bold significant results.

		Phe			Val		
		d.f.	T-ratio	*P*	d.f.	T-ratio	*P*
Paroster macrosturtensis	B	28	-4.497	**<.0005**	28	-2.163	**<.005**
Paroster mesosturtensis	M	28	-4.846	**<.0001**	28	-3.297	**<.005**
Paroster microsturtensis	S	28	-0.149	0.8829	28	1.967	0.0592
Paroster macrosturtensis larvae	Blv	28	-4.218	**<.0005**	28	-4.42	**<.0005**
Paroster mesosturtensis larvae	Mlv	28	-9.657	**<.0001**	28	-9.16	**<.0001**
Paroster microsturtensis larvae	Slv	28	-10.933	**<0.001**	28	-9.73	**<.0001**
Scutachiltonia axfordi	AM1	28	-18.4	**<.0001**	28	-10.2	**<.0001**
Yilgarniella sturtensis	AM2	28	-11.383	**<.0001**	28	-11.067	**<.0001**
Stygochiltonia bradfordae	AM3	28	-0.037	0.9704	28	0.282	0.7797
Tubificidae sp.	OL	28	-7.418	**<.0001**	28	-2.389	**<0.05**
Oribatida sp.	OR	28	6.594	**<.0001**	28	7.252	**<.0001**

4.3.2 *δ¹⁵N and trophic levels*

$\delta^{15}N_{Glu}$ average values varied between 15.40±0.40‰ ($AM3_{[HR]}$) and 22.31±0.29‰ ($M_{[HR]}$), while $\delta^{15}N_{Phe}$ values ranged from 10.67±0.45‰ ($AM3_{[HR]}$) to 14.53±0.06‰ ($M_{[HR]}$). When converted to trophic positions, the stygofaunal community at Sturt Meadows shows a truncated trophic chain, clustering around the secondary consumer level (Figure 4.4).

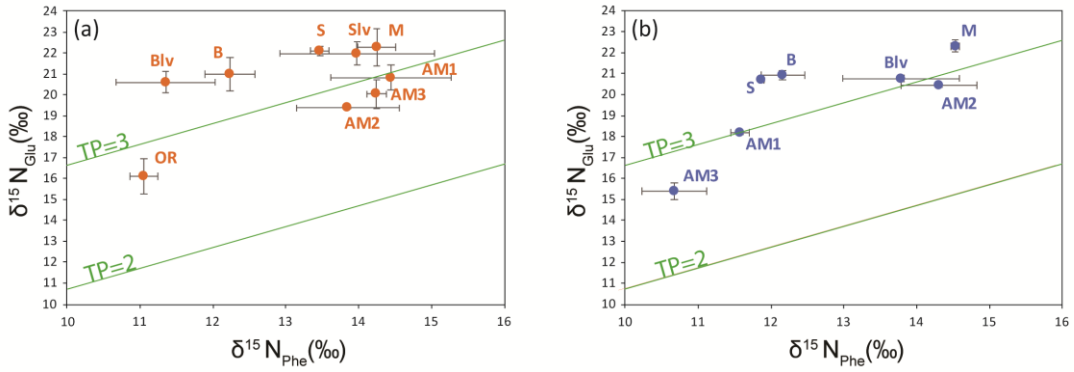

Figure 4.4. Calculated trophic positions (TP) of the stygofaunal specimens studied under LR (a) and HR (b) conditions.

Under LR conditions, *P. macrosturtensis* larvae (Blv) show the highest trophic position (TP=3.33±0.02), while water mites (OR) sit at the lowest (2.78±0.09). Under HR conditions, *P. microsturtensis* adults (S) have the highest trophic position (3.27±0.01), whilst *S. bradfordae* (AM3) show the lowest value (TP=2.73±0.01). Due to the low abundances it was not possible to analyse biochemical fingerprints from water mites ($OR_{[HR]}$: 37 individuals) and *P. microsturtensis* larvae ($Slv_{[HR]}$: 10 individuals) during the wet season (HR) (Table S4.4).

Overall, adult beetles (B, M and S) revealed higher trophic levels (TP>3) than amphipods (AM1, AM2 and AM3, TP<3). However, B, M and S did not show statistically higher values than AM1 under LR, the same pattern seen in *P. mesosturtensis* (M) under HR. *S. bradfordae* (AM3) and *P. macrosturtensis* larvae (Blv) are the only organisms to show a statistically significant change in their TP values between LR and HR (Table 4.3), both with decreasing trends.

Table 4.3. $\delta^{15}N_{Glu}$, $\delta^{15}N_{Phe}$ and TP values (±SD) during LR and HR regimes. Pairwise comparisons within taxa from the same rainfall conditions and between rainfall periods (in bold significant patterns) for the same taxa are also illustrated. Taxa sharing the same letter do not differ significantly (Tukey's HSD test, $P < 0.05$).

	$\delta^{15}N_{Glu}$(‰)		$\delta^{15}N_{Phe}$(‰)		TP		TP pairwise comparison		
	LR	HR	LR	HR	LR	HR	LR	HR	LRvsHR
B	20.99±0.79	20.93±0.23	12.23±0.34	12.16±0.30	3.26±0.06	3.26±0.01	de	e	0.9712
M	22.29±0.89	22.31±0.29	14.25±0.26	14.53±0.06	3.17±0.08	3.13±0.03	bcde	cde	0.5415
S	22.12±0.23	20.69±0.08	13.47±0.13	11.87±0.03	3.25±0.01	3.27±0.01	de	e	0.6698
Blv	20.61±0.5	20.77±0.14	11.35±0.68	13.79±0.80	3.33±0.02	3.03±0.09	e	bcd	**<.0001**
Slv	21.99±0.55	Na	13.98±1.06	Na	3.16±0.21	Na	bcde	Na	Na
AM1	20.84±0.62	18.19±0.1	14.44±0.83	11.57±0.13	2.95±0.03	2.98±0.01	abcd	bc	0.6193
AM2	19.38±0.01	20.45±0.08	13.85±0.7	14.31±0.52	2.84±0.09	2.92±0.08	abc	b	0.135
AM3	20.04±0.7	15.4±0.4	14.24±0.13	10.67±0.45	2.87±0.07	2.73±0.01	ab	a	**<.05**
OR	16.11±0.85	Na	11.05±0.19	Na	2.78±0.09	Na	a	Na	Na

4.3.3 *Food web dynamics*

CSIA-based TP correlated significantly with SIA $\delta^{15}N$ and $\delta^{13}C$ values both under LR ($P < 0.05$ in both cases) and HR conditions ($P < 0.01$ and $P < 0.05$ respectively). Under the latter conditions, $\delta^{13}C_{Val}$ values correlated significantly with CSIA-based TP ($P < 0.05$). Copepods are generally thought to sit at the base of the food web [53,54]. However, these were analysed only *via* bulk SIA due to organism and sample size constraints, and so could not be included in the TP analysis. They showed more ^{13}C-depleted $\delta^{13}C$ (cyclopoids: $\delta^{13}C_{LR}$ = -20.5‰, $\delta^{13}C_{HR}$= -21.9‰; harpacticoids: $\delta^{13}C_{LR}$= -20.6‰, $\delta^{13}C_{HR}$= -23.5‰) and enriched $\delta^{15}N$ (cyclopoids: $\delta^{15}N_{LR}$ = 13.9‰, $\delta^{15}N_{HR}$ = 14.5‰; harpacticoids: $\delta^{15}N_{LR}$ = 11.9‰, $\delta^{15}N_{HR}$ = 15.8‰) values under HR (Figure 4.5a and b), indicating that the change in rainfall regimes could play a role in stygobiotic meiofaunal biochemical incorporations.

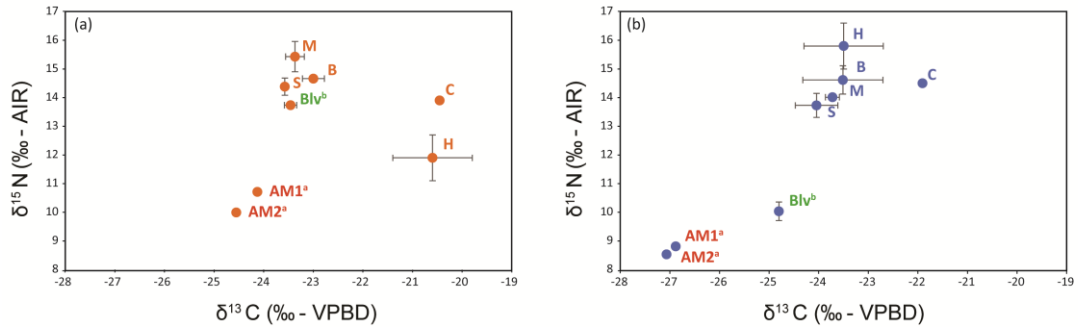

Figure 4.5. SIA biplots of adults *P. macrosturtensis* (B), *P. mesosturtensis* (M), *P. microsturtensis* (S), *P. macrosturtensis* larvae (Blv), *S. axfordi* (AM1), *Y. sturtensis* (AM2), Cyclopoida sp. (C) and Harpacticoida sp. (H) under low rainfall (a) and high rainfall (b). AM1^{a} and AM2^{a} (in red): taxa showing the biggest depletion in $\delta^{13}C$ values for essential amino acids (phenylalanine and valine) across rainfall conditions; Blv^{b} (in green): taxon showing the biggest drop in trophic position value (TP) between LR and HR. Refer to Table S4.6 for $\delta^{13}C$ and $\delta^{15}N$ values of the taxa.

Amphipods AM1 and AM2 sat at the base of the trophic web under both rainfall conditions (TPs always below three, Table 4.3), and SIA carbon values ($\delta^{13}C$) confirmed a shift – already pinpointed *via* CSIA - towards more ^{13}C-depleted carbon sources under HR. AM3, the smallest and rarest amphipod species in the calcrete, did not allow bulk SIA analyses due to the low abundances detected (LR (average value between LR1 and LR2): 27; HR: 19, Table S4.4).

With respect to dietary preferences, for the amphipod *S. axfordi* (AM1), mixing models suggest that roots (and hosted microbial flora) contributed the greatest proportion (50%) during low rainfall conditions (LR) (Figure 4.6). The remaining diet was composed of POC (16.1%, derived from allochthonous carbon incorporations, a potential organic source for microbes), copepods (harpacticoids (13.9%) and cyclopoids (11.9%)) and sediment (8.1%) (i.e. OM laying at the bottom of the aquifer or epilithic biofilms).

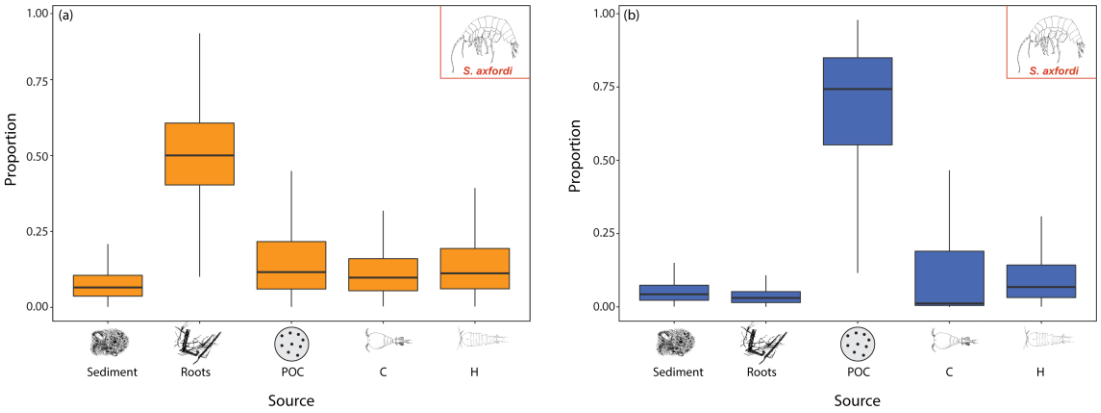

Figure 4.6. Modelled contributions to the diet of amphipod *S. axfordi* (AM1) under a) LR and b) HR conditions. POC: particulate organic carbon, C: Cyclopoida sp.; H: Harpacticoida sp. Medians and quartiles of each prey category are represented in the boxplot, see Table S4.6 for SIA $\delta^{13}C$ and $\delta^{15}N$ data. AM2 illustrated the same dietary preferences as AM1 under both rainfall conditions.

Under HR conditions, the POC dietary contribution reached 66.1%, while roots plummeted to 3.3% (Figure 4.6). Overall, amphipod *Y. sturtensis* (AM2) showed the same dietary patterns as AM1.

Adult beetles *P. macrosturtensis* (B) and *P. mesosturtensis* (M) show only slight depletions in their isotopic values during HR in bulk $\delta^{13}C$ and $\delta^{15}N$ SIA, in contrast to the larger changes seen in the CSIA data. *P. microsturtensis* (S), which showed an isotopic enrichment in CSIA, counter to the rest of the community, shows similar behaviour to *P. macrosturtensis* (B) and *P. mesosturtensis* (M) in the SIA (Table S4.6). All the three species show similar dietary preferences in mixing models across the rainfall periods (Table S4.7). While diets were dominated by amphipods AM1 and AM2 during the LR period (B: 39.9%, M: 49.3% and S: 47.9 % (Figure 4.7)), predation/scavenging of sister beetle species accounted for the biggest dietary proportions during the wet season (B: 52.9%; M: 49.4%; S: 41.9% (Figure 4.7)).

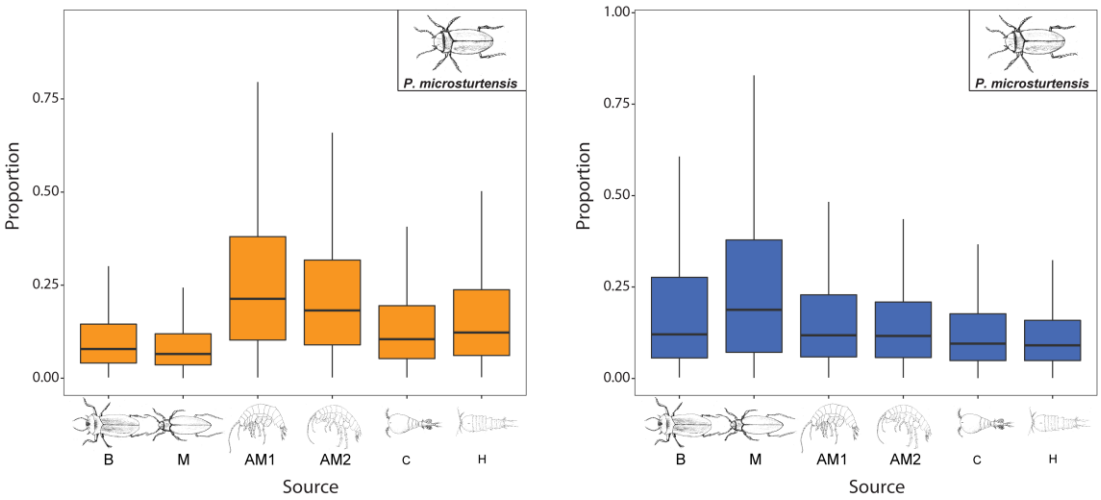

Figure 4.7. Contributions of *P. microsturtensis* adults' diet for a) LR and b) HR. Diet sources: *P. macrosturtensis* (B), *P. mesosturtensis* (M), *S. axfordi* (AM1), *Y. sturtensis* (AM2), Cyclopoida sp. (C) and Harpacticoida sp. (H). Medians and quartiles of each prey category are represented in the boxplot, see Table S4.6 for $\delta^{13}C$ and $\delta^{15}N$ bulk data. *P. macrosturtensis* (B) and *P. mesosturtensis* (M) illustrated same trends of dietary contributions across rainfall periods (Table S4.7). In these analyses, sister species *P. mesosturtensis* (M) and *P. microsturtensis* (S) were considered as *Paroster* prey items for diet reconstruction of *P. macrosturtensis* (B), while contributions from *Paroster* diet sources *P. macrosturtensis* (B) and *P. microsturtensis* (S) were used for *P. mesosturtensis* (M).

Mixing models indicate that *P. macrosturtensis* larvae (Blv), which showed the biggest shift in trophic position, has a preference for amphipods *S. axfordi* (AM1) and *Y. sturtensis* (AM2) under LR conditions (accounting for 52% of the diet contributions), but also consumes a range of other organisms (Figure 4.8). During HR, Blv's diet is dominated by the two amphipod species, accounting for 79.6% of food sources (Figure 4.8). Overall, these results indicate changes in amphipods (AM1 and AM2) diet preferences linked with different OM inputs, coupled with enhanced species-specific predatory pressures from Blv under HR conditions.

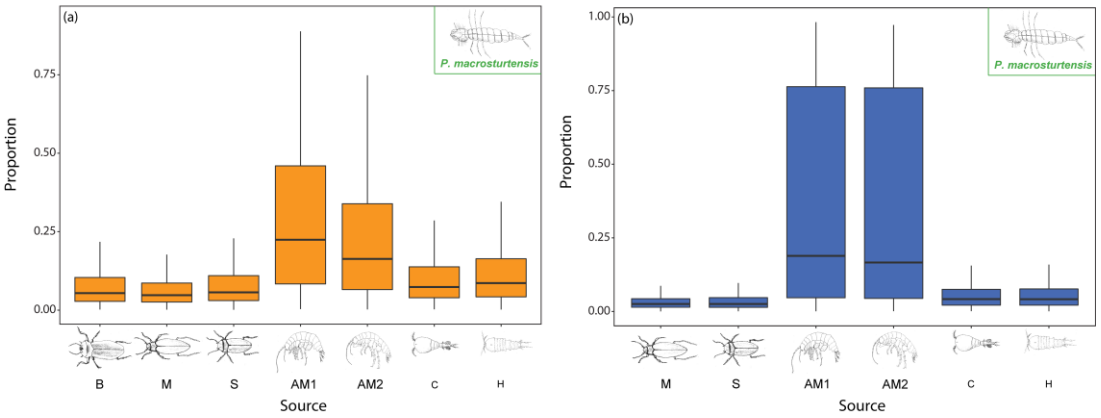

Figure 4.8. Stygofaunal contributions to the diet of *P. macrosturtensis* larvae for a) LR and b) HR. Diet sources: *P. macrosturtensis* (B), *P. mesosturtensis* (M), *P. microsturtensis* (S) *S. axfordi* (AM1), *Y. sturtensis* (AM2), Cyclopoida sp. (C) and Harpacticoida sp. (H). During HR, diet source *P. macrosturtensis* (B) was discarded as the Gelman-Rubin diagnostic reported values exceeding the corresponding upper confidence limits at the 95% confidence level. Medians and quartiles of each prey category are represented in the boxplot, see Table S4.6 for $\delta^{13}C$ and $\delta^{15}N$ bulk data.

4.4 Discussion

4.4.1 *Shifts in basal OM assimilation*

$\delta^{13}C_{EAA}$ data suggest that the stygofaunal community at Sturt Meadows experienced a seasonal shift in carbon flows during the wet season (HR). The overall tendency towards more ^{13}C-depleted $\delta^{13}C_{Val}$ and $\delta^{13}C_{Phe}$ values indicate general stygofaunal discrimination against ^{13}C sources in that season.

Three groups (OL, AM3 and S) showed a counter trend of ^{13}C enrichment in the EAAs during HR. Of these, the easiest to account for are the oligochaetes (OL) which also showed increased abundances (χ^2= 6.7698, $P < 0.05$) of individuals ranging from 2 (LR1) and 1 (LR2) to 21(HR) (Table S4.4), indicating ideal conditions for the taxon during the wet season. As detritovores, oligocheates may be expected to preferentially consume more degraded, and so ^{13}C-enriched, OM. The enrichment in *S. bradfordae* (AM3) and *P. microsturtensis* (S) is harder to explain at this stage. The low abundance of *S. bradfordae* (AM3) means that, like the oligochaetes, it was not included in the SIA analysis and less data are available. This taxon, together with *P. macrosturtensis* larvae (Blv), *Y. sturtensis* (AM2) and water mites (OR), lacked statistically significant differences when $\delta^{13}C$ values of EAA during LR are compared with those of NEAA involved in the glycolytic cycle. Newsome et al. [50] indicated

that $\delta^{13}C$ values of NEAA from diets of omnivorous animals reflect *de novo* synthesis but also dietary incorporations. Differential routing of macromolecules by consumers [55] are one possible contributor to our results. However, to date isotopic routing hypotheses have been tested only in vertebrates [30,56], with the study of metabolic pathways in aquatic invertebrates largely unexplored. Further CSIA investigations involving species-specific bio-assimilation processes within the stygofaunal community are needed to provide a more accurate understanding of the biochemical dynamics regulating this system.

In line with our general data trends, Hartland et al. [15], who reported consistent depletion in $\delta^{13}C$ stygofaunal values within OM-enriched groundwaters *via* sewage contamination, concluded that stress-subsidy gradients in groundwaters trigger profound changes in stygofaunal assemblages and have the potential to trigger shifts in feeding habits. Rainfall events trigger OM inflows which constitute high quality carbon sources for aquatic biota in groundwaters [16, 57].

Reiss et al. [26] demonstrated a strong link between nutrient inputs (mainly DOC) and groundwater microbe functional and metabolic richness after a major flooding event. Unfortunately, their methodology did not allow for corresponding macrofaunal trends to be identified. Nonetheless, microbially-derived OM incorporation by stygofauna has been reported in a number of groundwater ecology studies [16,21,58], and the biochemical importance of this linkage is widely accepted.

A key role in the observed trends at Sturt Meadows is played by amphipods which, together with copepods, are recognised as crucial actors in transferring OM to the upper stygofaunal trophic levels [16]. Specialized trophic habits in amphipods include epigean predation [10], detritivory [59], parasitism [60], biofilm grazing [61] and necrophagy [62]. Several studies have reported high degrees of trophic opportunism [54] and plasticity [63], allowing amphipods crucial shifts in feeding modes. Concurrently, niche partitioning has been addressed as a key mechanism to reduce intraspecific competition in ecosystems shaped by scarce nutrient availability [64]. However, our results do not show any conclusive evidence of epigean amphipod niche partitioning, with amphipods *S. axfordi* (AM1) and *Y. sturtensis* (AM2) showing the same dietary patterns. Overall, the isotopic data support the concept of opportunistic behaviours linked with changes in resource availability as a result of different rainfall regimes.

The HR event triggered substantial changes in the dietary proportions of *S. axfordi* (AM1) and *Y. sturtensis* (AM2), with notable decreases in root input and increases in POC. The extent of direct plant matter consumption by stygobionts reported in the literature – particularly by amphipods, which are facultative shredders – is both site and species-specific. Jasinska et al. [65] found that aquatic root mats were a key food source for a biodiverse cave fauna hosted by a shallow groundwater stream in Western Australia. Conversely, Navel et al. [66], reported the widely distributed amphipod species *Niphargus rhenorhodanensis* having preferential OM collector/gatherer feeding habits. In another study, Simon et al. [67] suggested that wood inputs played a role as indirect source of OM consumed by the ephilitic microbial mats which were ultimately targeted by common *Gammarus* amphipods.

At Sturt Meadows, a plausible explanation for the patterns observed is that during the dry season epigean amphipods rely on a more omnivorous diets where roots falling from the surface, and associated microbial and fungal biota, provide a substantial food source. Conversely, the wet season triggers inflows of replenished carbon (^{13}C-depleted POC) that might fuel biological turnovers in microbiological activity, and POC-attached microflora may be ultimately targeted by epigean amphipods. These assumptions are in line with the finding reported by Brankvotis et al. [16], and support the concept that grazers play a crucial role in sustaining the functional diversity in groundwaters. The importance of plant matter input during at least part of the year is supported by a previous bulk SIA investigation at Sturt Meadows [40], which also suggested that terrestrial sources of carbon, mainly DOC, reached the aquifer *via* percolation and play a crucial role in energy flows within the system. It is worth noting that our δ^{13}C values of decarbonated sedimentary fractions (referred above as 'sediment') were less ^{13}C-depleted than those in other groundwater investigations [68,69,70], and had ranges close those for dissolved organic carbon (DIC) in the region [71,72,73]. Portillo et al. [74] reported karst microbial growth induced by both carbonate precipitation and dissolution, suggesting the inclusion of inorganic carbon within the estimation of global carbon budgets in groundwaters. In line with this work, Chapelle [75] reported *in situ* DIC production as a result of microbial metabolism involved in the dissolution of carbonate material in the black Creek aquifer (California, USA). Our results suggest that carbonate assimilation and/or dissolution processes are likely to occur in the sedimentary deposits of the aquifer, transferring an inorganic carbon isotopic fingerprint into the decarbonated and organic fractions. This can

be tested in future by further functional studies on the microbial community [76] at the site.

Copepods (C and H) showed high $\delta^{15}N$ values compared to the rest of the stygofaunal community (Table S4.6), suggesting alternative nitrogen sources linked to different microbial baselines. Copepods act as energy drivers in recycling nitrogen *via* ingestion of sediment and attached bacteria [12], with ammonia (NH_3), together with nitrates (NO_3^-), being an essential nutrient and energy source for subterranean microorganisms [77]. At Sturt Meadows aquifer, where ammonia levels are considerably higher than the natural concentrations [38], proliferation of selectively grazed ammonia-oxidising bacteria (AOB) might have played a key role in triggering the enriched $\delta^{15}N$ values in copepods.

The present study is constrained by its focus on stygofauna and therefore cannot provide direct evidence of microbially-derived ecological shifts. Future research needs to combine stygofaunal and microbial investigations to create a complete picture of the ecosystem. CSIA and functional genetic studies on microbes and copepods would also help define transitions from microflora to stygofauna, a process that has been understudied so far. Recent promising investigations in surface terrestrial [34] and aquatic [78] environments suggest a design - carbon fingerprinting - based on the incorporation of isotopic data into multifactorial mixing models that allow specific elucidation of bacterial sources in diets. Overall, despite the methodological challenges posed by groundwaters, isotopic data on stygofaunal carbon fluxes provides baseline knowledge that help untangle the intricate biochemical dynamics regulating subsurface environments.

4.4.2 *Trophic interactions*

Our data on nitrogen CSIA pinpointed two main trophic levels marked by a small but clear separation between the top predators - adult beetles (B, M and S) - and primary consumer amphipods (AM1, AM2 and AM3) under both rainfall conditions. Compared to other ecosystems [29], the Sturt Meadows aquifer shows a very simple and truncated trophic web dominated by omnivorous habits. This is consistent with previous assumptions [67] due to the lack of primary producers [9] and scarce nutrient availability [79].

Within subterranean beetles, the smallest species *P. microsturtensis* (S), together with *P. macrosturtensis* (B), sit at the top of the trophic chain during HR (Table 4.3). Under those

conditions, increased oxygen levels [38] may play a role in shaping changes in stygofaunal niche occupation. Subterranean beetles' body size has been found to drive differential physiological responses to increased exoskeleton respiration rates (inversely proportional to the body size) which ultimately affect the ability to allocate energy for breeding and foraging [80]. As the smallest species *P. microsturtensis* (S) can adapt their metabolism more quickly than direct competitor sister species *P. mesosturtensis* (M) under favourable conditions - such as HR regimes – they are more likely to show shifts in ecological niche occupation [38]. This trend, combined with the group feeding tendency of *P. microsturtensis* (S) beetles [40], indicates higher efficiency in activating more intensified predatory strategies when compared to *P. mesosturtensis* (M).

Dytiscidae beetle larval stages - commonly referred as 'water tigers' - are ferocious carnivores [81] with extremely opportunistic feeding behaviours involving scavenging and cannibalism [82]. At Sturt meadows, the third instar of blind *P. macrosturtensis* larvae (Blv) has a considerably bigger head capsule - paired with elongated mouthparts - than adult stages (Figure 4.9). These morphological features are likely to provide ethological advantages for non-visual predacious habits within light-less environments such as groundwaters [83]. This is consistent with stable isotope data from LR conditions positioning Blv at the top of the trophic web (Table 4.3).

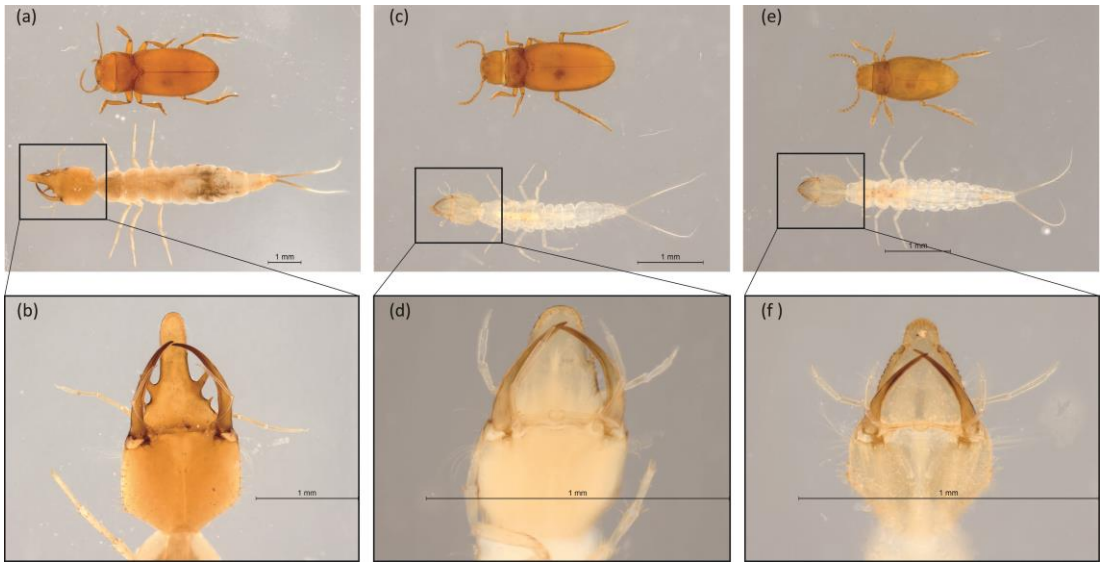

Figure 4.9. Comparisons between adult and larvae (whole body and head capsule) of *P. macrosturtensis* (a and b), *P. mesosturtensis* (c and d) and *P. microsturtensis* (e and f). All the scale bars in the photos refer to a length of 1 mm. Values of the lengths of the head capsules estimated through photographic calculation: *P. macrosturtensis* adult: 0.65 mm, *P.*

macrosturtensis larvae: 1.87 mm, *P. mesosturtensis* adult: 0.38 mm; *P. mesosturtensis* larvae: 0.71 mm, *P. microsturtensis* adult: 0.24 mm, *P. microsturtensis* larvae: 0.70 mm.

Overall, modelled dietary contributions of *P. macrosturtensis* larvae (Blv) indicated a preference for amphipods (AM1 and AM2) coupled with residual cannibalism/scavenging (B, M and S) and predation of copepods (C and H) (Figure 4.8). Under HR, *P. macrosturtensis* larvae (Blv) showed the biggest drop in TP compared to LR (TP_{LR-HR} = - 0.3), which can be explained by an increased predatory focus on amphipods, and reduced feeding on secondary consumer sister species.

Previous work on surficial dytiscids larval stages published by Inoda et al. [84] stressed the importance of prey recognition through smell. According to their results, prey density was a key factor in shaping feeding behaviours, and self-other recognition played a role in group feeding. Overall, these findings indicated prevention of cannibalism through scent recognition. In groundwater, with total darkness and high influence of OM inputs on population dynamics [85,86], these patterns are likely to be strengthened. We suggest that the shifts in Blv predation seen in our results are dictated by a combination of chemical recognition and increased likelihood of encountering prey (amphipods) driven by enhanced resource availability (OM) during HR periods.

The role of bottom-up vs top-down forces in natural communities has been a cornerstone issue in the field of trophic ecology since the first empirical investigations [87]. Despite the controversy generated by the debate, there is now consensus that both forces act simultaneously on populations. This reinforces the need for whole system studies considering the interaction between heterogeneous (biotic and abiotic) forces and their effect on communities [88,89,90].

Our study, in line with a number of other investigations in the field [26,91] confirms that rainfall events *via* water advection are key drivers in defining energy flows and ecological patterns within resource-limited environments. We suggest that OM-driven bottom-up regulations, increasingly accepted as driving factors shaping population dynamics in aquifers [92], shaped the shifts in feeding behaviours among amphipod taxa in the calcrete. However, despite the beneficial conditions for primary consumers triggered by increased nutrient availability (i.e. microbial biofilms) and better environmental settings (i.e.

increased oxygen, [38]), a decrease in amphipod populations under HR indicates the existence of additional ecological factors.

Top-down forces (i.e. natural predators), widely studied in surface aquatic ecosystems [93,94], have hardly been addressed in groundwater. Previous genetic investigations at Sturt Meadows pinpointed predatory pressures from beetles on amphipods and copepods [95] and reported a lack of trophic niche partitioning among the *Paroster* species. In another study, Hyde [96] reported evidence from metagenomics data suggesting that subterranean blind beetles at Sturt Meadows feed on both prey invertebrates and their sister species. Our isotope results support these hypotheses, indicating opportunistic predaceous habits in the calcrete, mixed with scavenging/cannibalism. However, substantial uncertainty remains about the magnitude of interspecific predatory pressures among *Paroster* sister species, and further species-specific lab experiments are needed to investigate these ethological aspects.

Biochemical functional role interpretation coupled with abundance data suggests that bottom-up population dynamics are counterbalanced in the system by top-down forces. Increased numbers of top predators (adult beetles B, M and especially S) were paired with a decrease of key prey items (amphipods AM1, AM2 and AM3) when HR is compared with the dry season (LR1 and LR2) (Figure 4.10).

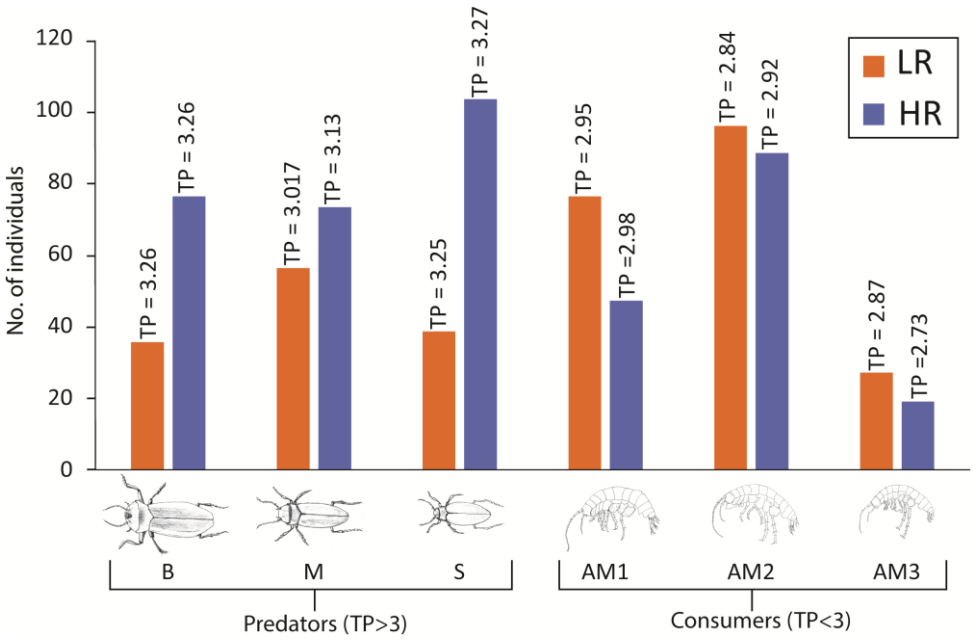

Figure 4.10. Bar chart graphs comparing dry season abundances (as the average value of LR1 and LR2) with HR conditions for top predators (beetles B, M and S) and key prey items

(amphipods AM1, AM2 and AM3). See Table S4.4 for detailed abundance data. None of the abundances of these taxa changed significantly between LR and HR. See Saccò et al. [38] for detailed statistical analyses across LR1, LR2 and HR.

In light of the dynamics shown by our isotope data, we suggest that the reported shift in amphipods (AM1 and AM2) carbon incorporation during HR might have triggered changes in their ecological behaviour, exposing them to increased predatory pressures from the top predator *Paroster* beetles (B, M and S). However, given the high degree of opportunistic behaviour reported by stygofauna [14], further investigations on species-specific ethological dynamics would be helpful to infer community dynamics.

The number of third instar dytiscidae larvae 'Blv' did not vary across sampling campaigns, suggesting differential ecological niche occupations. Previous investigations on *Paroster* larvae detected three instars before pupating, with the first two occupying a reduced proportion of their lifetime [83]. Future investigation of early stages of larval developments are needed to establish if potential population blooms (i.e. mass reproduction) are linked with contrasting recharge periods.

4.5 Conclusions

The application of CSIA and SIA allows elucidation of the trophic dynamics shaping stygofaunal communities in an arid zone calcrete aquifer. Rainfall acts as a driver in regulating both top down and bottom up changes in dietary habits. Subterranean invertebrate population dynamics are notoriously hard to investigate due to sampling obstacles and a current lack of knowledge around stygofaunal biological cycles [7,36]. However, our isotopic results allow a greater insight into the food web dynamics and the biogeochemical forces that shape them than has previously been possible. Further investigations involving higher numbers of samples from more biodiverse systems or complex trophic assemblages (i.e. alluvial aquifers) will help refine the approach. The incorporation of qualitative analyses such as DNA metabarcoding would also complement quantitative isotopic methods to provide crucial insights into processes (i.e. cannibalism) and key driving forces (i.e. niche partitioning) that are hard to detect *via* one method alone. Lastly, investigation of nitrogen sources and their isotopic changes would open up the nitrogen data collected to interpretation beyond trophic position.

Groundwater environments are fundamentally important to ecosystems, communities and industry, and a robust understanding of their ecosystem dynamics is essential to accurately assess environmental impacts, whether anthropogenic, or climatic. Isotopic data, especially if combined in multidisciplinary studies with other parameters [22] has a key role to play in elucidating previously hard to investigate function within these cryptic systems.

Acknowledgements

We wish to acknowledge the traditional custodians of the land, the Wongai people, and their elders, past, present and emerging. We acknowledge and respect their continuing culture and the contribution they make to the life of Yilgarn region. The authors acknowledge NERC group at Bristol University for the Life Sciences Mass Spectrometry Facility (LSMSF) and Dr Alice Mora for the support with the facilities at La Trobe Institute for Molecular Sciences (LIMS). The authors thank Flora, Peter and Paul Axford of the Meadows Station are thanked for their kindness and generosity in providing both accommodation and access to their property.

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Supplementary material

Table S4.4. Abundance data of the different stygofaunal taxa at Sturt Meadows detected during the sampling campaigns LR1, LR2 and HR.

RAINFALL PERIOD		LR1																																
GEOLOGICAL ZONE		A1						A2							A3					CD							A4								
TAXON	ID	A8	C5	F4	H5	J7	N4	L4	S2	U2	W2	Q2	W3	W4	C1	D1	E1	G1	H2	A11	D13	E10	E11	Q3	Q4	S8	B13	C13	E12	E13	F13				
Tubificidae sp.	TU														2																			
Oribatida sp.	OR			2			2					1	1									1		1		3		1	1		2			
Harpacticoida sp.	H	1	3	9	17	18	5	7	13	17	12		8	27	5	15	12	3	72	21	8	19		2	8	27	7	1		6	11			
Cyclopoida sp.	C	18	6	1	11	16	36	8	9	14	2	5	3	3	2	6	1	5	131	92	1	33	11	19	14	55	2	269	3	26	1			
Scutachiltonia axfordi	AM1		19	1	14		13		1	7		1		1		1		1	9	4	5		2						6		7			
Yilgarniella sturtensis	AM2		1	1	23		13		21		2		2	2				3	2	2	7	2			2			3						
Stygochiltonia bradfordae	AM3			1	3				6	3			1					1	4			2							2					
Paroster macrosturtensis	B		1		1	1							1				1		2								1		11	1	6			
Paroster mesosturtensis	M			3	1		3		9	1	1		1	1	4	1	4	1	3	9	1				1				1	9			
Paroster microsturtensis	S		1		2		1		6							3	2	1	1		6			1					1	5	1		
Paroster macrosturtensis larvae	Blv		1										1				1	1	2	1	1									1			
Paroster mesosturtensis larvae	Mlv								1																		1							
Paroster microsturtensis larvae	Slv		1						1	1	1		3			1	1	1	1		2		2	1	1				1	2			
		LR2																																
Tubificidae sp.	TU																								1								
Oribatida sp.	OR			1			2		1	1	8	123		3		1		1		1		2			2		1		1	1			
Harpacticoida sp.	H	82	26	5	12	2	18	2	8	2	17	7	4	29	259	7	22		11	6	1	2	3	34	3	3	12		1	2	1			
Cyclopoida sp.	C	86	29	22	24	4	16	2	16	129	7	97	87	19	68	16	31	4	22	35	31	9	29	66	1		6	33	7	81	38			
Scutachiltonia axfordi	AM1	3	1		3	1	1		1	6	1		12	4			2	2		2				1			8	1	9	1	1			
Yilgarniella sturtensis	AM2	28	11		1	2				6		2	11	2		2	6		4	6	1	5		1	1		4		6	4	2			
Stygochiltonia bradfordae	AM3	2	2		1	1	3			1			1				1	1	2	2	1	5	2	1				2	2		1			
Paroster macrosturtensis	B	1				2			2	1	2		7					3		3	1						4		7	3	9			
Paroster mesosturtensis	M	3				1	1			7		3	6	2	2		3			5	22						1			2				
Paroster microsturtensis	S	1				1	3	1			1		7	1		1				1	17						7		2	3				
Paroster macrosturtensis larvae	Blv		1				1						3							6							1							
Paroster mesosturtensis larvae	Mlv																				2													
Paroster microsturtensis larvae	Slv	1	2			3						2	1			2	1			5	6			4			2			1				
		HR																																
Tubificidae sp.	TU	14	1			1													3			1									1			
Oribatida sp.	OR											29						2	3											3				
Harpacticoida sp.	H	64	44	13	1	32	17	8	4	83	6		5	4	3	7	154	11	19	13	12	2	26	9	2	13	8	3	7	14	4			
Cyclopoida sp.	C	54	91	23	17	22	12	4	8	19	4	2	23	13	15	2	87	1	29	148	77	66	76	15	11	16	137	41	39	36	47			
Scutachiltonia axfordi	AM1				2		4			13			2	2			2	1	1	1			1				4	2	8		4			
Yilgarniella sturtensis	AM2	2	3		9	4	2			12			7	1			13		2	4		3	4				2	8		5	7			
Stygochiltonia bradfordae	AM3		1							1										1		8						7		1			
Paroster macrosturtensis	B	4			3					3	9		28			3				6			1	3			1		2	5	8			
Paroster mesosturtensis	M	7	2		6	1	3	1	1	1		6	4	2	1		1		1	11		1	6	1	1		4	1		11				
Paroster microsturtensis	S	22	3	3	6	1	3			14	1	3	9	7				1		8			2				7		2	6	5			
Paroster macrosturtensis larvae	Blv									1				1			1		1		2									1				
Paroster mesosturtensis larvae	Mlv																			2		1						1						
Paroster microsturtensis larvae	Slv		1																	2	1						2			4				

Table S4.5. Sample weights (mg) for SIA and CSIA analyses. n/a: not available.

ID	SIA		CSIA			
	$\delta^{13}C$ and $\delta^{15}N$		$\delta^{13}C$		$\delta^{15}N$	
	LR	HR	LR	HR	LR	HR
B	0.65	0.82	1.21	1.27	5.12	5.02
M	0.67	0.68	1.31	1.42	5.15	5.01
S	0.63	0.75	1.27	1.22	5.11	5.05
Blv	0.81	0.69	1.46	1.34	5.19	5.09
Mlv	n/a	n/a	0.32	1.11	n/a	n/a
Slv	n/a	n/a	1.24	0.86	3.97	n/a
AM1	1.19	1.25	1.18	1.37	5.03	5.02
AM2	0.69	1,27	1.23	2.89	4.55	2.58
AM3	n/a	n/a	1.15	2.61	4.38	2.52
OL	n/a	n/a	0.16	0.52	n/a	n/a
OR	n/a	n/a	1.03	0.53	2.47	n/a
C	2.42	2.45	n/a	n/a	n/a	n/a
H	2.79	2.77	n/a	n/a	n/a	n/a
Roots	0.14	0.08	n/a	n/a	n/a	n/a
Sediment	1.28	1.28	n/a	n/a	n/a	n/a
POC	0.6	0.6	n/a	n/a	n/a	n/a

Table S4.6. $\delta^{13}C$ and $\delta^{15}N$ values of stygofauna, roots, sediment and POC during LR and HR.

ID	$\delta^{13}C$		$\delta^{15}N$	
	LR	HR	LR	HR
B	-23±0.22	-23.51±0.81	14.66±0.04	14.62±0.49
M	-23.37±0.19	-23.72±0.14	15.43±0.53	14.01±0.05
S	-23.6±0.3	-24±0.43	14.4±0.3	13.07±0.42
Blv	-23.46±0.12	-24.8±0.06	13.74±0.08	10.03±0.32
AM1	-24.14±0.3	-26.88±0.05	10.71±0.3	8.81±0.09
AM2	-24.55±0.3	-27.1±0.3	9.99±0.3	8.5±0.3
C	-20.45±0.3	-21.91±0.3	13.9±0.3	14.5±0.3
H	-20.60±0.3	-23.50±0.3	11.9±0.8	15.8±0.8
Roots	-20.57±0.3	-20.90±0.3	5.1±2.0	12.1±0.3
Sediment	-10.33±0.3	-9.65±0.3[1]	11.0±1.2	11.4±1.2
POC	-21.58±0.1	-26.47±0.1	10.73±0.1	8.35±0.1

Table S4.7. Dietary proportions of *P. macrosturtensis* (B), *P. mesosturtensis* (M) and *P. microsturtensis* (S) under LR and HR conditions.

Taxon	ID	Rainfall	Dietary proportions (%)						
			P. macrosturtensis (B)	*P. mesosturtensis* (M)	*P. microsturtensis* (S)	*S.axfordi* (AM1)	*Y. sturtensis* (AM2)	Cyclopoida sp. (C)	Harpacticoida sp. (H)
P. macrosturtensis	B	LR	/	11.7±11.9	14.8±14.2	25±18.7	14.6±13	15.1±13.4	18.8±17.1
P. mesosturtensis	M	LR	10.4±10.8	/	11.1±11.3	27.4±20	21.8±17.2	13±11.7	16.3±15.3
P. microsturtensis	S	LR	11.2±10.8	9.6±9.4	/	25.4±18.8	22.5±16.4	14.5±12.4	16.8±15.3
P. macrosturtensis	B	HR	/	26.3±19.8	26.6±19.9	9.9±8.9	9.1±7.8	14.1±12	14±11.9
P. mesosturtensis	M	HR	21.1±17.7	/	28.3±20.4	12.7±11.4	12.1±10.8	13.4±12	12.4±10.3
P. microsturtensis	S	HR	18.2±16.4	23.7±19.3	/	17.6±15.9	16.1±14.2	12.8±11.1	11.6±9.6

Chapter 5 | Tracking down carbon inputs underground from an arid zone Australian calcrete

Submitted as a research article (2020).

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Abstract

Freshwater ecosystems play a key role in shaping the global carbon cycle and maintaining the ecological balance that sustains biodiversity worldwide. Surficial water bodies are often interconnected with groundwater, forming a physical continuum, and their interaction has been reported as a crucial driver for organic matter (OM) inputs in groundwater systems. However, despite the growing concerns related to increasing anthropogenic pressure and effects of global change to groundwater environments, our understanding of the dynamics regulating subterranean carbon flows is still sparse. We traced carbon composition and transformations in an arid zone calcrete aquifer using a novel multidisciplinary approach that combined isotopic analyses of dissolved organic carbon (DOC) and inorganic carbon (DIC) ($\delta^{13}C_{DOC}$, $\delta^{13}C_{DIC}$, $^{14}C_{DOC}$ and $^{14}C_{DIC}$), with fluorescence spectroscopy (Chromophoric Dissolved OM (CDOM) characterisation) and metabarcoding analyses (taxonomic and functional genomics on bacterial 16S rRNA). To compare dynamics linked to potential aquifer recharge processes, water samples were collected from two boreholes under contrasting rainfall: low rainfall ((LR), dry season) and high rainfall ((HR), wet season). Our isotopic results indicated limited changes and dominance of modern terrestrial carbon in the upper part of the bore field, but correlation between HR and increased old and ^{13}C-enriched DOC in the lower area. CDOM results showed a shift from terrestrially to microbially derived compounds after rainfall in the same lower field bore, which was also sampled for microbial genetics. Functional genomic results showed increased genes coding for degradative pathways - dominated by those related to aromatic compound metabolisms - during HR. Our results indicate that rainfall leads to different responses in different parts of the bore field, with an increase in old carbon sources and microbial processing in the lower part of the field. We hypothesise that this may be due to increasing salinity, either due to mobilisation of chloride from the soil, or infiltration from the downstream salt lake during HR. This study is the first to use a multi-technique assessment using stable and radioactive isotopes together with functional genomics to probe the principal organic biogeochemical pathways regulating an arid zone calcrete system. Further investigations involving extensive sampling from diverse groundwater ecosystems will allow better understanding of the microbiological pathways sustaining the ecological functioning of subterranean biota.

Key-words: carbon, groundwater, rainfall, stable isotope analysis, fluorescence, radiocarbon, functional genomics.

5.1 Introduction

The global carbon cycle fuels the processes that are responsible for maintaining the ecological functioning of ecosystems (e.g. Battin et al., 2009; Poulter et al., 2014). Terrestrial environments, together with oceans, play a key role in sequestering atmospheric carbon pools and allow fundamental recycling of biomass (Schimel, 1995). However, on-going global warming, mainly caused by increased greenhouse gases linked with anthropogenic activities, is putting at risk the maintenance of this ecological balance (Cox et al., 2000; Stassen, 2016).

During the last decade, carbon storage and fluxes in freshwater environments have gained prominence as key actors in the global cycling of organic matter (e.g. Catalán et al., 2016; Chambers et al., 2011; Kolmakova et al., 2019). Drake et al. (2017) estimated up to 5.1 Pg y^{-1} of carbon delivered from land to surficial inland aquatic systems (lakes, rivers, reservoirs). Kayranli et al. (2010) reported that soil and sediment from wetlands are amongst the world's most extensive carbon sinks, with peatlands accounting for a third of the organic soil worldwide (Weishampel et al., 2009). These observations are in concordance with Keiluweit, et al., (2017), who indicated that surficial soil and unsaturated zones provide the biggest carbon source within the terrestrial framework. However, while widely investigated in surficial ecosystems, carbon flows are understudied in groundwater environments (Dragoni and Sukhija, 2008; Monger et al., 2015).

Groundwater systems are often hydrologically interconnected with each other and/or to surface terrestrial environments and water bodies (Viaroli et al., 2018). Especially in arid environments, near-surface groundwaters (e.g. groundwater dependent ecosystems (GDEs)), provide a vital conceptual and physical continuum (Glanville et al., 2016). Surface water-groundwater exchanges (SW/GW) shape biogeochemical dynamics, including carbon cycling and nutrient circulation, which regulate the functioning of both surface and subterranean ecosystems (Stegen et al., 2016). However, dissolved carbon concentrations within aquatic subterranean environments are typically orders of magnitude lower than lakes and rivers (Chapelle and Lovley 1990; Downing and Striegl, 2018; Hofmann and

Griebler, 2018), where concentrations of dissolved organic matter usually range between 2 and 10 mg L^{-1} (Kalbitz et al., 2000). McDonough et al. (2019) reported average subterranean global dissolved organic (DOC) concentrations of ~ 1 mg L^{-1} f, while the global flux of inorganic carbon (DIC) into groundwater is estimated to be 0.2 GtC y^{-1} (Kessler and Harvey, 2001).

Subterranean DOC replenishment can occur either *via* SW/GW and/or *via* rainfall recharge through soils containing high organic matter (OM) content (e.g. Meredith et al., 2016; Meredith et al., 2018). Baker et al. (2000) suggested that seasonal saturation of sediments overlying unconfined groundwater plays a key role in regulating organic carbon dynamics underground. Similar site-specific models have been suggested over the last two decades (e.g. Neilson et al., 2018; Vesper and White, 2004), emphasising the importance of the vadose zone as a source of carbon for groundwater biological communities and biogeochemical cycling (Manna et al., 2019).

Groundwater communities are thought to be bottom-up regulated by the availability of OM which drives ecological functioning (i.e. energy flows, trophic cascade effects) in groundwater ecosystems (Foulquier et al., 2011). Microbial diversity and productivity are considered to be limited by the concentration and bioavailability of organic matter in groundwater (Portillo et al., 2009). While heterotrophic metabolism is commonly considered a major process for sustaining food web interactions in a typically low-energy system (Simon et al., 2003), chemolithoautotrophic strategies have also been extensively reported (e.g. Hutchins et al., 2016 and references therein; Wegner et al., 2019). Microbially-processed OM, together with detrital fractions (Hancock et al., 2005), are transferred to higher trophic levels of subterranean biota (Brankovits et al., 2017) by higher primary consumers (i.e. terrestrial (troglofauna) and obligate aquatic (stygofauna)). As a result, subterranean carbon turnovers, often linked with recharge regimes (Reiss et al., 2019), are ultimately responsible for cascading effects on energy flows and food web interactions (Datry et al., 2005).

The Sturt Meadows (SM) calcrete in Western Australia hosts a stygofaunal community composed of 18 macroinvertebrate species including blind dytiscid beetles and chiltoniid amphipods, and is a hotspot for subterranean aquatic invertebrate diversity (Guzik et al., 2011; Humphreys et al., 2009). Recent investigations into the ecological functioning of the

calcrete stygofaunal assemblages (Saccò et al., 2019b, Saccò et al., 2019c) have indicated that rainfall input dynamics play a vital role in shaping cascade effects. Here, we extend this research by investigating carbon input dynamics and microbial processing under contrasting rainfall periods *via* hydrochemistry, stable and radiocarbon isotope ecology and DNA metabarcoding analyses. Through this multidisciplinary approach, we aim to 1) elucidate the nature of the carbon inputs under differential rainfall regimes, 2) provide isotope-based tracking of the organic and inorganic carbon sources in the groundwater, and 3) identify metabolic and functional microbial patterns coupled with organic inflows linked to rainfall percolation. The study of carbon inputs and their microbial incorporation has the potential to expand our understanding of the ecological dynamics sustaining biodiversity in this taxonomic hotspot.

5.2 Material and methods

5.2.1 *Study area*

Field work was carried out at the Sturt Meadows calcrete (SM calcrete), located within the Sturt Meadows pastoral station in the northeast side of the Yilgarn region (28°41'S 120°58'E), Western Australia (Figure 5.1a). The Yilgarn craton is one of the most important Late Archaean metallogenic provinces in the world (Czarnota et al., 2010), and constitutes the bulk of this West Australian land mass. The area hosts calcretes formed by the precipitation of calcium carbonate along palaeodrainage channels (Morgan, 1993), which have been the focus of research for more than a century (e.g. Lamplugh, 1902; Lintern, 2001; Mabbutt, 1969). The SM aquifer is located upstream of Lake Raeside, covering an area of ~43 km^2, and has a strong biogeochemical gradient comparable to estuarine systems (Humphreys et al., 2009). Previous studies of the depth and lithography of the calcrete (Bradford, 2010; Bradford et al., 2013) identified two main geological sectors: calcretes and clayey formations (Figure 5.1b).

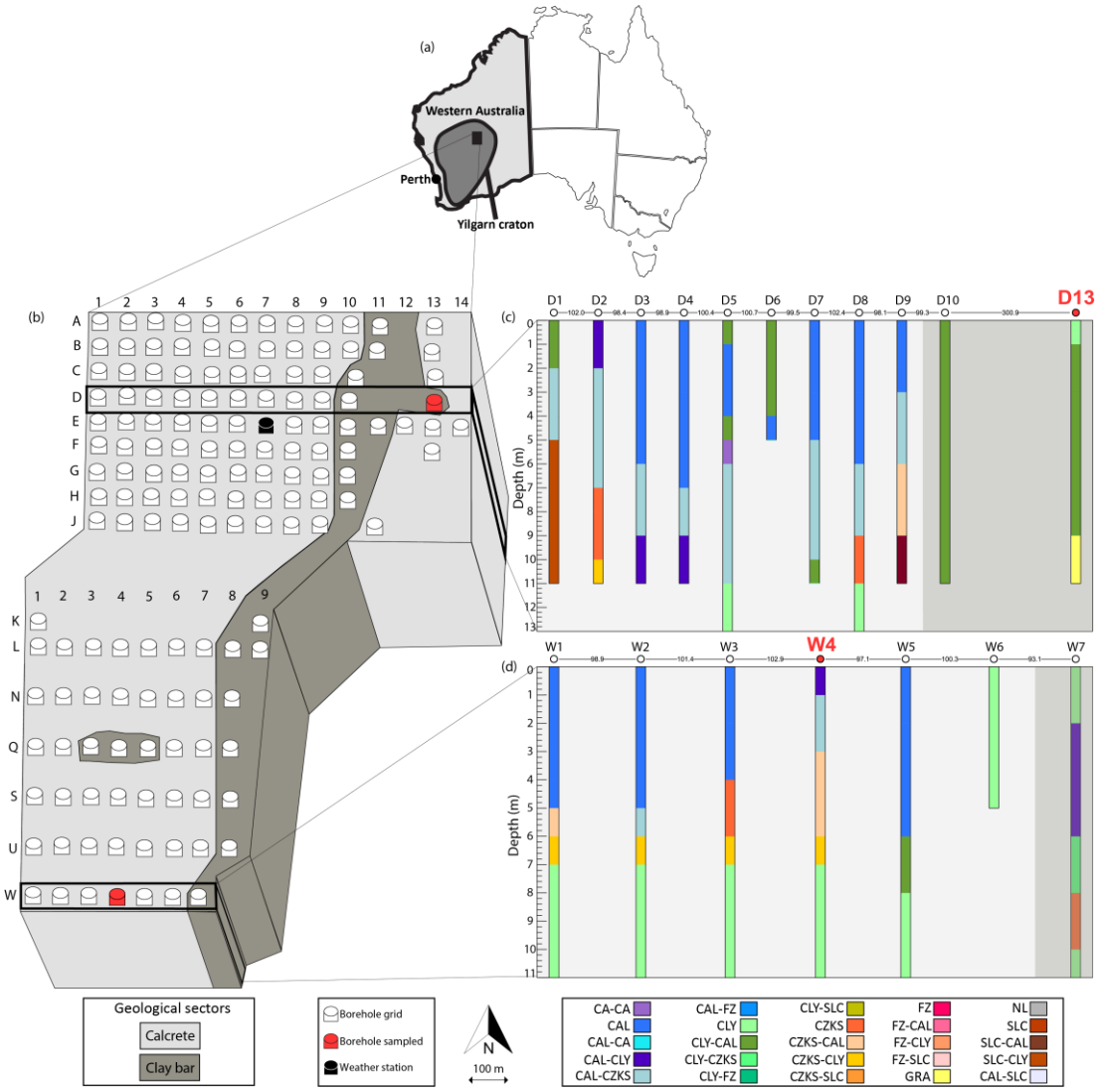

Figure 5.1. Borehole grid showing its location within the Yilgarn region (a), the geological sectors (b) and the bores sampled (D13 and W4, in red), together with the lithological profiles ((c) and (d)). CAL: soft calcrete, CZKS: siliceous calcrete, FZ: ferruginous zone, CLY: clay, GRA: granite, NL: no geology log, SLC: silcrete, GDR: granodiorite, ASB: asbestos, CA: cavity.

The mean permeability of the SM calcrete is similar to that of sand (1.9–4.6 × 10^{-4} m s–1 (Anaconda, 2001)), suggesting an average porosity of ~25% (Allford et al., 2008). The average yearly rainfall of the area is low, at around 200 mm and pan evaporation is 2400 mm $year^{-1}$ (BoM). The aquifer is very shallow, located two to four metres below the surface, and is accessible through boreholes, initially drilled for mineral exploration, along a grid that can be divided into two sections. The northern grid is 0.9 × 1.4 km with bore spacing at 100 m in north-south and east-west directions, while the southern section is 1.2 × 0.9 km, containing bores separated from each other 100 m east–west and 200 m north–south

(Figure 5.1b). These bores are capped but unlined, except within about 0.5 m from the surface, where they are lined with PVC pipe, to stabilise the surface (Allford et al., 2008).

5.2.2 *Field work procedures and sample preparation*

Groundwater samples were collected from the unlined bores at D13 (zone CD) and W4 (zone A2) using a submersible centrifugal pump (GEOSub 12V Purging Pump) after three well-volumes were purged and stabilisation of in-field parameters was observed. The selected bores are representative of the two main geological units of the area, W4 being in calcrete and D13 in clayey formations (Fig 1b,c,d). Preliminary investigations on the hydrology of the SM aquifer indicated that these two bores are the most reliable (i.e. lowest risk of drying) to test biogeochemical and ecological patterns across time (Saccò et al., 2019b).

Rainfall and groundwater level fluctuations were monitored for one year (from 18/06/2017 to 17/06/2018) through a weather station installed near bore E7 (Figure 5.1b) and indicated very patchy rainfall events and unpredictable recharge dynamics (Figure 3.2). However, monitoring of groundwater chemistry at SM calcrete revealed infiltration of rainfall from the surface together with increased inputs of ammonia after rain precipitation (Saccò et al., 2019b). For this study, two sampling campaigns, corresponding to contrasting rainfall periods as categorised by Hyde et al. (2018), were carried out on the 7/11/2017 (low rainfall, LR; <10 mm of rain during the 30 days prior to sampling) and on the 17/03/2018 (high rainfall, HR; >30 mm of rain during the 30 days prior to sampling).

Changes in carbon content in water after different levels of rainfall were investigated using dissolved organic (DOC) and inorganic (DIC) carbon and their isotopes ($\delta^{13}C_{DOC}$ and $\delta^{13}C_{DIC}$) coupled with radiocarbon analysis ($^{14}C_{DOC}$ and $^{14}C_{DIC}$). These techniques were complemented by measuring the DOC fluorescence. Samples for $\delta^{13}C_{DOC}$ were filtered through 0.2 µm glass fiber filters, collected in 60 mL HDPE bottles and frozen after sampling. The $^{14}C_{DOC}$ samples were filtered through 0.2 µm filters, collected in 1 L HDPE bottles and frozen after sampling. The $\delta^{13}C_{DIC}$ samples were filtered through 0.2 µm filters, collected in 12 mL glass vials (Exetainers) and refrigerated after sampling. Samples for $^{14}C_{DIC}$ analysis were filtered through 0.45 µm filters and collected in 1 L HDPE, with no further treatment. The DOC fluorescence samples were collected in 1 L HDPE bottles and kept refrigerated in darkness

until further tests. Other hydrochemistry parameters such as water isotopes (^{3}H, $\delta^{18}O$ and $\delta^{2}H$) and chloride concentration (Cl^{-}) were measured in water samples collected in 1 L HDPE bottles that were immediately frozen until further analyses. All samples were sealed with sealing tape after collection to limit atmospheric exchange, and kept in the dark.

Water samples for functional genomic investigations on the microbial community were collected from the bore W4 and stored in 1 L HDPE bottles and frozen immediately after collection. Samples were then filtered through 0.4 µm nitrocellulose membrane filter (Millipore, Sigma, Burlington, MA, USA) using a vacuum system, and the filtered content was kept frozen (-20°C) until further analyses. Temperature, pH, ORP, salinity, DO, and depth were measured *in situ* (bores D13 and W4) using portable field measurement equipment (Hydrolab Quanta Multi-Probe Meter®).

5.2.3 *Instrument methods and data analysis*

5.2.3.1 *Biogeochemical measurements*

DOC was determined by the non-purgeable organic carbon (NPOC) method using a Shimadzu high temperature combustion TOC-L/TNM-L analyser. DIC was obtained through a total organic carbon (TOC) configuration which measured the total carbon, followed by inorganic carbon. The TOC analysis was based on a standard method 5310-B (APHA, 2012) with detection by NDIR detector. Both DOC and DIC analyses were run in duplicates and the combustion temperature was 720 °C.

$\delta^{13}C_{DOC}$ isotopic ratios of waters were calculated by a Liquid Chromatography Isotope Ratio Mass Spectrometer (LC-IRMS) at the La Trobe Institute for Molecular Sciences (LIMS, La Trobe University, Melbourne, Australia) composed by a Accela 600 pump connected to a Delta V Plus Isotope Ratio Mass Spectrometer *via* a Thermo Scientific LC Isolink (Thermo Scientific). $\delta^{13}C_{DIC}$ isotopic ratios in water were analysed by Isotope Ratio Mass Spectrometer - Western Australia Biogeochemistry Centre at The University of Western Australia using a GasBench II coupled with a Delta XL Mass Spectrometer (Thermo-Fisher Scientific) - and results, with a precision of ± 0.10 per mil (‰), were reported as ‰ deviation from the NBS19 and NSB18 international carbonate standard (Dogramaci and Skrzypek, 2015). $\delta^{13}C_{DOC}$ and $\delta^{13}C_{DIC}$ values were reported ‰ relative to the Vienna Peedee Belemnite (VPDB).

For radiocarbon analyses of both the DOC and DIC ($^{14}C_{DOC}$ and $^{14}C_{DIC}$), pre-treated samples were subjected to CO_2 extraction and graphitization following the methodology published by Hua et al. (2001) and Bryan et al. (2017). ^{14}C content of samples was determined by means of the Accelerator Mass Spectrometry (AMS) at ANSTO (Australian Nuclear Science and Technology Organization) in Sydney, Australia (Fink et al., 2004). Radiocarbon results were reported in conventional age before present (BP, with BP being 1950), percent of modern carbon (pMC) and $\Delta^{14}C$ value in per mil (‰) relative to the absolute radiocarbon standard activity in 1950 (Stuiver and Polach, 1977).

Absorbance scans and excitation emission matrices (EEMs) were recorded simultaneously using an Aqualog® (Horiba Scientific). Fluorescence intensities were measured at excitation wavelengths 250-500 nm (1 nm increments) and emission wavelengths 250-575 nm (3 nm increments). The composition of DOM was characterised by a range of indices (BIX, HIX_{EM}, and A_{254}, Table S5.2) and by identifying individual fluorescent components using parallel factor analysis (PARAFAC) (Stedmon and Markager, 2005).

5.2.3.2 *Genetic analyses*

Three 1 litre water sample replicates collected from the bore W4 (zone A2) during both rainfall periods (LR and HR) were used for bacterial 16S metabarcoding and microbial functional analysis. Water samples were filtered using two Sentino peristaltic microbiology pumps (Pall Life 126 Sciences, New York, USA), through 0.45 µm sterile membrane filters (Pall Life Sciences, New York, USA). All water filtering equipment was soaked for a minimum of 10 minutes in 10% sodium hypochlorite solution and treated with UV light prior to use and between samples. Immediately post-filtering, half of the filter membrane was used for DNA extraction, while the remaining half was frozen at -20°C.

Water membranes, inclusive of laboratory controls, were extracted using DNeasy Blood and Tissue Kit (Qiagen; Venlo, Netherlands), with the following modifications to the manufacturer's protocol. For the DNA digest, both the ATL buffer (360 µL) and Proteinase K (40 µL) solutions were doubled to ensure that the membranes were adequately exposed to the lysis solution to optimise DNA yield. The DNA digests were incubated (56°C) overnight in a rotating hybridisation oven. The digest was transferred into a clean tube and loaded into a QIAcube (Qiagen; Venlo, Netherlands) automated DNA extraction system for the

remainder of the extraction process. The DNA was eluted off the silica column in 100 µL AE buffer.

The quality and quantity of DNA extracted from each water membrane was measured using quantitative PCR (qPCR), targeting the bacterial 16S gene. PCR amplifications to assess the quality and quantity of the DNA target of interest *via* qPCR (Applied Biosystems [ABI], USA) were in 25 µL reaction volumes consisting of 2 mM $MgCl_2$ (Fisher Biotec, Australia), 1 x PCR Gold Buffer (Fisher Biotec, Australia), 0.4 µM dNTPs (Astral Scientific, Australia), 0.1 mg bovine serum albumin (Fisher Biotec, Australia), 0.4 µM of each primer (Bact16S_515F and Bact16S_806R; Caporaso et al., 2011; Turner et al., 1999), and 0.2 µL of AmpliTaq Gold (AmpliTaq Gold, ABI, USA), and 2 µL of template DNA (Neat, 1/10, 1/100 dilutions). The cycling conditions were: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 52°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 10 minutes.

Extracts that successfully yielded DNA of sufficient quality, free of inhibition, as determined by the initial qPCR screen (detailed above), were assigned a unique 6-8bp multiplex identifier tag (MID-tag) with the bacterial 16S primer set. Independent MID-tag qPCR for each water membrane were carried out in 25 µL reactions containing 1X PCR Gold Buffer, 2.5 mM $MgCl_2$, 0.4mg/mL BSA, 0.25 mM of each dNTP, 0.4 µM of each primer, 0.2 µL AmpliTaq Gold and 2-4 µL of DNA as determined by the initial qPCR screen. The cycling conditions for qPCR using the MID-tag primer sets were as described above. MID-tag PCR amplicons were generated in duplicate and the library was pooled in equimolar ratio post-PCR for DNA sequencing. The final library was size selected (160-600bp) using Pippin Prep (Sage Sciences, USA) to remove any MID-tag primer-dimer products that may have formed during amplification. The final library concentration was determined using a QuBitTM 4 Fluorometer (Thermofischer, Australia) and sequenced using a 300 cycle V2 kit on an Illumina MiSeq platform (Illumina, USA).

MID-tag bacterial 16S sequence reads obtained from the MiSeq were sorted (filtered) back to the water sample based on the MID-tags assigned to each DNA extract using Geneious v10.2.5 (Drummond et al., 2011). MID-tag and primer sequences were trimmed from the sequence reads allowing for no mismatch in length or base composition. Filtered reads were input into a containerised workflow comprising USEARCH (Edgar, 2010) and BLASTN

(Altschul et al., 1990). The fastx-uniques, unoise3 (with minimum abundance of 8) and otutab commands of USEARCH were applied to generate unique sequences, ZOTUs (zero-radius OTUs) and abundance table, respectively. The ZOTUs were compared against the nucleotide database using the following parameters in BLASTN: perc_identity >= 94, evalue <= 1e-3, best_hit_score_edge 0.05, best_hit_overhang 0.25, qcov_hsp_perc 100, max_target_seqs = 5. An in-house Python script was used to assign the ZOTUs to their lowest common anscestor (LCA). The threshold for dropping a taxonomic assignment to LCA was set to perc_identity >= 96 and the difference between %identity of the two hits when their query coverage is equal was set to 1.

To investigate functional activity involved in carbon cycling, the 16S metabarcoding data were fed to the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) software package to generate predicted metagenome profiles (Langille et al., 2013). PICRUSt2 Python script picrust2_pipeline.py (Douglas et al., 2019) was run with default options using Zotu fasta files and Zotu tables (i.e. number of reads per sample per zotu) to predict functional abundances per each taxon, which were clustered into Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs (KOs) (Kanehisa and Goto, 2000) and MetaCyc pathway abundances (Caspi, 2006) focusing on carbon metabolism and degradation pathways, respectively. Regarding the comparisons across rainfall periods (LR and HR), higher functional abundances were referred as overrepresented, a widely employed terminology in functional genomic studies (e.g. González-Mercado et al., 2020; Yurgel et al., 2019; Zeng et al., 2015).

5.2.4 *Statistical analysis*

The statistical analyses on isotope, fluorescence and absorbance data were performed in R software version 3.6.0 (Development-Core-Team, 2016). DOC, DIC, $\delta^{13}C_{DOC}$ and $\delta^{13}C_{DIC}$ values (two independent replicates per each parameter) per each bore (W4 and D13) were compared across the two rainfall events using ANOVAs (R-package 'stats'; outliers were identified using box plot methods (package 'rstatix'), homogeneity of variances was tested through the Levene's test (function leveneTest()) and normality was tested through the Shapiro-Wilk test (function shapiro.test())). Radiocarbon results were unique per bore and sampling campaign, therefore data were not analysed statistically.

The R package staRdom (version 1.1.1) (Pucher et al., 2019) was used to correct EEMs, calculate all fluorescence/absorbance indices and for conducting PARAFAC modelling. EEMs were corrected for blanks (Milli-Q water), inner filter effects, Raman normalised (Lawaetz and Stedmon, 2009), and scatter (Raman and Rayleigh) were removed and interpolated prior to PARAFAC. Our PARAFAC model was split-half validated (Murphy et al., 2013) and recognized five fluorescent components (Figure S5.6). These components are reported as maximum fluorescence intensity of each component (Fmax) in each sample. Principal Components Analysis (PCA) was conducted on fluorescence/absorbance indices to assess differences between sites and rainfall period. The R studio (version 3.6.1) 'prcomp' function was used to carry out the PCA and results are presented in two dimensions (PC1 and PC2) along with eigenvectors. Differences in HIX_{EM}, BIX, FI and A_{254} between sites and rainfall periods were tested using 2-way ANOVA, where site and rainfall period (and their possible interaction) were treated as fixed factors. Tukey's HSD tests were performed to determine which of the means were significantly different when significant main effects were found. Data were log transformed to achieve normality when required.

Beta diversity patterns - the variations in species composition among rainfall periods - were analysed through the calculation of the Ochiai index (Ochiai, 1957) (R-package 'adespatial') and the quotient of the temporal turnover (Simpson pairwise dissimilarity) and total dissimilarity (measures as Sorensen pair-wise dissimilarity) (R-package 'betapart', function *beta.pair*). Pielou's evenness index (J) was calculated to infer the degree of dominant species in abundance, with values ranging from 0 (no evenness at all) to 1 (complete evenness). The Phyloseq package in R (McMurdie and Holmes, 2013) was used to plot the ZOTU abundance at the family and genus level for low rainfall (LR) and high rainfall (HR) periods from the bore W4. The Statistical Analysis of Metagenomic Profiles (STAMP) bioinformatics software package was used to visualize and determine statistically significant results from the PICRUSt2 output (Parks et al., 2014). For comparison of potential microbial metabolic shifts across rainfall periods, the White's non parametric t-test ($P < 0.05$) was used for both carbon metabolism and degradation pathways with confidence intervals of 95%, and visualized with extended error bar plots.

5.3 Results

5.3.1 *Organic inputs across rainfall periods*

The DOC concentrations ranged from 0.39 ± 0.21 mg/L (mean ± SD for W4 under LR) to 1.94 ± 0.75 mg/L (mean ± SD for D13 during HR), while concentrations of dissolved inorganic carbon (DIC) ranged from 63.5 ± 0.14 mg/L (mean ± SD for W4 under LR) to 87.44 ± 0.66 mg/L (mean ± SD for D13 during HR). DOC concentrations for bore W4 significantly increased under HR compared to LR (ANOVA, $P < 0.05$). Concurrently, W4 had significantly more positive $\delta^{13}C_{DOC}$ values under HR conditions than under LR conditions (ANOVA, $P < 0.001$) (Figure 5.2a). Groundwater from bore D13 also had higher DOC concentrations under HR (Figure 5.2a) than those during LR, but this was not statistically significant.

$\delta^{13}C_{DOC}$ values in D13 did not change after rainfall (Table S5.1), while its $^{14}C_{DOC}$ ages also remained similar between LR and HR, being younger than those in W4 (Figure 5.2c). Compared to W4, DIC concentrations were higher in D13, and $\delta^{13}C_{DIC}$ values were less enriched, but these differences were not statistically significant (Figure 5.2b). Similar increasing trends were found for DIC concentration and $\Delta^{14}C_{DIC}$ values for the two bores when LR was compared to HR (Figure 5.2d).

Increasing trends for water temperature, DO (bore D13) and chloride concentrations were coupled with decreasing patterns for pH, DO (bore W4), ORP and depth (Table S5.4) when LR was compared to HR. $\delta^{18}O$ and $\delta^{2}H$ values did not vary across rainfall periods within the two bores analysed, while Tritium values from bore D13 were slightly lower during HR (0.53 TU) when compared to LR (0.77 TU).

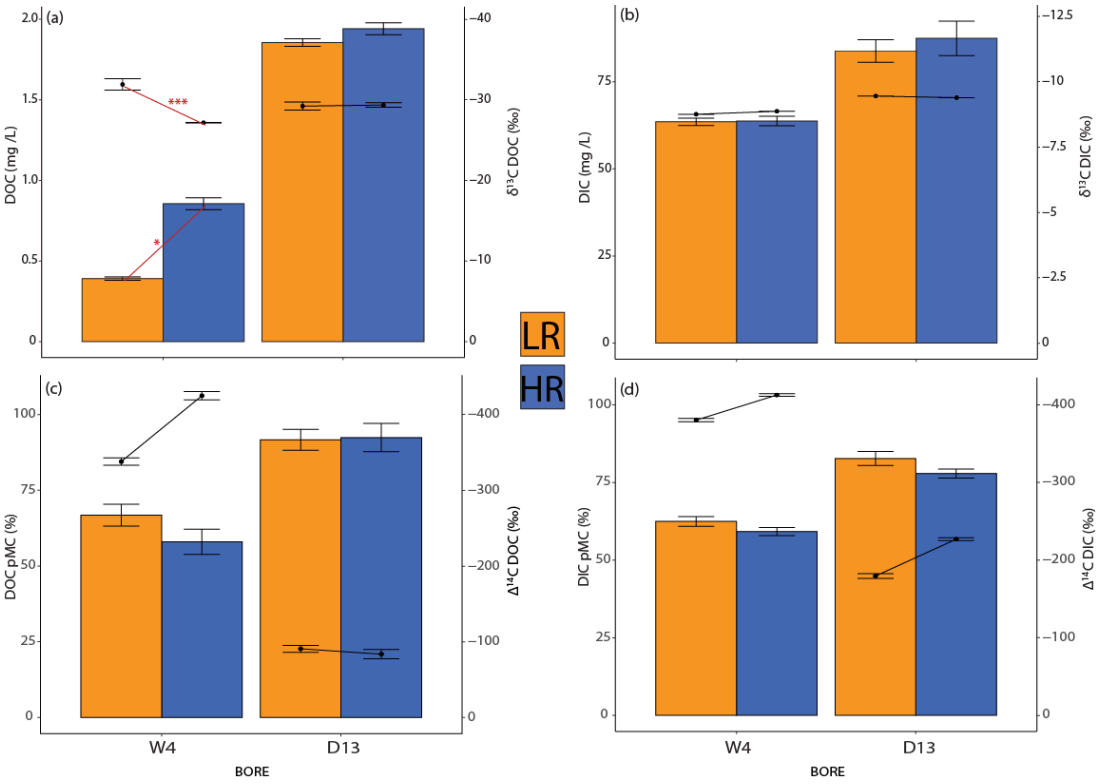

Figure 5.2. Bar charts illustrating DOC and DIC concentrations (a and b), and respective percent of modern carbon (pMC, with modern defined as 1950) (c and d) from the bores W4 and D13 across LR (dark yellow) and HR (blue). The whiskers of the bars refer to standard deviation values. Combined line graphs refer to δ^{13}C DOC (a), δ^{13}C DIC (b), Δ^{14}C DOC (c) and Δ^{14}C DIC (d). * significant trend with *P* value < 0.05; ** significant trend with *P* value < 0.005; *** significant trend with *P* value < 0.0005. Refer to Table S5.1 for the specific values of the parameters.

5.3.2 *Fluorescence and absorbance characterization*

Parallel factor analysis (PARAFAC) identified five unique humic-like fluorescent components (Figure S5.6). Component 1 (C1) had a primary excitation peak at <250 nm and secondary peak at 330 nm with a broad emission peak from 370 to >575 nm (Em. max at 415 nm). Component 2 (C2) had an excitation peak at <250 nm and at 300 nm with a broad emission peak from 350 to >575 nm (Em. max at 395 nm). Component 3 (C3) had an excitation peak at 268 nm and at 386 nm with a broad emission peak from 400 to >575 nm (Em. max at 446 nm). Component 4 (C4) had an excitation peak at 260 nm and at 370 nm with a broad emission peak from 420 to >600 nm (Em. max at 493 nm). Component 5 (C5) had an excitation peak at 250 nm and at 318 nm with an emission peak from 310 to >410 nm (Em. max at 364 nm). C1-C4 were all considered aromatic and derived from terrestrial plant sources while C5 represented a lower molecular weight, UVA humic-like compound

(Fellman et al., 2010). The rainfall affected the fluorescence intensity of all PARAFAC components. For site D13, the fluorescence maximum (Fmax) of all components increased after HR, while site W4 displayed the opposite trend, with elevated fluorescence after low rainfall (LR) (Figure 5.3a). During both rainfall periods, the Fmax of all components at site D13 was greater compared to site W4 (Figure 5.3a). Further, the relative composition of components changed between bores. C1 was most predominant across both bores and recharge periods explaining 37-50% of the CDOM signal. The contribution of C3 and C4 was consistent across samples and rainfall regimes ranging from 20-25% and 12-15% respectively. C5 had the largest change in contribution between the bores; contributing 7-8% at bore D13 and 13-18% at bore W4. Finally, during HR there was no presence of C2 at bore W4 (Figure 5.3a).

Optical indices (HIX_{EM}, A_{254}, BIX) varied between sites and rainfall period (Figure 5.3b). Overall, PCA of optical indices revealed a marked shift in CDOM composition for site W4, from more terrestrially derived compounds during LR to compounds with a lesser degree of humification during HR (i.e., microbial derived, autochthonous) (Figure 5.3b). In contrast, site D13 displayed negligible changes in CDOM composition, displaying slightly greater intensity of humic-like/terrestrial compounds during HR compared to during LR (Figure 5.3b). The humification index (HIX_{EM}) ranged from 0.89 to 0.99, indicating that CDOM for both bores and rainfall periods was largely comprised of humic compounds, as HIX_{EM} values above 0.9 indicated a greater degree of humification (Hansen et al., 2016; Ohno, 2002; Zsolnay et al., 1999). During HR, both sites showed a marginal decrease in their HIX_{EM} values, especially for site W4, however both remained close to 0.9 (Figure 5.3c). Greater A_{254} absorbance at bore D13 indicated more aromatic content than site W4 (Figure 5.3b and c). Interestingly, BIX was greater at site W4 ($\mu = 1.53 \pm 0.11$) during HR than during LR ($\mu = 1.07 \pm 0.04$) and compared to site D13 ($\mu = 0.87 \pm 0.01$) for either rainfall period ($P < 0.05$, Figure 3c). The fluorescence index (FI) indicated CDOM was of terrestrial origin (FI ~ 1.4; McKnight et al., 2001) and did not change between bores or rainfall periods ($\mu = 1.46 \pm 0.02$; Figure 5.3c).

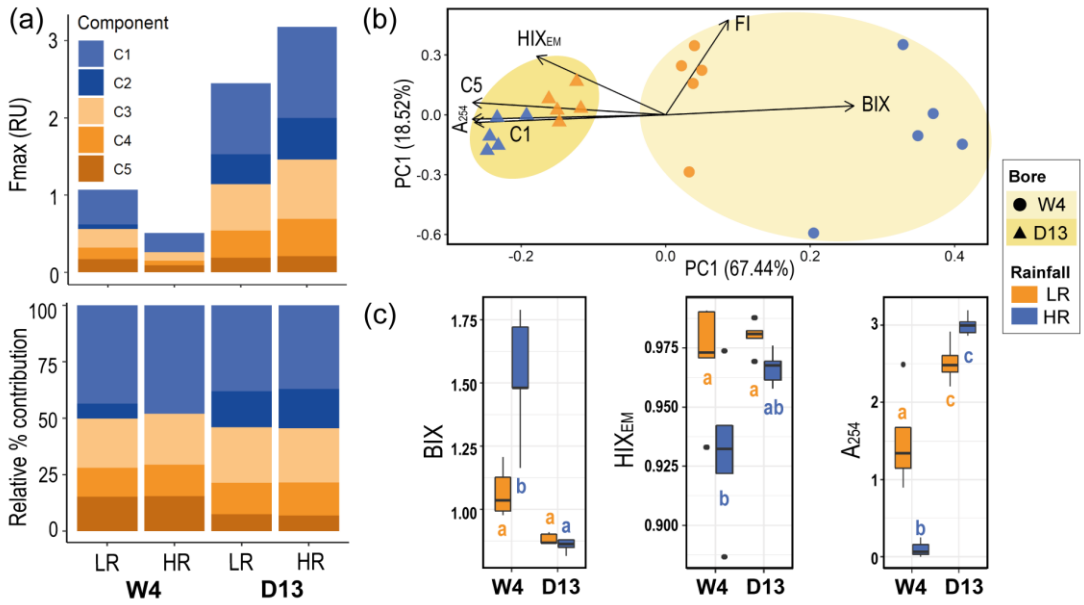

Figure 5.3. CDOM characterisation between bores and recharge periods. (a) Fluorescence maximum in Raman units (Fmax) and percent contribution of the five PARAFAC components (i.e. C1, C2, C3, C4 and C5), (b) PCoA ordination discriminating PARAFAC components, fluorescence indices (HIX_{EM}, BIX) and absorbance indices (A_{254}) where correlations of indices with axes are visualised as arrows, and, (c) BIX, HIX_{EM}, and A_{254} values. Significant post hoc comparisons ($P < 0.05$) are indicated by lowercase letters.

5.3.3 *Microbial patterns*

The 16S rRNA sequencing yielded 7503 sequences clustered into 87 ZOTUs (37 ZOTUs either belonged to uncultured bacteria or no reference was available). After the removal of the ZOTUs associated with the lab controls (N=16), 25 ZOTUs were unique to LR, 25 ZOTUs belonged to HR, and both rainfall periods shared the other 21 ZOTUs. During LR, the dominant ZOTUs belonged to the families Rhodobacteraceae (*Paracoccus* sp. and *Roseivarius* sp.), Pseudomonadaceae (*Pseudomonas* sp.), Planococcacea (*Planomicrobium* sp.) and Caulobacteraceae (*Brevundimonas* sp.). Under HR the dominant ZOTUs corresponded to the families Rhodobacteraceae (*Stappia* sp. and *Roseibacterium* sp.), Phyllobacteriaceae (*Nitratireductor* sp.) and Rhodospirillaceae (*Thalassobaculum* sp. and *Tageae* sp.) (Figure S5.7). All the genera experienced turnovers between LR and HR (Ochiai index, $P < 0.05$), suggesting that a shift in community assemblages across the two rainfall events has occurred. Specifically, 81.5% of the dissimilarity was due to genus replacement between rainfall periods (turnover), with the rest (18.5%) explained by the nestedness (species loss from rainfall period to rainfall period). Values of the Pielou's evenness index (J) during LR and HR ranged from 0.71 to 0.74.

Predictions of the quantitative proportion of individual metabolic pathways related to carbon turnover revealed a dominance of carbon fixation (46%) and methane metabolism (40%), followed by carbohydrate (8%) and lipid metabolisms (6%) (Figure 5.4a). Despite being more abundant under LR for the former two, none of the four main metabolic categories changed significantly between LR and HR. Pairwise tests indicated that 4 out of the 10 carbon processing pathways (Figure 5.4b) and 10 out of the 76 degradative pathways examined (Figure 5.4c) were significantly ($P < 0.05$) overrepresented in one of the two rainfall periods (either LR or HR). For carbon metabolism, the dicarboxylate-hydroxybutyrate cycle was more abundant during LR, whereas the reductive pentose phosphate cycle, pentose phosphate pathway and reductive acetyl-CoA pathway were more abundant during HR. With the exception of the glycogen degradation pathway, all the degradative pathways (arginine, purine, catechol, glucose, salicylate and aromatic compounds) were more abundant during HR. All pathways tested can be found in Table S5.3.

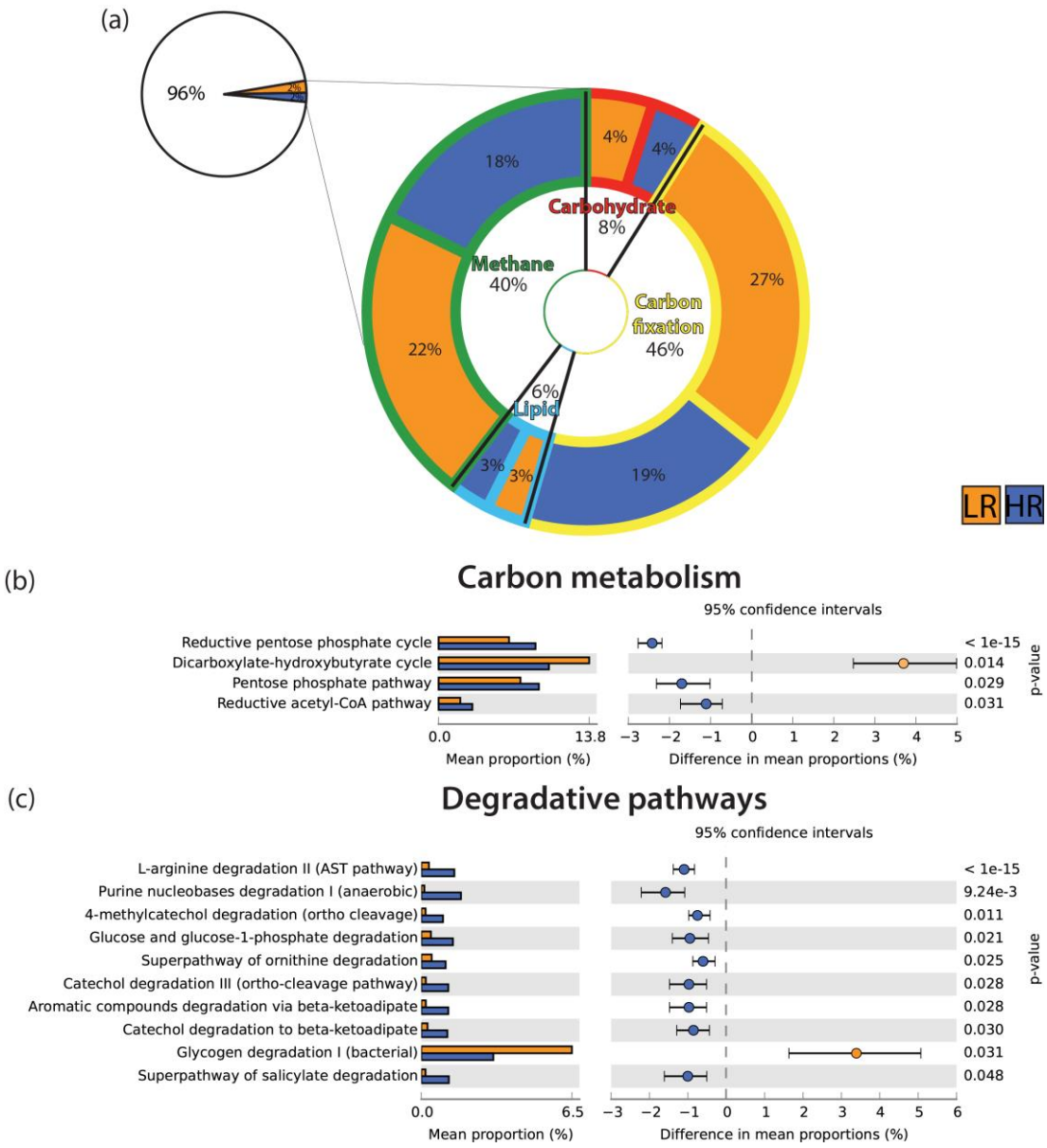

Figure 5.4. Prediction of the microbial community metabolic status based on 16S rRNA amplicon sequencing and functional genomics analyses between LR and HR periods from the bore W4. (a) doughnut chart showing the proportion of the metabolisms considered compared with the total pathways detected and the specific proportions of methane (green), carbohydrates (red), lipid (light blue and carbon fixation (yellow) metabolisms (derived from KOs). (b) and (c) extended error bar plots of predictive metagenome pathways differentially abundant between rainfall periods (P < 0.05, White's non parametric t-test).

5.4 Discussion

5.4.1 *Carbon replenishment in groundwater systems*

In groundwater systems, carbon is replenished either through diffuse recharge through the unsaturated zone and/or *via* direct recharge from surface waters (Meredith et al., 2019). These processes are linked to rainfall conditions (i.e. wet/dry periods) and the hydrology of the system. SW/GW interactions drive OM incorporation into the ecosystem, which is typically characterised by low carbon content (McDonough et al., 2019).

Aquifer recharge indicators such as tritium, oxygen-18 and deuterium did not vary much in the Sturt Meadows system between rainfall periods, suggesting limited recharge during our study. Conversely, chloride concentrations increased under HR (Table S5.4), suggesting intrusion of hypersaline water from the surface during this period. These results indicate that carbon and nutrient inflows occur despite low recharge after rainfall, suggesting that soil zone processing plays a key role in regulating the biochemical flows at SM aquifer (Saccò et al., 2019b).

DOC concentrations revealed increasing trends after rainfall (HR), although only statistically significant for W4, indicating some carbon inputs to the system. At bore W4, older ($^{14}C_{DOC}$) and enriched DOC ($\delta^{13}C_{DOC}$) was found under HR, suggesting a sedimentary organic matter source, likely subject to microbial reprocessing causing stable isotope enrichment. In contrast, bore D13 showed stable trends characterised by more modern DOC inputs. This difference in biochemical patterns suggests that *in situ* carbon sources play a central role at bore W4, possibly in tandem with changes in microbial activity occurring during HR. Meanwhile, bore D13 is receiving steady inflows of younger (and less microbially recycled) OM.

Patterns of DIC concentrations and $\delta^{13}C_{DIC}$ were steady across rainfall regimes. Inorganic dissolution was higher in D13, and input from younger carbonates (inorganic fraction of carbon in calcretes) was detected. Overall, our isotopic data from organic and inorganic carbon indicated different responses in the upper (D13) and lower (W4) catchments, with D13 showing more modern carbon but less response to the rainfall event.

Groundwater CDOM quality depended on the bore and rainfall period. Humification (HIX_{EM}) and fluorescence index (FI) values indicated that CDOM from both bores, regardless of rainfall period, were dominated by high molecular weight molecules (humic-like fluorophores) associated with the presence of terrestrially derived organic matter (i.e. FI ~ 1.4, HIX_{EM}>0.9; McKnight et al., 2001; Ohno, 2002). Furthermore, most CDOM components (C1-C4) were identified as large molecular weight humic-like compounds derived from terrestrial plant material, with the exception of C5 which was identified as UVA humic-like, a lower molecular weight component that is associated with autochthonous production and microbial processing (Fellman et al., 2010). The intensity of all components (C1-C4) was greater in the upper catchment (i.e. bore D13), which is consistent with the presence of the more modern and less ^{13}C enriched terrestrial carbon at this site shown by the isotopic data (i.e. >DOC). Fellman et al. (2011) also showed an overall decrease in fluorescence characteristics from the upper to lower catchment in pools of the semiarid Pilbara (Western Australia). The fluorescence results indicate that the dominant source of groundwater carbon at Sturt Meadows aquifer is the terrestrial soil. However, during HR, bore W4 shows elevated BIX (>1.5) values indicated the presence of CDOM with an autochthonous origin (i.e. microbially derived; Helms et al., 2008), along with an increased relative contributions of a lower molecular weight component (i.e. C5) at this bore. This is again consistent with the isotopic results, and suggests that the HR event stimulated specific microbial activity at this site, leading to changes in the recycling of older organic matter, and stable isotopic enrichment.

One potential explanation for this is the infiltration of ions from hypersaline surficial soils into the groundwater during HR, as well as potential mixing with the adjacent lake Raeside (i.e. >-Cl, Table S5.4), forming a groundwater estuary (Humphreys et al., 2009). Autochthonous CDOM is more common in estuarine and marine environments compared to freshwater bodies (Santos et al., 2016) and has been reported across microtidal subterranean estuaries (Couturier et al., 2016). The occurence of this at W4 but not D13 may relate to either its geology (W4 is in calcrete, while D13 has a higher proportion of clays), or its position in the lower half of the bore field which is hydrologically nearer the neighbouring saline systems.

One alternative explanation for the CDOM results would be an influx of photochemically altered older carbon from the overlying soils, as the non-mineralized fraction of

photobleached CDOM has optical properties that are similar to estuarine and marine CDOM (Hansen et al., 2016; Helms et al., 2008). However, there is no obvious explanation as to why this should occur only around bore W4.

Overall, our results suggest that rainfall events play a role in regulating carbon stocks at the SM calcrete, but that the resultant changes are not straightforward. The rainfall events measured were not substantial enough to trigger a full hydrological recharge of the system - something that will become more common with the declining rainfall in the Yilgarn region - but nonetheless sufficiently affected the lower part of the bore field to drive changes in the OM type. To understand the details of this change, a better understanding of the microbiome of the system and its interaction with changes in water chemistry is required.

Several investigations have stressed the importance of rainfall events as ecological drivers leading to shifts in biotic community assemblages in groundwater environments (e.g. Reiss et al., 2019; Wu et al., 2018). The current climate change scenario predicts reduced rainfall events linked to increased droughts, events that are likely to affect the biochemical balance sustaining biota in groundwater (Green et al., 2011; Mammola et al., 2019a). Modelling of current ecological dynamics will allow prediction of future effects to the vital (and too often taken for granted) ecosystem services provided by groundwater environments.

5.4.2 *Microbial trends and carbon pathways*

The studied rainfall events triggered shifts in the microbial community assemblages in bore W4. Under both rainfall conditions, the microbial community was typical of saline and hypersaline environments (Stepanov et al., 2014; Unno et al., 2015). Rhodobacteracea, the most widely distributed bacteria in marine environments (Pujalte et al., 2014), was the most dominant family on site. Interestingly, families that were highly abundant under HR (i.e. Phyllobacteriaceae and Rhodospirillaceae) were scarce under the LR period, indicating that rainfall provides conditions for their proliferation. Conversely, the vast majority of other families - especially Pseudomonadacea, Planococcaceae and Caulobacteraceae - were only present during LR. Genus level analysis indicated a more abundant and diverse community under LR than during HR. Decline in biodiversity during recharge events has been ascribed to dilution processes caused by water inflows linked to storm events decreasing the density of micro-organisms and thus their detectability (Pronk et al., 2009),

and low recharge regimes have been suggested hosting the autochthonous microbial community (Farnleitner et al., 2005). While dilution of bacterial density processes may play a role at Sturt Meadows, a more comprehensive understanding of the microbial ecological dynamics and their variation over time is needed, requiring further long-term investigation.

The putative assessment of pathways related to cells' carbon metabolisms provided evidence for inorganic carbon fixation and methane pathways (i.e. methane oxydation), two of the most common biochemical routes reported in groundwater systems (e.g. Brankovitz et al., 2017; Rightmire and Hanshaw, 1973; Ullman et al., 2003). No significant changes in the proportions of each of the main pathways were detected between LR and HR. In a recent study, Hofmann and Griebler (2018) tested the 'priming effect' - the activation of microbial growth and OM transformation under increased OM availability - in groundwater. After a series of laboratory experiments under increasing nutrient concentrations, no evidence of priming could be observed. While in overall agreement with these findings, our investigation of specific metabolic pathways revealed a substantial increase in degradative pathways under HR in W4, which is again consistent with our fluorescence and isotopic results. Volatile organic compounds, such as toluene, catechol and phenyl acetate, can be very abundant in contaminated aquifers (e.g. Abbai et al., 2012; Langwaldt et al., 2006; Shinoda et al., 2004) and may also occur naturally in the hypersaline lakes of Western Australia (Ruecker et al., 2014). Aromatic compounds have been found leaching into groundwater after rainfall and shifting the character of DOM in sandy aquifers (McDonough et al., 2019), confirming their importance as biochemical drivers in typically low energy systems (e.g. Filip and Smed-Hildmann, 1992). After rainfall, the microbial community in W4 seemed to profit from these new inflows of OM, as indicated by the high abundances of taxa potentially involved in aromatic compound degradation such as *Stappia* sp., *Roseibacterium* sp., *Tageae* sp. and *Thalassobaculum* sp. (e.g. Dutton and Evans, 1978; Pujalte et al., 2014 and references therein). Taxa with a high affinity to denitrification processes such as *Paracoccus* sp., *Roseovarius* sp., *Brevundimonas* sp. and *Planomicrobium* sp. (e.g. Chen et al., 2015; Pujalte et al., 2014 and references therein; Tsubouchi et al., 2014), dominated under LR. However, denitrifying *Nitratireductur* sp. was also present under HR, suggesting that nitrogen (nitrates, nitrites and ammonia) provides basal energy sources under both rainfall conditions. However, additional investigations on specific nitrogen pathways of SM calcrete bacteria will be necessary to elucidate this further.

Degradation of glucose also increased under HR, suggesting adaptations to the increased OM availability. During these conditions, abundances of *Pseudomonas* sp. - one of the most opportunistic and versatile bacteria on earth - plummeted, probably due to the repressing effect of glucose on the expression of several genes (Rojo, 2010). The other three degradative metabolic pathways which were more abundant after rainfall - arginine, ornithine and purine - constitute catabolic pathways whose main product is ammonia (e.g. Schneider et al., 1998). High ammonia concentrations were detected under HR (Saccò et al., 2019b), and might represent a compendium of nutrient inputs and metabolic production.

The only degradative pathway that was significantly overrepresented during LR was the degradation of glycogen, the primary carbon and energy storage compound of many bacteria (Park et al., 2011). Our results are in line with Yamamotoya et al. (2012), suggesting that this polysaccharide of glucose is key to long-term bacterial survival and is utilised when carbon sources become limiting, as per the case of LR period. Another pathway that followed this decreasing trend after rainfall was the dicarboxylate-hydroxybutyrate cycle. Characteristic for microaerophiles and anaerobes (Berg, 2011), this cycle is considered 'energetically efficient' in contrast to other autotrophic carbon fixation pathways (Lannes et al., 2019). A plausible explanation is that the dicarboxylate-hydroxybutyrate pathway is activated when OM is scarce (such as LR), and uncommon under HR when OM is more available. In addition to pathways involving OM processing, those involving inorganic carbon fixations, namely the reductive acetyl-CoA pathway and reductive pentose phosphate cycle, also increased after rainfall. Inorganic incorporation might play a role in natural carbon fixation and cycling in groundwater microbes (i.e. Nowak et al., 2017), an assumption that has rarely been tested.

5.5 Conclusions

A combination of biochemical and genetic data allowed preliminary untangling of the biochemical function regulating microbial communities at the SM calcrete (Figure 5.5). Given their importance in allowing the transition between abiotic to biotic frameworks, bacteria are vital in shaping the biochemical flows regulating subterranean biodiversity (Griebler and Lueders, 2009). However, despite their importance, many questions about subterranean microbial processes remain unresolved. Indeed, the fields of groundwater ecology and subterranean microbiology would mutually benefit from the integration of

respective insights. Due to increased natural and anthropic pressures, subterranean biotic communities are currently being exposed to increased losses of taxonomical and functional diversity, leading to poorer and more fragile groundwater ecosystems (Mammola et al., 2019b). Further medium to long term interdisciplinary studies monitoring the changes in groundwater ecological dynamics will allow to assess the impact of the climate changes on one of the most essential ecosystems in the world.

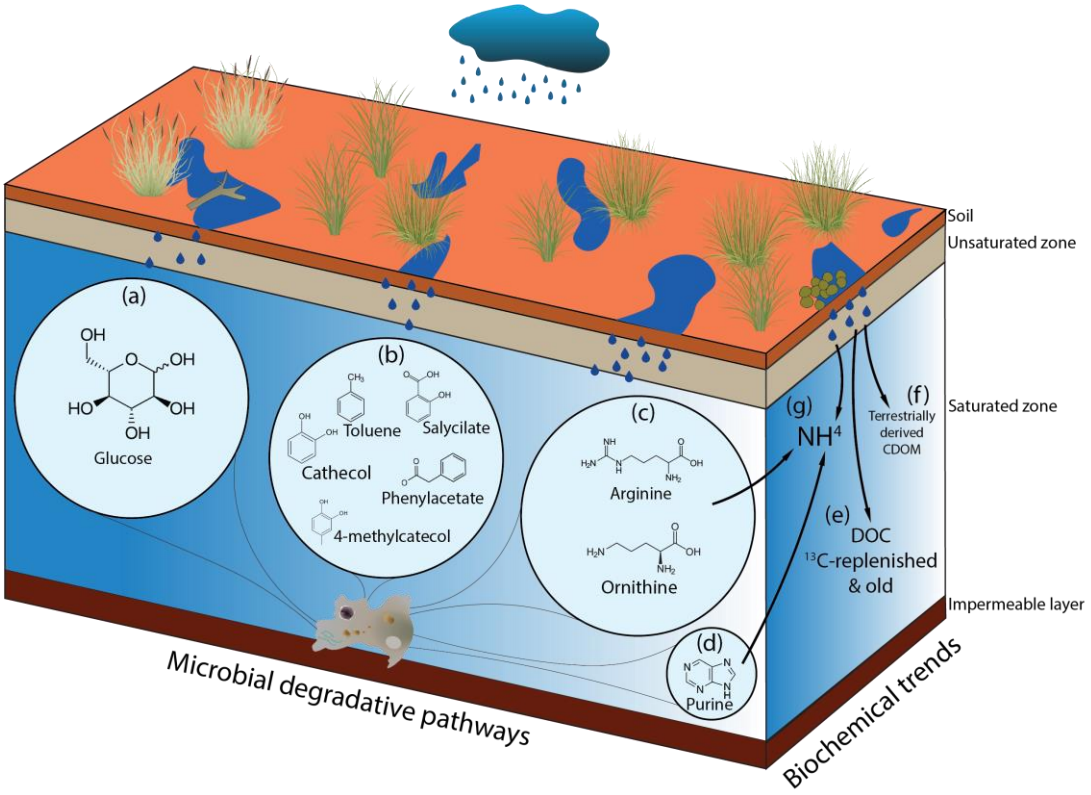

Figure 5.5. Scheme of the main degradative pathways and biochemical patterns under HR. (a) Glucose degradation, (b) aromatic degradation, (c) arginine and ornithine degradation, (d) purine degradation, (e) DOC replenishment inferred from isotopic data, (f) terrestrially derived CDOM inflows (fluorescence analysis) and (g) increase in ammonia concentrations as a result of nutrients inputs from the surface and microbial metabolic activities (purine and amino acid degradation).

Akcnowledgements

We wish to acknowledge the traditional custodians of the land, the Wongai people, and their elders, past, present and emerging. We acknowledge and respect their continuing culture and the contribution they make to the life of Yilgarn region. The authors thank Flora, Peter and Paul Axford of the Meadows Station for their kindness and generosity in providing both accommodation and access to their property.

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Supplementary material

Table S5.1. DOC (Dissolved Organic Carbon) and DIC (Dissolved Organic Carbon) concentrations (mg/L), $\delta^{13}C$ DOC, $\delta^{13}C$ DIC, pMC DOC, $\Delta^{14}C$ DOC, Conventional Age DOC, pMC DIC, $\Delta^{14}C$ DIC, Conventional Age DIC for the bores W4 and D13. BP: before present with present being 1950 AD; pMC: percent of modern carbon.

	Concentration (mg/L)		$\delta^{13}C$		pMC	$\Delta^{14}C$ (‰)	Age (BP)	pMC	$\Delta^{14}C$ (‰)	Age (BP)
	LR	HR	LR	HR	LR			HR		
DOC_{W4}	0.39±0.21	0.86±0.73	-31.91±0.5	-27.15±0.03	66.76±0.48	-337.9±4.84	3245±60	57.99±0.55	-426.1±5.5	4380±80
DOC_{D13}	1.86±0.46	1.94±0.75	-29.25±0.36	-29.35±0.2	91.7±0.46	-90.6±4.6	695±45	92.41±0.62	-87.4±6.1	630±60
DIC_{W4}	63.50±0.14	63.71±0.18	-8.75±0.1[2]	-8.87±0.1[2]	62.47±0.21	-380.5±2.1	3835±30	59.2±0.17	-412.8±1.7	4210±25
DIC_{D13}	83.81±0.43	87.44±0.66	-9.45±0.1[2]	-9.39±0.1[2]	82.73±0.3	-179.5±3	1575±30	77.9±0.19	-227.4±1.9	2005±20

[1] Accuracy of the GC-iRMS

Table S5.2. Fluorescence/absorbance indices and their definitions. Adapted from Coble et al. (2014). Note: em = emission wavelengths, ex = excitation wavelengths.

Fluorescence /absorbance index	Parameters/description	Interpretation	Reference
Humification Index (HIX_{EM})	At ex 254 nm, area of peak under em 435–480 nm divided by area under em 300–345 nm + 435–480 nm.	Higher numbers are indicative of lower H:C ratios, attributed to a greater degree of humification.	(Zsolnay et al., 1999; Ohno, 2002)
Freshness Index (BIX)	Intensity at em 380 nm divided by max intensity between em 420 nm and em 435 nm at ex 310 nm.	Indicates proportion of recently produced DOM.	(Huguet et al., 2009; Fellman et al., 2010)
Fluorescence Index (FI)	The ratio of em 450 nm and em 500 nm at ex 370 nm.	Indicates if precursor material for DOM is of a more microbial (FI ~ 1.8) in nature or more terrestrially derived (FI ~1.2).	(McKnight et al., 2001)
Spectral Slope Ratio (SR)	The ratio of S275-295 to S350-400.	Lower S275-295 correlated with higher aromatic content and higher molecular weight, i.e. if SR is above 1 then the CDOM is more marine-like.	(Helms et al., 2008)
Coble peaks (A, B, C, M, T)	A: Humic-like ex = 250–260 nm, em = 380–480 nm. B: Tyrosine-like ex = 270–280 nm, em = 300–320 nm. C: Humic-like ex = 330–350 nm, em = 420–480 nm. M: Marine humic-like ex = 310–320 nm, em = 380–420 nm. T: Tryptophan-like ex = 270–280 nm, em = 320–350 nm.	Identifies the intensity of protein-like and/or humic-like peaks.	(Coble, 1996)
C:A	The ratio of Peak C to Peak A intensity.	An indication of the amount of humic-like vs. fulvic-like fluorescence in a sample.	(Coble, 1996; Baker et al., 2008)
C:M	The ratio of Peak C to Peak M intensity.	An indication of the amount of diagenetically altered (blue-shifted) fluorescence in a sample.	(Coble, 1996; Helms et al., 2013)
$SUVA_{254}$	Absorption coefficient at 254 nm divided by DOC concentration.	Absorbance per unit carbon. Typically a higher number is associated with greater aromatic content	(Weishaar and Aiken, 2001)

Table S5.3. Abundances of PICRUSt2 outputs relating to carbon metabolism (KO, level 3) and degradative pathways (MetaCyc). Pathways in bold indicate the significantly ($P < 0.05$) overrepresented pathways in one of the two rainfall periods.

Carbon metabolism - Kegg

Pathway	HR_1	HR_2	HR_3	LR_1	LR_2	LR_3
Glycolysis	7475	6729	7102	10596	12413	13758
Pentose phosphate pathway	3345	2940	3142	4040	4867	4303
Citrate cycle	5124	3854	4489	7233	9925	8574
Methane metabolism	3660	3036	3348	3713	4661	6245
Reductive pentose phosphate cycle	3285	2791	3038	3255	3983	4206
Reductive citrate cycle	4498	4799	4649	7553	10444	10894
Reductive acetyl-CoA pathway	1137	980	1058	1093	1556	861
3-Hydroxypropionate bi-cycle	3259	2391	2825	3760	5563	3017
Hydroxypropionate-hydroxybutylate cycle	1675	497	1086	1848	2738	1597
Dicarboxylate-hydroxybutyrate cycle	3831	3092	3462	6206	8896	9555

Degradative pathways - MetaCyc

pathway	HR_1	HR_2	HR_3	LR_1	LR_2	LR_3
3-phenylpropanoate and 3-(3-hydroxyphenyl)propanoate degradation	0	0	76	15	50	12
3-phenylpropanoate and 3-(3-hydroxyphenyl)propanoate degradation to 2-oxopent-4-enoate	0	0	41	7	29	4
3-phenylpropanoate degradation	1	28	1	0	0	0
4-aminobutanoate degradation V	67	61	169	115	69	236
4-deoxy-L-threo-hex-4-enopyranuronate degradation	1	76	98	0	0	0
4-hydroxyphenylacetate degradation	4	41	38	8	6	22
4-methylcatechol degradation (ortho cleavage)	55	35	87	24	1	2
acetylene degradation	50	175	246	108	139	273
adenosine nucleotides degradation II	414	24	315	343	509	231
allantoin degradation IV (anaerobic)	0	0	0	28	2	0
allantoin degradation to glyoxylate III	72	5	35	59	4	4
aromatic biogenic amine degradation (bacteria)	0	0	82	17	55	23
Aromatic compounds degradation *via* beta-ketoadipate	93	35	88	25	1	3
catechol degradation I (meta-cleavage pathway)	0	0	105	25	67	29
Catechol degradation III (ortho-cleavage pathway)	93	35	88	25	1	3
Catechol degradation to beta-ketoadipate	87	32	92	24	2	15
chlorosalicylate degradation	0	0	0	3	2	12
cinnamate and 3-hydroxycinnamate degradation to 2-oxopent-4-enoate	0	0	41	7	29	4

creatinine degradation I	266	2	24	12	3	0
D-fructuronate degradation	42	111	222	50	86	156
D-galactarate degradation I	3	32	26	4	3	19
D-galacturonate degradation I	3	51	114	8	4	124
D-glucarate degradation I	3	33	27	5	4	32
fucose degradation	2	44	23	9	11	3
galactose degradation I (Leloir pathway)	121	223	373	177	136	360
gallate degradation I	0	0	0	5	3	12
gallate degradation II	0	0	0	5	3	12
Glucose and glucose-1-phosphate degradation	55	60	148	36	5	24
glycine betaine degradation I	168	0	15	10	5	0
Glycogen degradation I (bacterial)	89	207	249	269	390	379
guanosine nucleotides degradation III	397	64	321	338	506	246
L-1,2-propanediol degradation	0	2	52	145	37	149
lactose and galactose degradation I	34	101	110	5	53	59
L-arginine degradation II (AST pathway)	90	63	102	20	5	27
L-histidine degradation I	112	54	242	65	19	67
L-histidine degradation II	1	8	27	6	5	23
L-leucine degradation I	324	3	204	154	210	163
L-rhamnose degradation I	27	42	115	34	29	22
L-tryptophan degradation to 2-amino-3-carboxymuconate semialdehyde	0	0	72	0	0	0
L-tyrosine degradation I	108	3	181	30	10	19
mannan degradation	0	0	175	0	0	0
methylgallate degradation	0	0	0	6	3	15
methylphosphonate degradation I	172	47	127	27	41	18
myo-, chiro- and scillo-inositol degradation	10	41	89	71	42	72
myo-inositol degradation I	4	35	65	58	38	39
nicotinate degradation I	1	4	15	3	3	14
phenylacetate degradation I (aerobic)	65	44	46	26	1	4
protocatechuate degradation II (ortho-cleavage pathway)	206	50	201	57	16	59
Purine nucleobases degradation I (anaerobic)	130	50	140	12	8	0
purine nucleotides degradation II (aerobic)	380	44	371	404	583	185
purine ribonucleosides degradation	254	181	169	136	114	177
starch degradation V	85	183	229	217	135	333
sucrose degradation III (sucrose invertase)	53	182	237	57	89	309
sucrose degradation IV (sucrose phosphorylase)	53	58	133	81	76	164
superpathway of β-D-glucuronide and D-glucuronate degradation	31	81	109	12	6	126
superpathway of aerobic toluene degradation	0	0	24	15	1	4
superpathway of D-glucarate and D-galactarate degradation	3	32	26	4	3	19
superpathway of glucose and xylose degradation	125	180	308	225	101	128

superpathway of hexitol degradation (bacteria)	87	163	209	30	137	146
superpathway of hexuronide and hexuronate degradation	3	51	96	7	1	110
superpathway of L-arginine and L-ornithine degradation	5	54	48	9	8	36
superpathway of L-arginine, putrescine, and 4-aminobutanoate degradation	5	54	48	9	8	36
superpathway of N-acetylglucosamine, N-acetylmannosamine and N-acetylneuraminate degradation	7	75	55	10	45	138
superpathway of N-acetylneuraminate degradation	21	151	131	31	100	230
Superpathway of ornithine degradation	72	48	65	29	18	23
superpathway of phenylethylamine degradation	62	43	30	30	1	5
superpathway of purine deoxyribonucleosides degradation	230	149	230	128	171	187
superpathway of pyrimidine deoxyribonucleosides degradation	55	120	167	73	67	195
Superpathway of salicylate degradation	100	34	85	23	1	3
superpathway of taurine degradation	13	0	6	0	2	0
toluene degradation I (aerobic) (*via* o-cresol)	0	0	107	28	69	32
toluene degradation II (aerobic) (*via* 4-methylcatechol)	0	0	107	28	69	32
toluene degradation III (aerobic) (*via* p-cresol)	66	41	60	32	1	4
toluene degradation IV (aerobic) (*via* catechol)	0	1	60	12	2	6
urate biosynthesis/inosine 5'-phosphate degradation	478	395	478	584	767	361
vitamin B6 degradation	0	1	9	27	6	2

Table S5.4. Hydrochemical values of the bores D13 and W4 under LR and HR. Na: Not available. Units of $\delta^{18}O$ and ^{2}H in per mil (‰), and units of tritium in TU (Tritium Units).

Bore	Rainfall period	Temperature (°C)	pH	Salinity (PSS)	DO (mg L^{-1})	ORP	Depth (m)	Cl- (mg L^{-1})	$\delta^{18}O$	^{2}H	Tritium
W4	LR	23.77	8.11	20.11	4.89	91.00	6.30	8040	-4.49 ± 0.15	-37 ± 1	Na
D13	LR	22.85	7.33	19.67	3.14	68.00	7.50	9410	-5.30 ± 0.15	-42.4 ± 1	0.77
W4	HR	26.22	6.71	19.96	4.71	80.00	6.00	11800	-4.49 ± 0.15	-38 ± 1	0.08
D13	HR	26.75	6.76	16.84	3.89	59	7.2	9850	-5.28 ± 0.15	-42.6 ± 1	0.53

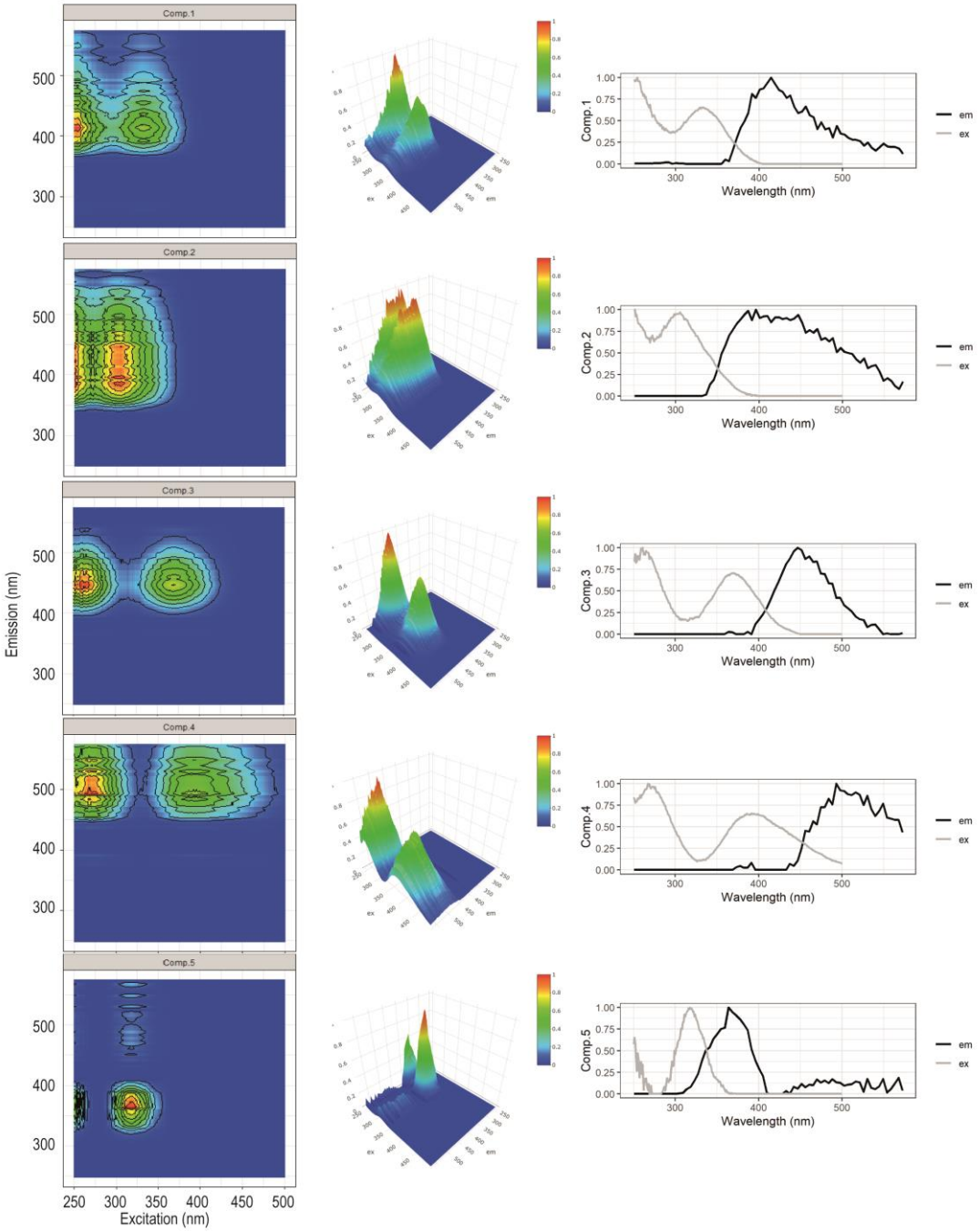

Figure S5.6. Two-dimensional (left panel) and three-dimensional (mid panel) fluorescence landscapes, and the excitation (grey line) and emission (black line) spectra (right panel) for the five different components identified by the PARAFAC model. Intensity is scaled to a maximum fluorescence of 1.

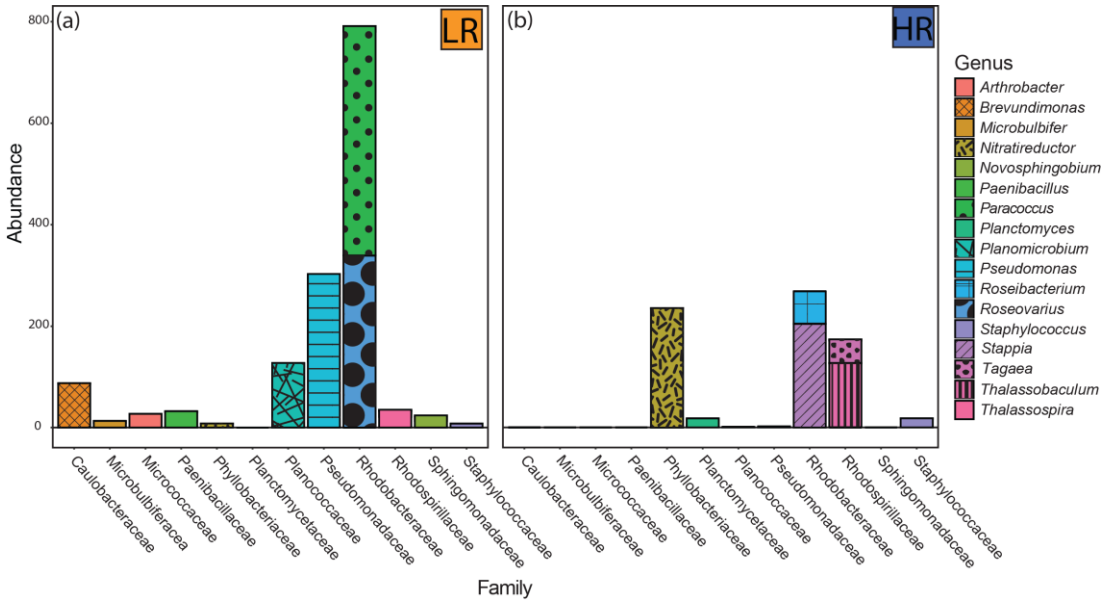

Figure S5.7. Bar plots illustrating the abundances of genus and families under LR (a) and HR (b). Abundances corresponding to the 37 ZOTUs without a reference/belonging to uncultured bacterium were removed from the figure for clarity purposes.

Chapter 6 | What's going on down (under) there? Unravelling biochemical flows under differential rainfall periods in a Western Australian calcrete

Submitted as a research article (2020).

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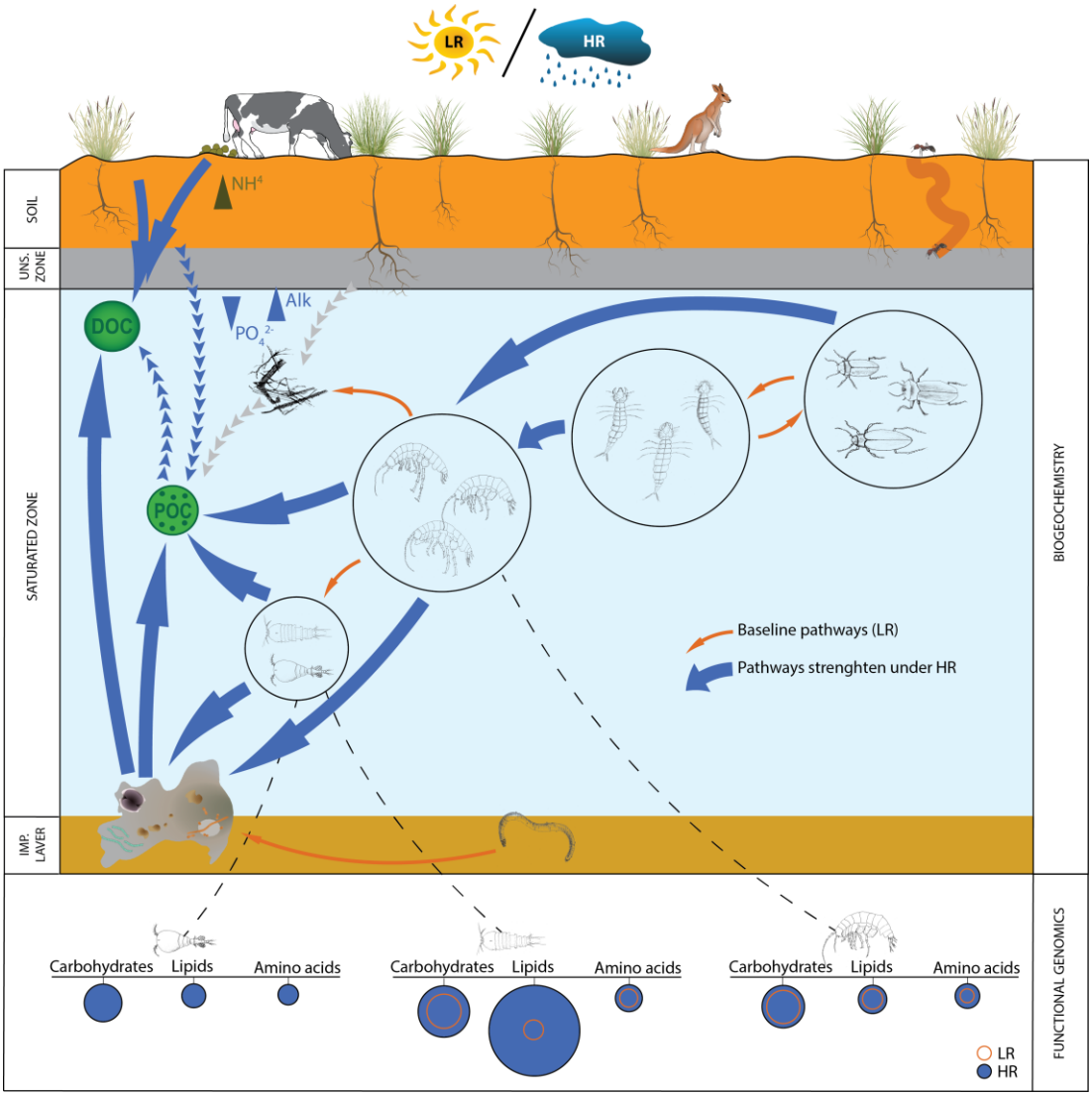
Abstract

Groundwaters host vital resources – 97% of unfrozen freshwater on the planet – playing a key role in the near future of humanity. However, our knowledge about their ecosystem functioning is limited, while subterranean environments are increasingly exposed to anthropic impacts and climate change-related processes. Novel biochemical (e.g. isotopic ecology) and genetic (e.g. eDNA) techniques, increasingly employed in freshwater studies, have the potential to unravel the complex dynamics shaping subsurface ecosystems, providing new insights to the small but quickly growing field of groundwater ecology. Stygofauna, together with microbes, are crucial in shaping and maintaining the organic matter (OM) cycles in environments characterized by low energy and scarce carbon availability. Here we investigate calcrete stygofaunal dynamics linked with contrasting rainfall periods (low rainfall (LR), dry season; high rainfall (HR), wet season) through an interdisciplinary design integrating isotope ecology and genetics. Our results indicate that the microbial gut community of copepods and amphipods experienced a shift in taxonomic diversity and predicted organic functional metabolic pathways after rainfall (HR). The HR regime triggers a cascade effect driven by microbes (OM processors) and exploited by copepods and amphipods (primary and secondary consumers), which is finally transferred to the aquatic beetles (top predators). Overall, and in line with related work, the inflow of rainfall triggered shifts towards more deterministic dynamics, revealing a complex web of interactions in a seemingly simple environmental setting. This study provides a novel approach to untangling the biochemical flows shaping the biotic community within a calcrete aquifer. More investigations applying multidisciplinary approaches to other subsurface ecosystems, i.e. alluvial aquifers, will help to understand present ecological patterns and predict future scenarios in groundwaters. This will help manage and preserve one of the most vital but underrated ecosystems in the world.

Key-words: groundwater ecology, carbon flows, rainfall, stygofauna, microbes, stable isotope analysis, functional genomics.

Graphical abstract



6.1 Introduction

Groundwaters, together with deep sea environments, are some of the least explored ecosystems in the world. Despite the recent upsurge in groundwater investigations, the subsurface ecological framework still suffers from a lack of knowledge, both in terms of biological diversity and ecological functioning, notwithstanding groundwater's environmental importance (Gleeson et al., 2012; Griebler et al., 2014). During the last twenty years, several efforts to improve the level of groundwater protection and develop conservation plans have been implemented worldwide (e.g. EPA, 2003; EU-GWD, 2006; US Fish and Wildlife Service, 2002). However, aquifers still face increasing threats from impacts linked with anthropic activities, such as over-extraction and/or contamination, and climate change (i.e. saline intrusion, alteration of recharge and discharge regimes, invasive species) (Griebler et al., 2019; Mammola et al., 2019; Mazza et al., 2014).

Groundwaters are inaccessible, cryptic, environments shaped by a complex web of biotic-abiotic interactions in a characteristically low-energy system (Griebler and Lueders, 2009). Subsurface obligate aquatic fauna - namely stygofauna - display arrays of specific environmental adaptations (loss of eyes, transparent body colours, long antennae, etc.) (Humphreys, 2006). Stygofauna are perceived as adapted to a stable physical-chemical environment, and there is evidence of high degrees of resilience to the fluctuations of the environmental conditions, i.e. groundwater recharge, source of organic matter and energy (Martin et al. 2009).

Rainfall events are considered major drivers in shaping hydrological dynamics in aquifers *via* processes like percolation or lateral flow (Jan et al., 2007), and stygofauna respond both in function and community composition to these hydraulic shifts (Datry et al., 2005; Saccò et al., 2019c). Several groundwater investigations indicate that inflows of terrestrial organic material (OM) cause ecological shifts within subsurface communities (Stegen et al., 2016). Reiss et al. (2019) found that temporally variable dissolved organic carbon (DOC) coming from the surface after an extreme recharge episode, triggered changes in groundwater bacterial biodiversity (DOC-derived microbial loops). In another study, Brankovits et al. (2017) suggested that grazers (i.e. amphipods, shrimps, etc.) fuel the transfer of organic compounds (DOC, methane, etc.) to the higher levels of the food web. These outcomes are in line with several groundwater theoretical models (e.g. Boulton et al., 2008; Hancock et

al., 2005; Humphreys, 2009), and indicate that terrestrial OM is a vital biodiversity driver in an environment characterized by low nutrient content and scarce carbon availability (Stegen et al., 2016).

However, the interpretation of carbon flows and trophic web interactions within groundwater biota is far from straightforward (Nielsen et al., 2018). In a recent study Žutinić et al. (2018) reported that shifts in food web structure from the community of an intermittent karstic spring were linked, together with the influence of the environmental oscillations, to evolutionary molecular clock patterns. In another study, Francois et al. (2016) described high degrees of trophic specialization in two species of cave isopods, and subsequently proposed a substantial review of the classic archetype of generalist feeding strategies in low energy environments. These results suggest there are composite pathways for the incorporation of organic matter in groundwaters, highlighting the need for interdisciplinary research that allows refinement of the ecological patterns shaping the subsurface framework (Saccò et al., 2019a).

Stable isotope chemistry (SIA, CSIA) and genetics (eDNA, DNA metabarcoding, etc.) are two disciplines providing new perspectives in the study of ecological dynamics in freshwater environments (Nielsen et al., 2018). The understanding of both species-specific patterns (e.g. Niemiller et al., 2018) and whole system modellings (e.g. Chikaraishi et al., 2014) has been improved by the application of approaches like $\delta^{13}C$ and $\delta^{15}N$ Compound Specific Stable Isotope Analysis (CSIA) and DNA metabarcoding. While the former enables elucidation of carbon/nutrient flows and detailing of trophic web interactions (Chikaraishi et al., 2007; Larsen et al., 2015), the latter identifies dietary input, and biological transfers along the food web, with a refined level of accuracy (Asmyhr et al., 2014). However, these techniques are still mainly employed in marine and surface terrestrial environments, and their application in groundwaters is in its infancy.

During the last 30 years, principally due to the high number of still undiscovered (and/or undescribed) stygofaunal species and regional diversity (Humphreys, 2009; Maurice and Bloomfield, 2012), the field of groundwater ecology has mainly focused on taxonomic diversity studies based on genetics (e.g. Guzik et al., 2009; Leijs et al., 2012). The arid western side of Australia, with its array of calcrete environments (Humphreys 1999, 2001) sustaining unique stygofaunal communities (Guzik et al., 2011), has been the focus of a

large number of studies on taxonomy, biogeography and evolutionary patterns (e.g. Cooper et al., 2008; Leys et al., 2003). Particularly, the Yilgarn region, one of the most ancient cratons in the world, has been found to host a plethora of endemic species including the highest diversity of subterranean beetles worldwide (Langille et al., 2019). Numerous studies have focused on the Sturt Meadows calcrete (e.g. Bradford et al., 2010; Bradford et al., 2013), and the information gathered during the last 15 years of studies from this biodiversity hotspot, once combined with data from isotope ecology and DNA barcoding, has the potential to elucidate for the first time the ecological functioning of one of these ecosystems. Previous studies on microbial and stygofaunal ecological patterns across different rainfall periods at Sturt Meadows indicated that - despite the lack of hydrological recharge *sensu stricto* - the inflow of nutrients after rainfall triggers shifts in microbial metabolisms (chapter 5), stygofaunal niche occupations (Saccò et al., 2019b) and invertebrate trophic interactions (Saccò et al., 2019c).

This investigation extends prior research by focusing on the carbon end energy flows regulating and sustaining stygofaunal dynamics at Sturt Meadows calcrete. In order to accomplish this goal, we apply a research design combining isotopic (SIA, CSIA and ^{14}C) and genetic investigations (DNA metabarcoding on bacteria from stygofaunal specimens) carried out on samples collected under contrasting rainfall periods (low rainfall, LR; high rainfall, HR). This investigation has three specific objectives: 1) unravel the biochemical paths that regulate the microbially-mediated nutrient assimilation among the stygofaunal community, 2) elucidate the flow of carbon and energy fluxes among primary/secondary consumers and predators and 3) understand the ecological functioning of the calcrete biotic community under two contrasting rainfall periods.

6.2 Methodology

6.2.1 *Study area*

The field work was carried out at the Sturt Meadows calcrete aquifer (28˚41‘S 120˚ 58‘E) located on Sturt Meadows pastoral station, Western Australia, ~42 km from the settlement of Leonora (833 km northeast of Perth, see Figure 6.1a). The study area is a calcrete aquifer lying in the Raeside paleodrainages in the Yilgarn region of Western Australian (Figure 6.1a). The vegetation of the area is Acacia woodlands, primarily *Acacia aneura* (F.Muell. ex

Benth.), and is subjected to combined grazing pressure from domestic stock, feral animals and macropods. The aquifer is accessible through a bore grid comprising 115 bore holes of between 5-11 m depth (Figure 6.1b).

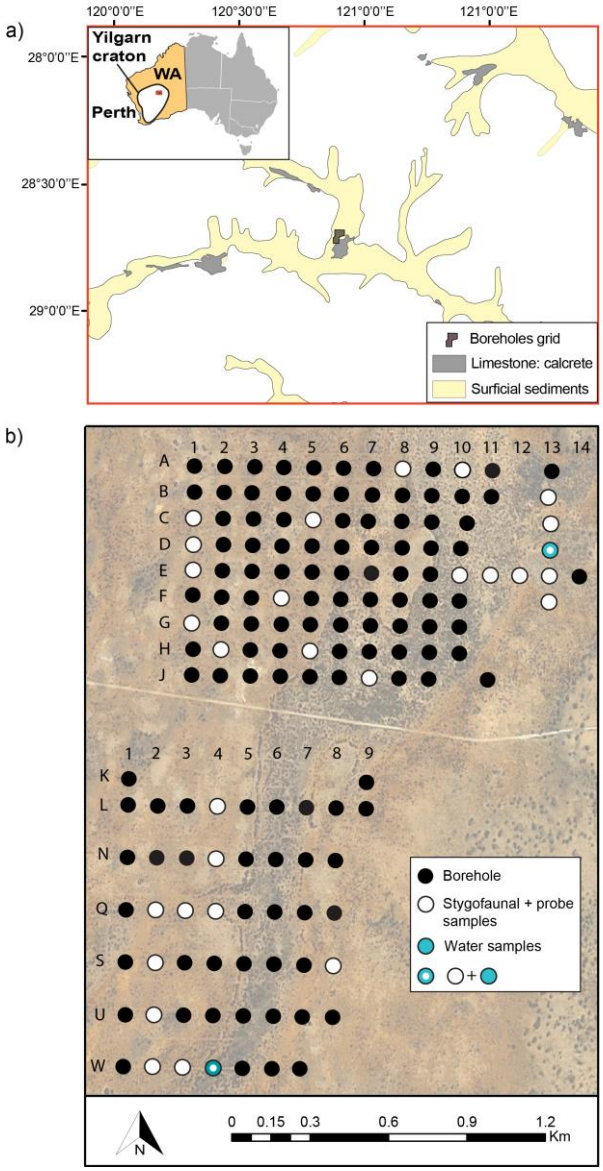


Figure 6.1. Map of the Sturt Meadow calcrete illustrating (a) the location within the Yilgarn craton region and detailed paleodrainages/cacretes in the area and (b) the grid map showing the location of the boreholes sampled for stygofaunal together with probe samples, water samples (in light blue) and the combination of both.

Three sampling campaigns were carried out, two of them (LR1: 26/07/2017 and LR2: 07/11/2017) corresponding to low rainfall periods (see Hyde et al., 2018) and one during the wet season (high rainfall, HR; two consecutive days of sampling collection: 17/03/2018 and 18/03/2018) (Figure S6.7). The well-studied stygofaunal community of the area is

composed of 11 main stygofaunal taxa belonging to five Classes: Oligochaeta (family Tubificidae (Vejdovský 1884)), subcohort Hydrachnidia, Maxillopoda (two species of harpacticoids: *Novanitocrella cf. aboriginesi* (Karanovic, 2004), *Schizopera cf. austindownsi* (Karanovic, 2004) and four species of cyclopoids: *Halicyclops kieferi* (Karanovic, 2004), *Halicyclops cf. ambiguous* (Kiefer, 1967), *Schizopera slenderfurca* (Karanovic & Cooper, 2012) and *Fierscyclops fiersi* (De Laurentiis et al., 2001)), Malacostraca: Amphipoda (species *Scutachiltonia axfordi* (King, 2012), *Yilgarniella sturtensis* (King, 2012) and *Stygochiltonia bradfordae* (King, 2012)) and Insecta: Coleoptera: Dytiscidae (species *Paroster macrosturtensis* (Watts & Humphreys 2006), *Paroster mesosturtensis* (Watts & Humphreys 2006) and *Paroster microsturtensis* (Watts & Humphreys 2006) and respective larvae).

6.2.2 *Field work procedures and sample preparation*

Given the delicacy of the hydrological dynamics in shallow calretes (Gray et al., 2016; Saccò et al., 2019b), extensive water extractions along the bores were avoided, and preliminary tests on the bores within the highest water depth were carried out to quantify potential risk of dewatering the calcrete. During the field campaigns LR2 and HR, 20 water samples in total (two samples for stable isotope analysis on DOC and DIC, three samples for radiocarbon analysis on DOC, one sample for radiocarbon analysis on DIC, and two samples for stable isotope and radiocarbon analyses on POC (Particulate Organic Carbon)) were collected from bores D13 and W4 (Figure 6.1b), which are representative of the two main geological conformations of the area - clacretic (W4) and clayey (D13) areas (Figure S6.8) - and host stable hydrological and biotic conditions (Saccò et al., 2019c). Water samples were collected using a submersible centrifugal pump (GEOSub 12V Purging Pump) after wells were purged of three well-volumes and stabilisation of in-field parameters was observed, according to the methodology detailed in Bryan et al. (2017).

Samples for $^{14}C_{DIC}$ analysis were filtered through 0.45 µm filters and collected in 1 L high density poly-ethylene (HDPE) bottles. $\delta^{13}C_{DIC}$ samples were filtered through 0.2 µm filters, collected in 12 mL glass vials (Exetainers) and refrigerated after sampling. $\delta\ ^{13}C_{DOC}$ samples were filtered through 0.2 µm filters, collected in 60 mL HDPE bottles and frozen after sampling.$^{14}C_{DOC}$ samples were filtered through 0.2 µm filters, collected in 3 L HDPE bottles and frozen after sampling.

In order to investigate ^{14}C and $\delta^{13}C$ content of POC, two extra litres were collected from the same bores (D13, W4) and kept frozen (–20°C) until further analyses. $^{14}C_{POC}$ $\delta^{13}C_{POC}$ samples were then filtered through pre-combusted GF/F filters (12 hours at 450°C), washed with 1.2 N HCl to remove any inorganic carbon, and subsequently dried at 60°C for 24 hours. All samples were sealed with sealing tape after collection to limit atmospheric exchange and kept in darkness.

Adult and larval stygofaunal specimens were collected from the 30 bores sampled for water parameters investigations (for LR1, LR2 and HR), by haul netting, with 5 hauls of a weighted plankton net (mesh 100 µm, Allford et al., 2008) through the water column. All biological samples were kept frozen (–20°C) in darkness until further processing in the laboratory. Individual organisms were counted and identified (and consequently separated) to the lowest taxonomic level *via* optical microscopy and reference to specific taxonomic keys. Roots/leaves and sediment samples were also separated during the sorting in the laboratory. Sediment samples, roots/leaves and all the individuals pertaining to the same taxon were joined in the same vial according to the sampling campaign (LR1, LR2 or HR) and subsequently washed with Milli-Q water to remove impurities from their bodies. Sediment samples were soaked in acid (0.1 N HCl) to remove inorganic carbon, and together with the other samples were then oven dried at 60°C overnight and ground until obtaining a homogeneous fine powder and stored at –20°C until further analyses.

Previous investigations on the ecological niche trends at Sturt Meadows indicated that stygofauna characterize similar niche occupations under low rainfall regimes (LR1 and LR2) (Saccò et al., 2019b). In isotopic ecology there are competing requirements between isotopic detection limits, analytical replicates and cost. Therefore, to enable the investigation of the ecosystem's isotopic patterns between dry vs wet season, samples from LR1 and LR2 were combined prior to analyses so as to maintain the main taxonomic and biological classifications pertaining to a unique low recharge category, namely LR (Saccò et al., 2019c).

6.2.3 *Bulk isotope and ^{14}C analyses*

Water $\delta^{13}C_{DIC}$ and $\delta^{13}C_{POC}$ isotopic ratios were analysed by Isotope Ratio Mass Spectrometer - WABC at The University of Western Australia using a GasBench II coupled with a Delta XL

Mass Spectrometer (Thermo-Fisher Scientific) - and results, with a precision of ± 0.10 ‰, were reported as ‰ deviation from the NBS19 and NSB18 international carbonate standard (Dogramaci and Skrzypek, 2015).

$\delta^{13}C_{DOC}$ isotopic ratios of waters were analysed *via* Liquid Chromatography Isotope Ratio Mass Spectrometer (LC-IRMS) at the La Trobe Institute for Molecular Sciences (LIMS, La Trobe University, Melbourne, Australia) composed by a Accela 600 pump connected to a Delta V Plus Isotope Ratio Mass Spectrometer *via* a Thermo Scientific LC Isolink (Thermo Scientific).

C and N bulk SIA on homogenised samples of sediment, roots, stygofauna and copepods (cyclopoids and harpacticoids) were performed at the Australian Nuclear Science and Technology Organisation (ANSTO, Sydney, Australia). Samples were loaded into tin capsules and analysed with a continuous flow isotope ratio mass spectrometer (CF-IRMS), model Delta V Plus (Thermo Scientific Corporation, U.S.A.), interfaced with an elemental analyser (Thermo Fisher Flash 2000 HT EA, Thermo Electron Corporation, U.S.A.) following the procedure published by Mazumder et al. (2017).

For radiocarbon analyses, samples (sediment, roots, copepods, ants, stygofauna, $^{14}C_{POC}$, $^{14}C_{DOC}$ and $^{14}C_{DIC}$) were subjected to CO_2 extraction and graphitization following the methodology published by Hua et al. (2001). ^{14}C content of samples was determined by means of the Accelerator Mass Spectrometry (AMS) at ANSTO (Australia's Nuclear Science and Technology Organization) in Sydney, Australia.

6.2.4 *Carbon Compound Specific Stable Isotope Analysis*

Samples from roots and stygofaunal specimens were hydrolysed under vacuum with 0.5 to 1 mL of amino acid-free 6M HCl (Sigma-Aldrich) at 110˚C for 24 h. The protein hydrolysates were dried overnight in a rotary vacuum concentrator and stored in a freezer. Prior to analysis, the samples were resolved in Milli-Q water and 10 µL of 1-mmol solution of 2-aminoisobutyric acid (Sigma-Aldrich) as internal standard. The sample stock had a concentration of approximately 8 to 10 mg/mL, which was further diluted as needed. Single amino acid carbon isotope analysis was carried out at the La Trobe Institute for Molecular Sciences (LIMS, La Trobe University, Melbourne, Australia) using an Accela 600 pump

connected to a Delta V Plus Isotope Ratio Mass Spectrometer *via* a Thermo Scientific LC Isolink (Thermo Scientific).

The amino acids were separated using a mixed mode (reverse phase/ion exchange) Primesep A column (2.1 x 250 mm, 100˚C, 5 µm, SIELC Technologies) following the chromatographic method described in Mora et al. (2017), after Smith et al. (2009). Mobile phases are those described in Mora et al. (2018). Percentage of Phases B and C in the conditioning run, as well as flow rate of the analytical run and timing of onset of 100% Phase C were adjusted as needed. Samples were injected onto the column in the 15 µL - partial loop or no waste - injection mode, and measured in duplicate or triplicate.

Once obtained the amino acidic spectrum per each sample, to elucidate carbon flows along the stygofaunal community, we focused on essential amino acids Valine (Val), Phenylalanine (Phe) and Arginine (Arg), as these compounds cannot be synthetised internally by the fauna but must be integrated through diet (McMahon and Newsome, 2019 and references therein; Saccò et al., 2019a). In addition, to distinguish between terrestrial vs aquatic carbon sources, the ratio between Val and Phe signals ($\delta^{13}C_{Val\text{-}Phe}$), a widely employed index in archaeology and freshwater biology (e.g. Webb et al., 2018), was calculated for roots, water mites, aquatic worms, amphipods and beetles (larvae and adults).

6.2.5 *Microbial taxonomic and functional gene analyses*

Consumers amphipods (*Scutachiltonia axfordi* (AM1), *Yilgarniella sturtensis* (AM2), *S. bradfordae* (AM3)), cyclopoids and harpacticoids, together with predator stygobiotic beetles (*Paroster macrosturtensis* (B), *P. mesosturtensis* (M) and *P. microsturtensis* (S)), were used for gut microbiome bacterial 16S metabarcoding and microbial functional analysis. A total of 16 AM1, 16 AM2, 16 AM3, 20 cyclopoids and 20 harpaticoids and 20 of each one of the three *Paroster* species (B, M and S), were sorted into duplicates of stygobitic pools of 3-5 individuals from both LR and HR events for DNA extraction. Prior to DNA extraction stygobitic animals (3-5 individuals per pool; n=40) were placed in a petri dish containing ultrapure water and UV sterilised for 10 minutes to eliminate any potential bacterial species contained on the exoskeleton as this study targeted the microbiome. Immediately post-UV treatment, the animals were placed into tissue lysis tubes with 180 µL

tissue lysis buffer (ATL) and 20 µL Proteinase K and homogenised using Minilys® tissue homogeniser (ThermoFisher Scientific, Australia) at high speed for 30 seconds. Lysis tubes, inclusive of two laboratory controls, were incubated at 56°C using an agitating heat block (Eppendorf ThermoStat™ C, VWR, Australia) for 5 hours. Following incubation, DNA extraction was carried out using DNeasy Blood and Tissue Kit (Qiagen; Venlo, Netherlands eluted off the silica column in 30-50 µL AE buffer).

The quality and quantity of DNA extracted from each stygobitic pool was measured using quantitative PCR (qPCR), targeting the bacterial 16S gene. PCR reactions to assess the quality and quantity of the DNA target of interest *via* qPCR (Applied Biosystems [ABI], USA) were in 25 µL reaction volumes consisting of 2 mM MgCl2 (Fisher Biotec, Australia), 1 x PCR Gold Buffer (Fisher Biotec, Australia), 0.4 µM dNTPs (Astral Scientific, Australia), 0.1 mg bovine serum albumin (Fisher Biotec, Australia), 0.4 µM of each primer (Bact16S_515F and Bact16S_806R; Turner et al. 1999; Caporaso et al. 2011), and 0.2 µL of AmpliTaq Gold (AmpliTaq Gold, ABI, USA), and 2 µL of template DNA (Neat, 1/10, 1/100 dilutions). The cycling conditions were: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 52°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 10 minutes.

DNA extracts that successfully yielded DNA of sufficient quality, free of inhibition, as determined by the initial qPCR screen (detailed above), were assigned a unique 6-8 bp multiplex identifier tag (MID-tag) with the bacterial 16S primer set. Independent MID-tag qPCR for each stygobitic pool were carried out in 25 µL reactions containing 1 X PCR Gold Buffer, 2.5 mM $MgCl_2$, 0.4 mg/mL BSA, 0.25 mM of each dNTP, 0.4 µM of each primer, 0.2 µL AmpliTaq Gold and 2-4 µL of DNA as determined by the initial qPCR screen. The cycling conditions for qPCR using the MID-tag primer sets were as described above. MID-tag PCR amplicons were generated in duplicate and the library was pooled in equimolar ratio post-PCR for DNA sequencing. The final library was size selected (160-600 bp) using Pippin Prep (Sage Sciences, USA) to remove any MID-tag primer-dimer products that may have formed during amplification. The final library concentration was determined using a QuBitTM 4 Fluorometer (Thermofischer, Australia) and sequenced using a 300 cycle V2 kit on an Illumina MiSeq platform (Illumina, USA).

MID-tag bacterial 16S sequence reads obtained from the MiSeq were sorted (filtered) back to the stygobitic pool based on the MID-tags assigned to each DNA extract using Geneious v10.2.5 (Drummond et al. 2011). MID-tag and primer sequences were trimmed from the sequence reads allowing for no mismatch in length or base composition.

Then, filtered reads were input into a containerised workflow comprising USEARCH (Edgar, 2010) and BLASTN (Altschul et al., 1990). The fastx-uniques, unoise3 (with minimum abundance of 8) and otutab commands of USEARCH were applied to generate unique sequences, ZOTUs (zero-radious OTUs) and abundance table, respectively. The ZOTUs were compared against the nucleotide database using the following parameters in BLASTN: perc_identity >= 94, evalue <= 1e-3, best_hit_score_edge 0.05, best_hit_overhang 0.25, qcov_hsp_perc 100, max_target_seqs = 5. An in-house Python script was used to assign the ZOTUs to their lowest common anscestor (LCA). The threshold for dropping a taxonomic assignment to LCA was set to perc_identity >= 96 and the difference between %identity of the two hits when their query coverage is equal was set to 1. Results on the microbial taxonomic diversity detected on ground water samples from a previous study on carbon inputs in the aquifer (chapter 5) were incorporated in this work and allowed qualitative comparison with the stygofaunal gut diversity.

To investigate functional activity involved in carbon cycling, the 16S metabarcoding data were fed to the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) software package to generate predicted metagenome profiles (Langille et al., 2013). These profiles were clustered into Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs (KOs) (Kanehisa & Goto, 2000) and MetaCyc pathway abundances (Caspi, 2006) focusing on the relative abundances of four carbon metabolisms: carbon fixation in prokaryotes, carbohydrates, lipids and amino acid metabolisms. Relative abundance of pathways linked with methane, nitrogen and sulfur metabolisms were also investigated.

6.2.6 *Statistical analyses*

The Phyloseq package in R (McMurdie and Holmes, 2013) was used to plot the ZOTU abundance at the order level for the styfofaunal specimens under low rainfall (LR) and high rainfall (HR) periods. The Statistical Analysis of Metagenomic Profiles (STAMP)

bioinformatics software package was used to carry out Principal Components Analysis (PCA) to assess the differences between functional genomic fingerprints based on the KEGG orthologs function prediction between copepods (C and H) and amphipods (AM1, AM2 and AM3), and determine statistically significant results from the PICRUSt2 output (Parks et al., 2014). Clustering patterns according to rainfall periods (LR and HR) and major consumers taxonomic groups (cyclopoids, harpacticoids and amphipods) were assessed through Permutational multivariate analysis of variance (PERMANOVA, R-package 'vegan') and pairwise post hoc pairwise multilevel comparisons (Martinez, 2019).

For comparison of potential shifts in abundances of microbial metabolic pathways within groundwater samples, copepods and amphipods across rainfall periods, Analysis of variance (ANOVA) (R-package 'stats'; outliers were identified using box plot methods (package 'rstatix' in R software version 3.5.1), homogeneity of variances was tested through the Levene's test (function *leveneTest()* in R 3.5.1) and normality was tested through the Shapiro-Wilk test (function *shapiro.test()* in R 3.5.1)) was performed on the abundance data (duplicates per each group) on the predicted pathways depicting carbon fixation, carbohydrate, lipid, amino acid, methane, nitrogen and sulfur metabolisms. ANOVAs coupled with Tukey's HSD pairwise comparisons (R-package 'stats') were employed to inspect significant differences between bulk SIA ($\delta^{13}C$ and $\delta^{15}N$) and essential amino acid ($\delta^{13}C_{Phe}$, $\delta^{13}C_{Arg}$, $\delta^{13}C_{Val}$ and $\delta^{13}C_{Val\text{-}Phe}$) data from the stygofaunal taxa within the different rainfall conditions (LR and HR). PERMANOVAs (R-package 'vegan') were also performed to investigate the potential clustering trends within the stygofaunal taxa across rainfall periods from the combination of radiocarbon ($\Delta^{14}C$) and carbon SIA ($\delta^{13}C$) isotopic fingerprints.

SIMM (Stable Isotope Mixing Models, R-package 'simmr') were used to estimate dietary proportions of copepods and amphipods within a Bayesian framework. Due to the lack of species-specific trophic discrimination factors for stygofauna, we employed the widely accepted values of 3.4 ± 2 ‰ for nitrogen and 0.5 ± 1 ‰ for carbon (Post, 2002). Markov chain Monte Carlo (MCMC) algorithms were used for simulating posterior distributions in SIMM, and MCMC convergence was evaluate using the Gelman-Rubin diagnostic by using 1.1 as a threshold value for analysis validation.

6.3 Results

6.3.1 *Stygofaunal gut microbiome patterns*

The gut microbiome of cyclopoids was dominated by betaproteobacteria under both rainfall regimes (accounting for 81% under LR and 71% under HR), while the microbiome community of harpacticoids illustrated a shift towards alphaproteobacteria (reaching 70% of the total) under HR. During LR, gut microbiomes of amphipods were dominated by the classes Actinobacteria (94% in AM1) and Bacilli (reaching 83% together with Actinobacteria in AM2 and 93% together with Betaproteobacteria in AM3). Contrarily, the most abundant classes within amphipods under HR were Alphaproteobacteria (64% in AM1 and 36% in AM2) and Clostridia (ranging up to 95% together with Alphaproteobacteria in AM3) (Figure 6.2a).

The PCA considering the KEGG orthologs function prediction showed that cyclopoids from both rainfall periods (C[LR] and C[HR]) clustered close to the harpacticoids (H[LR]) and amphipods (AM1[LR], AM2[LR] and AM3[LR]) from the LR regime (Figure 6.2b). In contrast, the latter two taxa grouped separately to the rest of the primary and secondary consumers under HR. Overall, the community clustered differently during the two rainfall periods (PERMANOVA, $P < 0.05$) and also according to the separation in major consumers taxonomic groups (cyclopoids, harpacticoids and amphipods) across LR and HR (PERMANOVA, $P < 0.005$). However, pairwise comparisons discarded any significant change across taxa and between rainfall events.

Predictions on the quantitative proportion of individual carbon metabolic pathways showed that carbon fixation was the most abundant metabolism within the four main routes analysed, accounting on average for the 1.8% of the total, followed by carbohydrate (0.4%), lipid (0.3%) and amino acid (0.2%) metabolisms. Apart from AM3 (Figure 6.2c.1), all the taxa illustrated increasing trends in abundance of the cited carbon metabolisms activity after rainfall (HR). Carbohydrate, lipid and amino acids metabolic categories significantly increased in harpacticoids, AM1 and AM2 during HR (Figure 6.2c.2,3 and 4), whilst only abundances of predicted pathways associated with carbohydrate metabolism increased in AM3 (Figure 6.2c.2).

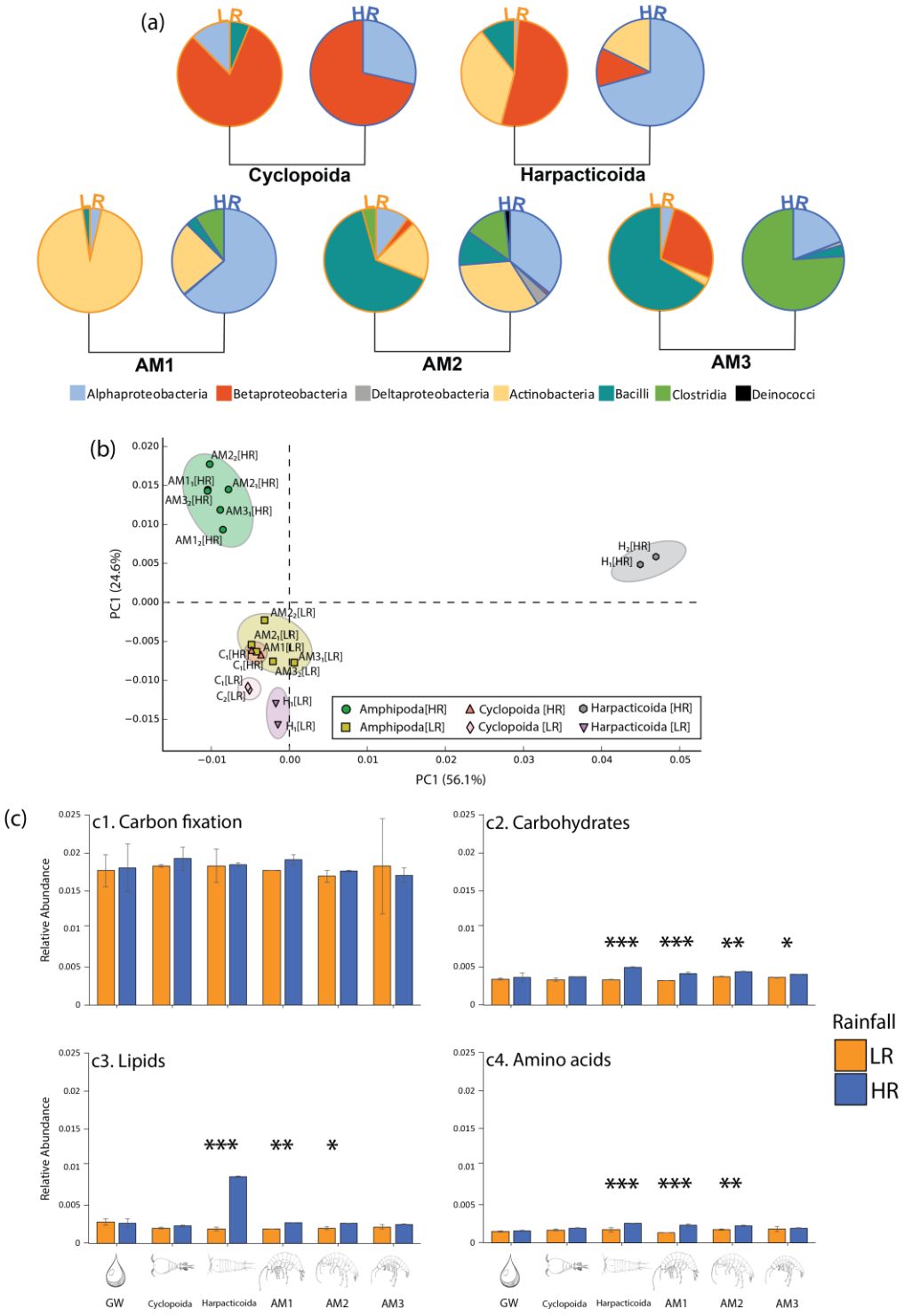


Figure 6.2. (a) Relative abundances (in %) of the classes found in copepods (cyclopoids and harpacticoids) and amphipods AM1, AM2 and AM3 under LR and HR. (b) PCA-based ordination analysis illustrating the distribution of taxa across rainfall periods (LR and HR) according to the KEGG orthologs metabolic functions. (c) Abundances of the major KEGG pathways associated with carbon metabolism (c1), carbohydrates metabolism (c2), lipids metabolism (c3) and amino acids metabolism (c4). *, P < 0.05; **, P < 0.005; ***, P < 0.0005.

6.3.2 *Biochemical flows on stygofauna*

6.3.2.1 *Organic inputs*

The $\delta^{13}C$ and $\delta^{15}N$ bulk values of sediment (the organic fraction from LR and HR) were close each other (Table 6.1) and both revealed very similar $\delta^{13}C$ values to the DIC (Figure 6.3b). Both groundwater sediment and DIC depicted old carbon sources within the two rainfall periods (Figure 6.3a)

Compared to sediment, roots had more depleted $\delta^{13}C$ values (LR: $\delta^{13}C$ = -20.6 ‰; HR: $\delta^{13}C$= -20.9 ‰) and modern ^{14}C fingerprints (Table 6.1), suggesting a recent terrestrial origin. In addition, a shift in $\delta^{15}N$ content can be observed in roots between LR and HR conditions, varying from $\delta^{15}N$ = 5.1±2.0 ‰ under LR to $\delta^{15}N$ = 12.1±0.3 ‰ after rainfall (HR). During HR, POC had more depleted $\delta^{13}C$ values (Figure 6.3a) than for the LR period, together with consistently older ages (Table 6.1).

Copepods (Cyclopoida (C) and Harpacticoida (H)) illustrated close $\delta^{13}C$ fingerprints to roots (cyclopoids: $\delta^{13}C$ = -20.5 ‰ during LR, $\delta^{13}C$= -21.9 ‰ under HR; harpacticoids: $\delta^{13}C$ = -20.6 ‰ during LR, $\delta^{13}C$= -23.5 ‰ under HR), while amphipods *S. axfordi* (AM1) and *Y. sturtensis* (AM2) showed more depleted values overall (Table 6.1). Moreover, copepods (C and H in one unique pool) and AM1 showed more depleted $\Delta^{14}C$ values under HR conditions than during the dry season (LR).

Within copepods, the highest proportion of carbon assimilations under LR was ascribable to sediment and attached bacteria (32.3% for cyclopoids and 31.9% for harpacticoids), while during the same rainfall regime DOC was the major organic driver (~50%) within amphipods *S. axfordi* (AM1) and *Y. sturtensis* (AM2). Under HR conditions, microbially-derived DOC was incorporated at considerably higher proportions for both groups (41.1% and 51% for copepods (C and H), 77.5% and 84.9% for amphipods (AM1 and AM2)) (Figure 6.3c). These results suggest that during HR the system gets an inflow of rainfall that triggers ‘pulses’ of carbon and nutrients ultimately profited by copepods (C and H) and amphipods (AM1 and AM2).

Table 6.1. Results from the $\delta^{13}C$, $\delta^{15}N$ and ^{14}C analyses on *Scutachiltonia axfordi* (AM1), *Yilgarniella sturtensis* (AM2) copepods (cyclopoids and harpacticoids), sediment, roots, particulate organic carbon (POC), dissolved inorganic carbon (DIC) and dissolved inorganic carbon (DOC). Mean values ± standard deviations are illustrated; pMC: percent of modern carbon; BP: before present (with present as 1950).

	$\delta^{13}C$		$\delta^{15}N$		pMC	$\Delta^{14}C$ (‰)	Age (BP)	pMC	$\Delta^{14}C$ (‰)	Age (BP)
	LR	HR	LR	HR	LR			HR		
Scutachiltonia axfordi	-24.14	-26.88±0.05	10.71	8.81±0.09	102.84±0.48	19.9±4.8	modern	99.86±1.01	-9.6±9.9	modern
Yilgarniella sturtensis	-24.55	-27.10	9.99	8.50	100.46±0.36	-3.7±3.6	modern	100.75±0.81	-0.9±8.1	modern
Cycopoida	-20.45±0.30[1]	-21.91±0.30[1]	13.90±0.30	14.5±0.30	100.27±0.56[3]	-5.6±5.6[3]	modern[3]	99.36±0.68[3]	-14.7±6.7[3]	modern[3]
Harpacticoida	-20.60±0.30[1]	-23.50±0.30[1]	11.90±0.80	15.8±0.80						
Roots	-20.57±0.30[1]	-20.90±0.30[1]	5.10±2.0	12.1±0.30	103.63±0.30	27.7±3.0	modern	103.34±0.47	24.9±4.7	modern
Sediment	-10.33±0.30[1]	-9.65±0.30[1]	11.0±1.20	11.4±1.20	22.16±3.23	-780.3±32.1	12100±1170	57.68±5.21	-428.0±51.7	4420±725
POC	-21.58±0.10[2]	-26.47±0.10[2]	10.73±0.10[2]	8.35±0.10[2]	86.55±0.76	-141.7±7.6	1160±70	63.86±0.28	-366.7±2.8	3605±35
DOC_{D13}	-29.25±0.36	-29.35±0.20	na	na	91.70±0.46	-90.6±4.6	695±45	92.41±0.62	-83.5±6.1	630±60
DOC_{W4}	-31.91±0.50	-27.15±0.03	na	na	66.76±0.48	-337.9±4.8	3245±60	57.99±0.55	-424.9±5.5	4380±80
DIC_{D13}	-9.45±0.10[2]	-9.39±0.10[2]	na	na	82.73±0.30	-179.5±3.0	1575±30	77.90±0.19	-227.4±1.9	2005±20
DIC_{W4}	-8.75±0.10[2]	-8.87±0.10[2]	na	na	62.47±0.21	-380.5±2.1	3835±30	59.20±0.17	-412.8±1.7	4210±25

[1] Accuracy of the CF-iRMS

[2] Accuracy of the GC-iRMS

[3] Calculated as overall copepods (cyclopoids mixed with harpacticoids)

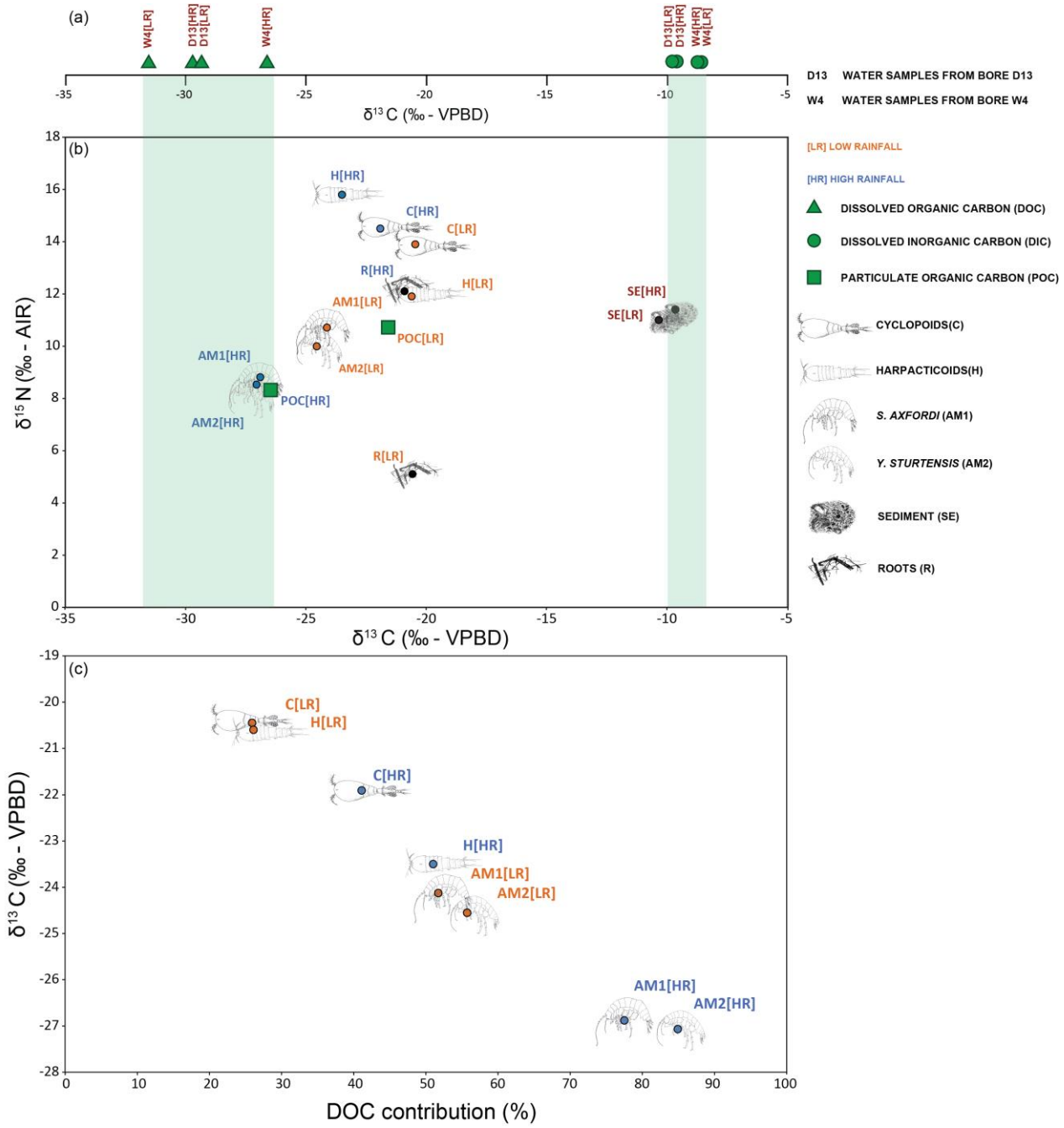


Figure 6.3. $\delta^{13}C_{DOC}$ and $\delta^{13}C_{DIC}$ from the bores W4 and D13 (during LR and HR) and their ranges (in light green) incorporated in graph (a), which illustrates the $\delta^{13}C$ and $\delta^{15}N$ for LR (low rainfall) and HR (high rainfall) of roots, sediment, POC (particulate organic matter), copepods (C and H) and amphipods (AM1 and AM2). Unless otherwise specified, standard deviations are calculated from analytical replicates. (c) Estimation of DOC contributions for the diets of copepods (C and H) and amphipods (AM1 ans AM2) during LR and HR. In red the old (considering present as 1950) carbon sources revealed by radiocarbon dating (refer to Table 6.1 for specific values).

6.3.2.2 *Carbon transfers*

$\delta^{13}C_{Phe}$, $\delta^{13}C_{Arg}$ and $\delta^{13}C_{Val}$ values indicated that almost all the taxonomic groups show a substantial and statistically significant change in their organic fingerprint (Table 6.2). *P. microsturtensis* (S) and *S. bradfordae* (AM3) were the only taxa that did not change significantly between the rainfall periods (LR vs HR) for all three essential amino acids (Val, Arg and Phe), with this trend potentially ascribable to coupled feeding habits (prey-predator interactions) or a highly conservative tendency in carbon assimilations for both groups.

The pattern unveiled by the analysis of $\delta^{13}C_{Val\text{-}Phe}$ values under LR and HR conditions confirms the shift in carbon source path (Table S6.3 and S6.4 and Figure 6.4). During the dry season (LR), amphipods (pool of AM1, AM2 and AM3) were not significantly different to roots signals. In contrast, beetles larvae and adults had significantly different $\delta^{13}C_{Val\text{-}Phe}$ values, suggesting a more aquatic (stygofaunal based) preference in carbon incorporation, as would be expected in predators. Under the high rainfall regime (HR), $\delta^{13}C_{Val\text{-}Phe}$ values for roots were statistically different (Figure 6.4) from all the five stygofaunal groups.

The combination of radiocarbon ($\Delta^{14}C$) and carbon SIA ($\delta^{13}C$) fingerprints indicated that roots, copepods, amphipods and beetles grouped differently (PERMANOVA, $P < 0.05$), suggesting tropic niche partitioning processes in OM assimilation. Roots clustered separately to the rest of the samples (Figure 6.5) and together with adult beetles (B, M and S) and AM2 were the only taxa that showed comparable $\delta^{13}C$ fingerprints across the rainfall regimes (LR and HR) (Table 6.1 and Table S6.5). Conversely, both amphipod AM1 and copepods (cyclopoids pooled together with harpacticoids) illustrated the biggest shifts in organic input preferences towards more depleted $\Delta^{14}C$ and $\delta^{13}C$ values (Figure 6.5 and Table 6.1).

Table 6.2. ANOVAs on recharges and taxonomic groups/roots with Tukey's pairwise comparisons between the values of Phenylalanine (Phe), Arginine (Arg) and Valine (Val) per each sample. Entire amino acids spectrums - together with the essential and non-essential AAs typification - for all the samples under LR and HR conditions are illustrated on Tables S6.6 and S6.7.

	Phe			Arg			Val		
	d.f.	T-ratio	*P*	d.f.	T-ratio	*P*	d.f.	T-ratio	*P*
B	28	-4.497	<.0005	28	-1.687	**0.1028**	28	-2.163	<.005
M	28	-4.846	<.0001	28	-4.208	<.0005	28	-3.297	<.005
S	28	-0.149	**0.8829**	28	2.015	**0.0536**	28	1.967	**0.0592**
Blv	28	-4.218	<.0005	28	-3.534	<.005	28	-4.42	<.0005
Mlv	28	-9.657	<.0001	28	-12.938	<.0001	28	-9.16	<.0001
Slv	28	-10.933	<0.001	28	-16.018	<.0001	28	-9.73	<.0001
AM1	28	-18.4	<.0001	28	-17.01	<.0001	28	-10.2	<.0001
AM2	28	-11.383	<.0001	28	-10.88	<.0001	28	-11.067	<.0001
AM3	28	-0.037	**0.9704**	28	1.208	**0.2372**	28	0.282	**0.7797**
OR	28	-7.418	<.0001	28	-4.306	<.0005	28	-2.389	<0.05
OL	28	6.594	<.0001	28	11.931	<.0001	28	7.252	<.0001
HM	28	11.894	<.0001	28	13.019	<.0001	28	15.977	<.0001
Ants	28	3.832	<.0005	28	2.436	<0.05	28	3.022	<.001
Roots	28	8.273	<.0001	28	10.428	<.0001	28	7.671	<.0001
Between recharges	1	206.553*	<.0001	1	141.566*	<.0001	1	80.240*	<.0001
Between taxa	10	126.724*	<.0001	10	57.473*	<.0001	10	33.585*	<.0001

* F-value instead of T-ratio

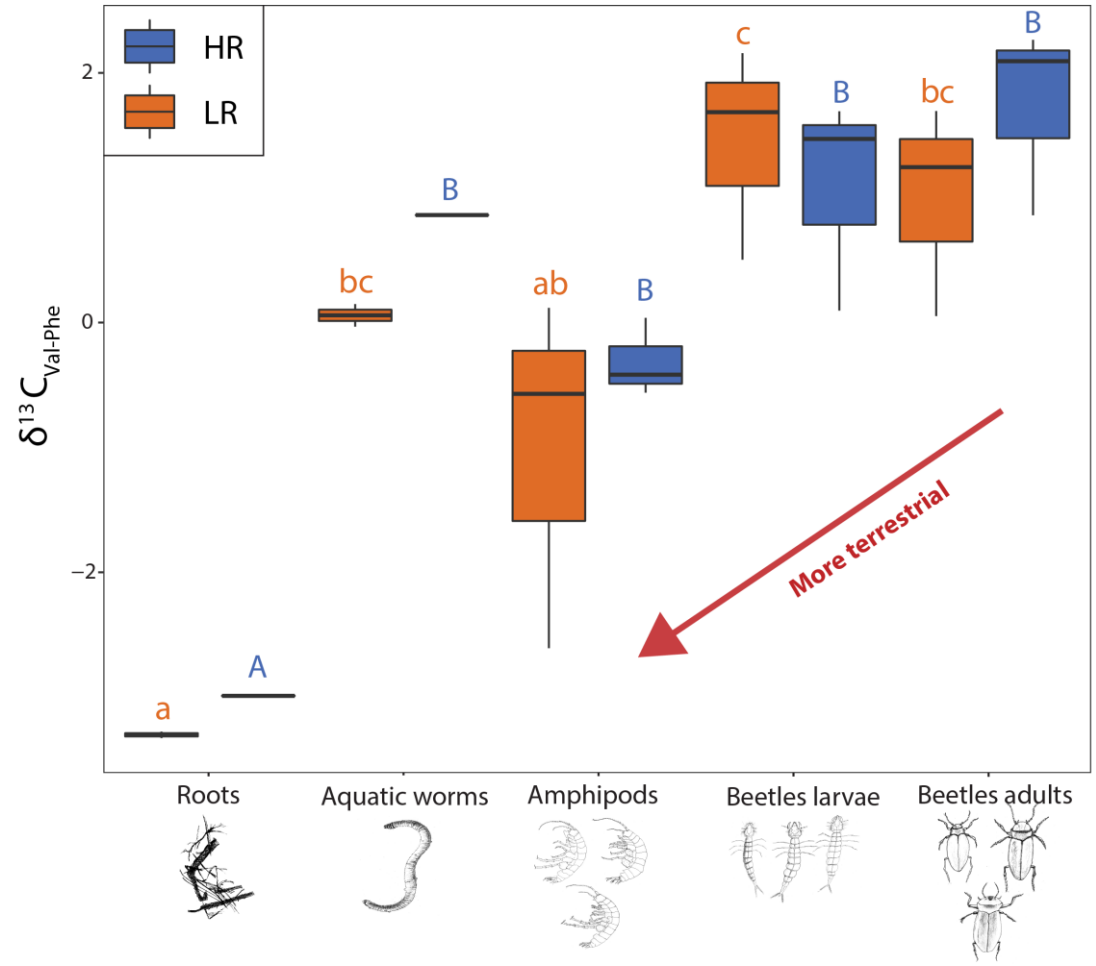


Figure 6.4. $\delta^{13}C_{Phe-Val}$ values calculated under LR and HR conditions for roots (R), aquatic worms (OL), amphipods (AM1, AM2 and AM3), beetles larvae (Blv, Mlv, Slv) and adults (B, M, S). More negative values indicate a more terrestrial carbon source. Refer to Table S6.3 and S6.4 for the significances of pairwise comparisons. Letters a,b and c are used for LR conditions, while letters A, B and C for HR.

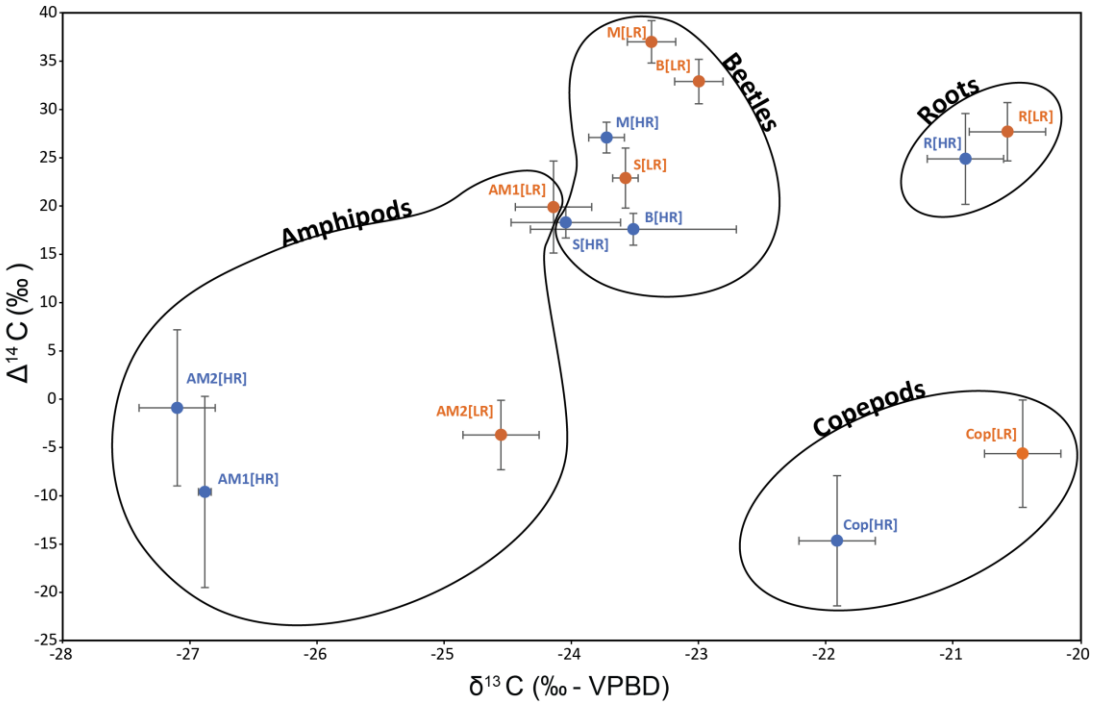


Figure 6.5. Biplot illustrating $\delta^{13}C$ and $\Delta^{14}C$ data from roots, beetles (B, M and S), amphipods (AM1 and AM2) and copepods (Cop, as a compendium of cyclopoids and harpacticoids). Refer to Table 6.1 and Table S6.5 for the specific isotopic values of each group.

6.4 Discussion

6.4.1 *Microbial/stygofauna transitions*

Rainfall events are responsible for carbon incorporation and nutrient inflows that play a key role in shaping biochemical dynamics in the Sturt Meadows calcrete aquifer (Saccò et al., 2019b; Saccò et al., 2019c). Metabarcoding and predicted metagenome results show that the gut microbiomes of primary consumers copepods, harpacticoids and amphipods changed dramatically both in community composition and metabolic functions under HR. The significant increase in oligotrophic bacteria (Alphaproteobacteria and Clostridia) during HR suggests that these two bacterial phyla become more prevalent when greater amounts of dissolved organic matter and nutrients become available. This coincides with previous studies that show these bacterial phyla to be the most common organic compound degraders found in aquifers (e.g. Alain et al., 2012; Geddes and Oresnik, 2014; Winderl et al., 2010).These phyla dominated the gut microbiota of the harpacticoids and all the three amphipods species AM1, AM2 and AM3 during HR.

Overall, amphipods hosted more abundant microbial communities (322 individuals on average between AM1, AM2, AM3 during LR, and 1182 under HR) when compared to the bacteria found in water (Figure S6.9) (Table S6.9). This is not surprising, in light of the dilution effect that water provides to the free-living bacteria (Pronk et al., 2009), and also considering that stygofauna act as vectors for prokaryotes (Smith et al., 2016).

Evidence from functional genomic analyses indicates that while carbon fixation pathways represent a stable metabolic baseline under both rainfall conditions, the abundance of carbohydrate, lipid and amino acid metabolisms significantly increased under HR within consumers harpacticoids and amphipods. Interestingly, within the primary consumer copepods, while harpacticoids showed consistent increased abundances of all the metabolisms studied after rainfall, those of cyclopoids remained steady. Galassi et al. (2009) reported that within low-water velocity karst systems, cyclopoids usually have free-swimming nektonic lifestyles, while harpacticoids prefer interstitial voids in the sediment (Galassi and Laurentiis, 2004). At Sturt Meadows, different ethological dynamics after rainfall would allow competency to be diminished in an environment with limited resource availability. In contrast to cyclopoids, the microbial gut microbiome community of harpacticoids experienced a shift towards more abundant alphaproteobacteria under HR. Concurrently, harpacticoids were the only taxa that illustrated increased methane, nitrogen and sulfur microbiome metabolisms during HR (Figure S6.10), suggesting that their feeding sources are markedly different form cyclopoids. However, our data from Bayesian mixing models showed little difference between the diets of the two groups (Table S6.8), and further mesocosm experiments will be necessary to confirm niche partitioning patterns.

Compared to copepods and amphipods, subterranean dytiscid species *P. mesosturtensis* (M) and *P. microsturtensis* (S) showed more uniform microbial gut communities (Figure S6.9) and more stable isotopic trends (Figure S6.5) across rainfall periods, indicating mitigated trophic shifts typical of constant predatory behaviours. Conversely, microbial gut diversity of *P. macrosturtensis* experienced a substantial shift from a Bacilli dominated environment under LR to an Actinobacteria-based community during HR, which might be ascribable to species-specific predatory pressures on AM1 and AM2 under HR (Saccò et al., 2019c).

Overall, our results from genetic analyses on stygofaunal gut microbiomes suggest that the inflow of OM at Sturt Meadows is exploited by microbes which are the potential direct (through biofilm grazing) and indirect (*via* POC assimilation) target of primary consumers, amphipods and copepods. A previous investigation on carbon inputs in water indicated a shift in microbial taxonomic assemblages coupled with increased degradative pathways after rainfall (HR) (chapter 5). In line with our work, Reiss et al. (2019) reported that rainfall inflows coupled with increased inputs of organic matter mediate changes in microbial diversity, abundances and respiration rates.

Meiofauna (copepods) and amphipods are commonly considered as filter-feeders and biofilm grazers (Boulton et al., 2008; Galassi et al., 2014), and have been depicted as crucial actors in the carbon fuelling to upper trophic levels (Hartland et al., 2011). In a recent study, Weitowitz et al. (2019) brought new light to the microbes-amphipods linkage, one of the most important associations in groundwater ecosystems, by providing empirical evidence of direct microbial ingestion by amphipods *Niphargus fontanus* (Bate, 1859) and *Niphargus kochianus* (Bate, 1859) and their resulting effects on biofilm assemblages.

Our study widens the understanding of these dynamics by incorporating novel information about the rainfall-driven shifts in functional metabolic activities of stygofaunal gut microbiomes. However, our genetic results, while interesting, are still indirect evidence of the 'DOC-microbes-stygofauna' ecological cascade, and community mesocosm experiments are urgently needed. Indeed, further species-specific investigations are required to elucidate the mechanisms of these interactions and bring crucial comprehension of the dynamics sustaining groundwater biodiversity.

6.4.2 *Faunal trends: carbon paths and food web interactions*

Australian shrubs potentially constitute a driver between surface and subsurface biochemical frameworks, especially arid soils (Kirschbaum et al., 2008, Shimp et al., 1993). Mulga roots have been reported penetrating deep into the soil to reach moisture, and frequently fall from the unsaturated zone into aquifers (Dunkerley, 2002). At Sturt Meadows, barcoding analyses revealed that root fragments in the water match with saltbush vegetation from the surface (Hyde's personal communication). Interestingly, while roots $\delta^{13}C$ values did not change between rainfall regimes, $\delta^{15}N$ showed more depleted

values under LR (Table 6.1). Termites, widely distributed in the area, are wood feeders that could play a key role in nitrogen fixation (Pate et al., 2012). Our results align to this hypothesis, with termites benefiting from the easily accessible nitrogen source from the nitrophilous mulga vegetation. In fact, we observed increased rates of nitrogen-depleted root material falling into the aquifer under environmental conditions (dry season, LR) which have been reported as favourable for termites' ethology (Coventry et al., 1988; Evans et al., 2011). Concurrently, moisturized vegetal material is highly likely to be targeted by fungi and microbes (e.g. Bärlocher et al., 2006) in the hyporeic zone, and enriched $\delta^{15}N$ values under HR might be a reflection of coexisting microbiological metabolisms (Saccò et al., 2019c).

$\delta^{13}C$ bulk values for roots, close to C3 photosyntetically-derived carbon fingerprints (Cerling et al., 1997), were almost identical (and not significantly different) to the ones of the meiofaunal and stygofaunal communities (Table 6.1). These results are in line with the lack of potential trajectories of trophic increments (from roots to the top predators) found for the same system by Bradford et al. (2010), and suggest other paths of carbon assimilation. The inorganic carbon component (DIC) in water showed very similar isotopic fingerprints to the sediment in both stable and radiocarbon (sediment: 4420±725 BP (HR) and $\delta^{13}C$ values ranging from -10.33 ‰ (LR) to -9.65 ‰ (HR)), suggesting that the DIC is sourced from calcrete bedrocks, and is only a marginal contributor to biological incorporation.

Several groundwater studies report terrestrially derived DOC as a primary factor in shaping ecological shifts under differential recharge conditions (e.g. Datry et al., 2005; Reiss et al., 2019). The $\delta^{13}C$ DOC values detected in this study (ranging from -31.91±0.5 ‰ to -27.15±0.03 ‰) were characteristic of surface derived carbon sources (-20‰ depleted if compared with atmospheric CO_2 values at -8 ‰, see O'Leary, 1988), suggesting that allochthonous material potentially drives the biochemical flows in the system. Interestingly, Sturt Meadows stygofaunal community illustrated differential OM incorporations under LR and HR regimes. During the dry period (LR), isotopic evidence from amphipods revealed that microbially-derived DOC incorporations were combined with sediment (~20% contribution), POC (~20% contribution) and their attached microbial communities (Table S6). This tendency towards opportunistic strategies shifts under HR, when biochemically enriched aquifers *via* rainfall inflows triggered a dominance of DOC-derived assimilations (ranging from 77.5 (AM1) to 84.9 (AM2)). Compared to amphipods, meiofauna (cyclopoids and harpacticoids) showed increased sediment ingestion under LR (~32% contribution),

however the consistent increase in DOC incorporations was confirmed after rainfall (Table S6.8). $\delta^{15}N$ stygofaunal signatures were consistent with a food web driven by soil-based OM incorporations (Simon et al., 2003) and meiofauna illustrated anomalously enriched $\delta^{15}N$ fingerprints compared to amphipods under both rainfall regimes. Moreover, cyclopoids and harpacticoids were the only groups which experienced increased $\delta^{15}N$ values coupled with rainfall under the HR regime, suggesting different nitrogen microbial baselines (Saccò et al., 2019c; Tiselius and Fransson, 2015) coupled with potential scavenging (e.g. Boxshall et al., 2016).

Stygobionts illustrate high resiliency rates to a lack of resources (Huppop, 2000). As reported by Gibert and Deharveng (2002), evolutionary trends in groundwater biota might have driven maximization of trophic plasticity coupled with low metabolic rates. Our results indicate that while under LR regimes omnivory might play a key role in maintaining stygofaunal assemblages, under HR conditions, prey-predator interactions - ultimately driven by shifts in trophic habits carried out by specific groups such as amphipods - are strengthened (Saccò et al., 2019c). Amphipods display a vast array of feeding modes - from facultative biofilm grazers to scavengers - which is thought to be strictly linked to high rates of resistance to starvation (Hartland et al., 2011; Hutchins et al., 2014). From an eco-biochemical perspective, amphipods - when present in groundwater - represent crucial components in microbial assimilation processes that fuel carbon transfers along the trophic chain (Brankovits et al., 2017). While we did not find direct isotopic evidence of biofilm assimilation from ephilitic microbial biofilms, amphipods did shift towards ^{13}C-depleted carbon inputs, and potentially more microbially-derived OM, under HR condition (Figure 6.3 and Figure 6.5). Aquatic worms (OL) showed a shift towards more depleted OM incorporations, however further isotopic and genetic analyses were constrained by the low abundances found in the field (Table S6.10).

6.5 Conclusions

The understanding of trophic flows within aquatic biota is fundamental to deciphering biochemical fluxes, but very few studies have attempted to fill this knowledge gap in subterranean environments (e.g. Simon et al. 2003). Some studies have attempted to model groundwater ecosystem ecological functioning (e.g. Hancock et al., 2005, Simon et al., 2007), but none of them has focused on wide multidisciplinary designs. Here we

combined information from previous food web investigation on stygofauna (Saccò et al., 2019c) and microbial patterns (chapter 5) with the information gathered *via* isotopic (carbon and nitrogen) and genetic data on stygofaunal gut biomes from this work (Figure 6.6). High rainfall events (see Figure S6.7 for rainfall categorisation) trigger ecological shifts characterized by a tendency towards more deterministic interactions. Bottom-up controlled microbial communities are proposed as major drivers regulating the trophic trajectories of stygofaunal specimens. The suggested modelling infers selective biofilm proliferation as a driver for increased biological activities in grazers (copepods and amphipods), which are the ultimate target of top predators (beetles, larvae and adults). Given the urgent need to widen the current knowledge of groundwater ecology trends, this investigation provides novel modelling that can bring further light to the processes regulating biodiversity in groundwater ecosystems. The understanding of these dynamics is crucial to evaluate the current state of conservation and investigate future trends both in pristine and contaminated groundwaters.

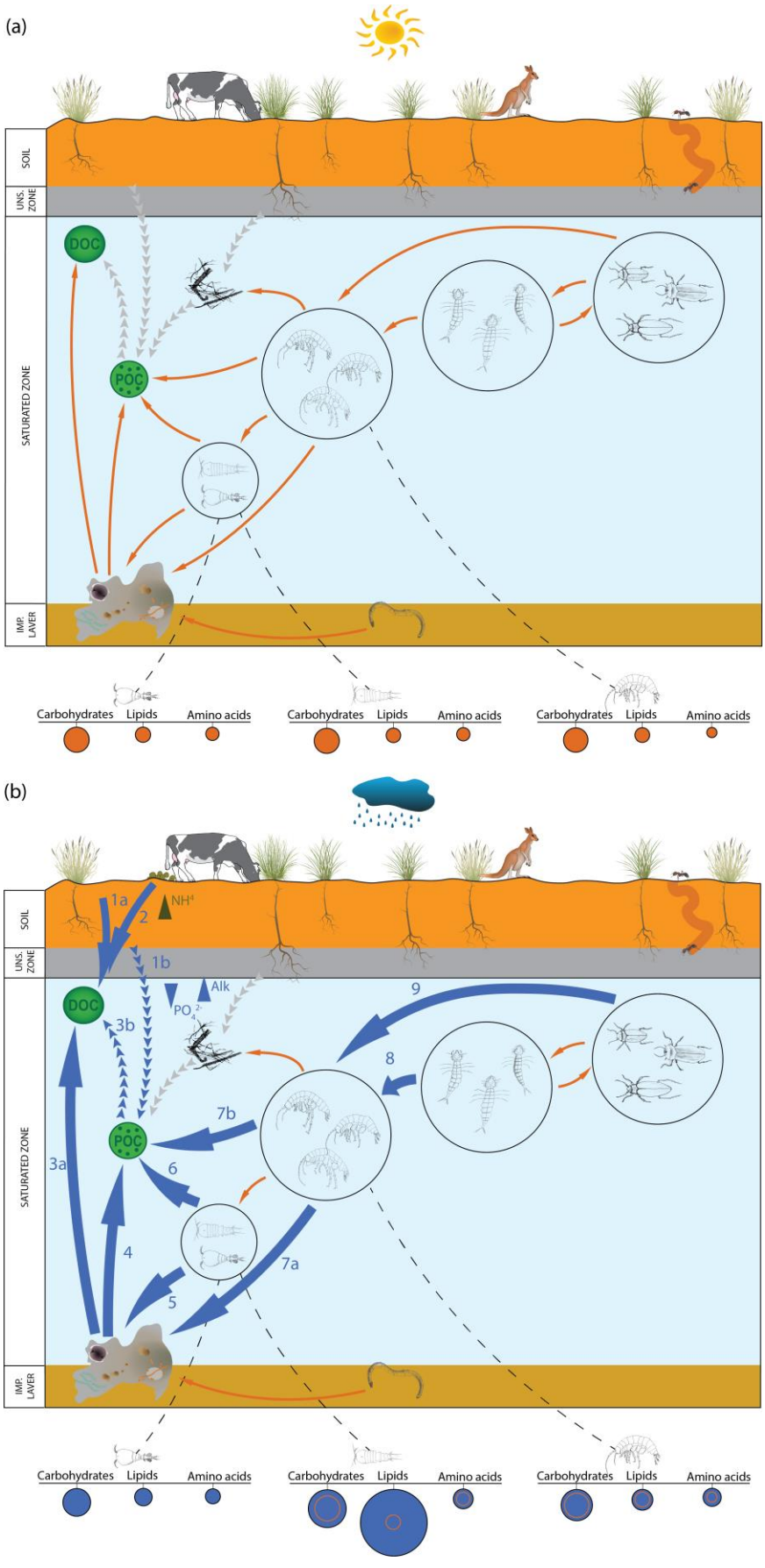

(a)
SOIL
UNS. ZONE
SATURATED ZONE
IMP. LAYER
DOC
POC
Carbohydrates
Lipids
Amino acids
Carbohydrates
Lipids
Amino acids
Carbohydrates
Lipids
Amino acids
(b)
SOIL
UNS. ZONE
SATURATED ZONE
IMP. LAYER
1a
2
NH4
1b
Alk
PO4 2-
DOC
3b
9
8
7b
POC
3a
6
4
7a
5
Carbohydrates
Lipids
Amino acids
Carbohydrates
Lipids
Amino acids
Carbohydrates
Lipids
Amino acids

Figure 6.6. Conceptual model of the principal biochemical flows at Sturt Meadows aquifer under LR (a) and HR (b) conditions. The dark yellow arrows illustrate the main biochemical paths, while bigger blue arrows underline those transitions strengthen under high rainfall period, and are numbered as follow: 1a, old and ^{13}C-replenished DOC leaches into the groundwater (Table 6.1); 1b, as a result of the rainfall infiltration, phosphates dilute, carbonates are released (higher alkalinity) (Saccò et al., 2019b) and old POC gets to the water; 2, ammonia concentrations increase as a combined effect of animal waste leaking from the surface and microbial metabolisms (Saccò et al., 2019b, chapter 5); 3, microbial biofilms consume the newly incorporated old DOC (partially derived from POC (route 3b)) (chapter 5); 4, biofilms decompose POC; 5, harpacticoids browse on biofilm and cyclopoids filter particulate organic matter (route 6); 7a, amphipods graze on microbial mats (and filter POC (route 7b)) and fuel the carbon to the upper trophic levels; 8, beetles larvae and adults (route 9) (top predators) exert a higher trophic pressure on amphipods after rainfall (Saccò et al., 2019c). Dashed lines led to the proportions of the carbohydrate, lipid and amino acid microbial metabolisms (diameter of the bubbles proportional to the relative abundances in cyclopoids, harpacticoids and AM1; inner dark yellow circles under HR (b) are illustrated for comparison with the significative lower relative abundances under LR (a)).

Acknowledgements

We wish to acknowledge the traditional custodians of the land, the Wongai people, and their elders, past, present and emerging. We acknowledge and respect their continuing culture and the contribution they make to the life of Yilgarn region. The authors thank Flora, Peter and Paul Axford of the Meadows Station are thanked for their kindness and generosity in providing both accommodation and access to their property.

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Supplementary material

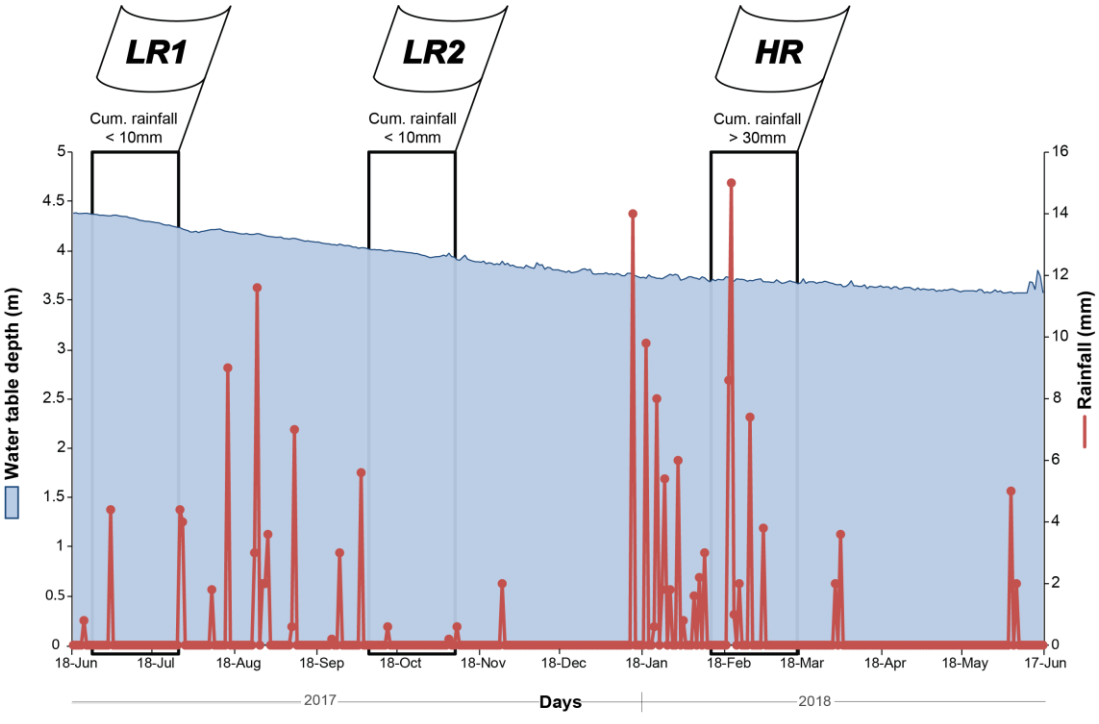

Figure S6.7. Weather station data from bore E7 extracted from Saccò et al., 2019b: water level (in mm, in light blue) and rainfall events (in mm, in red). Flags indicate the dates of LR1 (26/07/2017), LR2 (07/11/2017) and HR (17/03/2018) campaigns, and the black rectangles illustrate the cumulative rainfall and water depth trend for the 30 days before samplings (for rainfall periods category thresholds refer to Hyde et al. (2018).

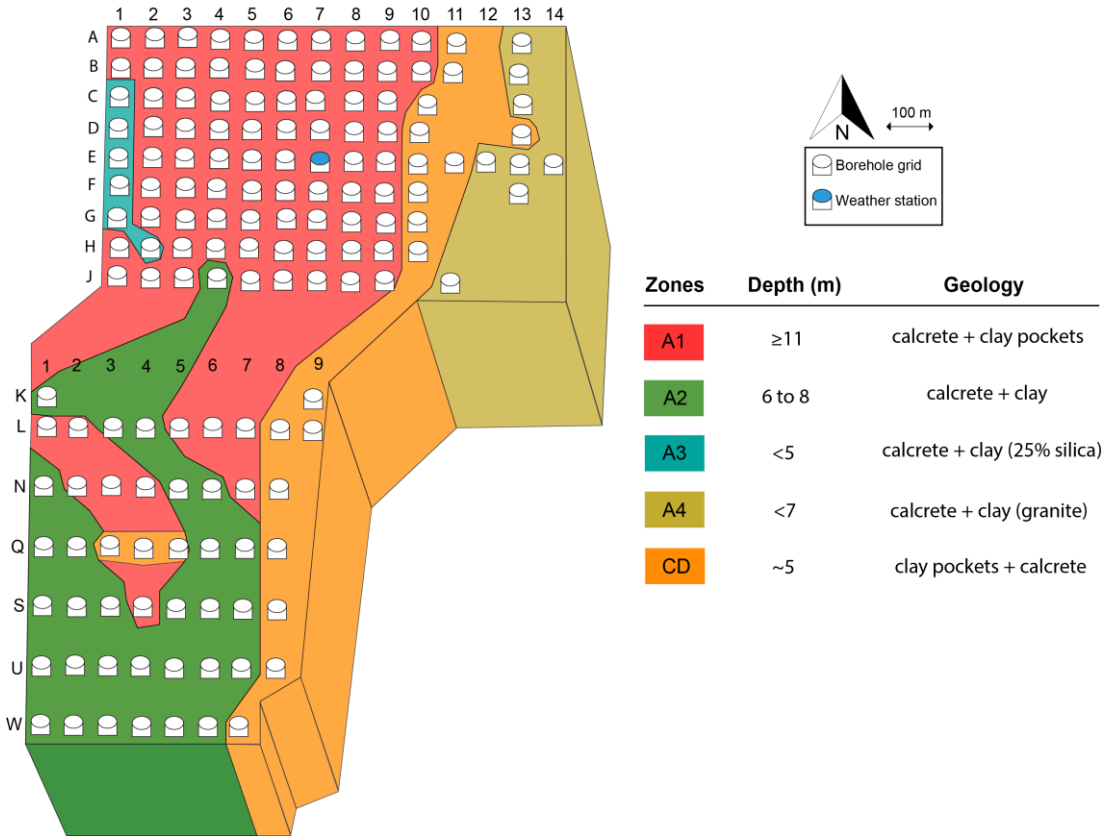

Figure S6.8. Sturt Meadows geological zones and location of the weather station (bore E7).

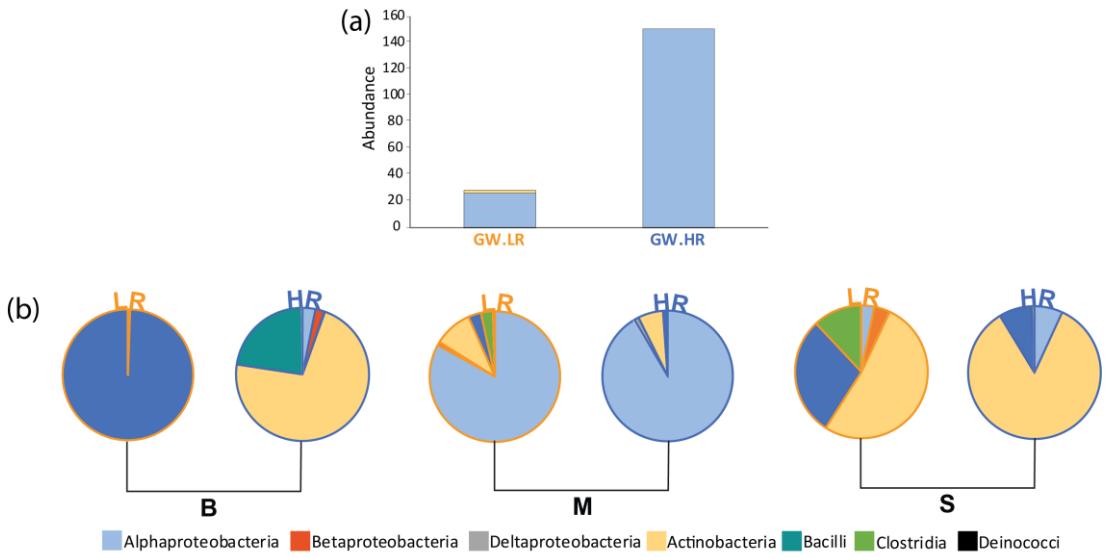

Figure S6.9. (a) Barplot illustrating the bacterial abundances in groundwater samples under LR (GW.LR, in dark yellow) and HR (GW.HR, in light blue). (b) Relative abundances (in %) of the bacterial classes in beetles B, M and S under LR and HR.

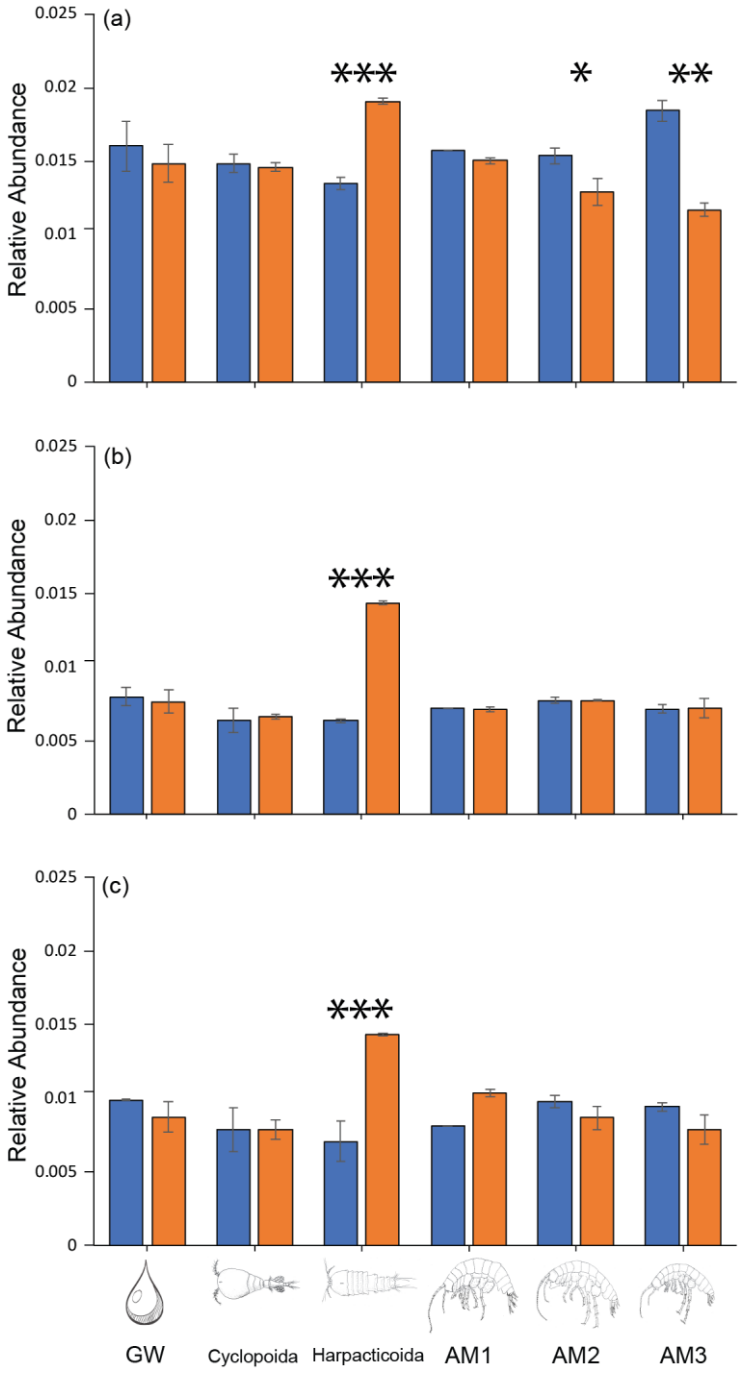

Figure S6.10. Abundances of the major KEGG pathways associated with methane metabolism (a), nitrogen metabolism (b) and sulfur metabolism (c). *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

Table S6.3. Tukey's pairwise comparisons of $\delta^{13}C_{Val\text{-}Phe}$ values of the groups pertaining to LR.

Groups	Roots	Water mites	Amphipods	Aquatic worms	Beetles larvae	Beetles adults
Roots		ns	ns	*	***	***
Water mites			ns	*	***	**
Amphipods				ns	*	ns
Aquatic worms					ns	ns
Beetles larvae						ns
Beetles adults						

Table S6.4. Tukey's pairwise comparisons of $\delta^{13}C_{Val\text{-}Phe}$ values of the groups pertaining to HR.

Groups	Roots	Water mites	Amphipods	Aquatic worms	Beetles larvae	Beetles adults
Roots		*	*	**	**	***
Water mites			ns	ns	ns	ns
Amphipods				ns	ns	ns
Aquatic worms					ns	ns
Beetles larvae						ns
Beetles adults						

Table S6.5. Results from the $\delta^{13}C$, $\delta^{15}N$ and ^{14}C analyses on beetles (B, M and S) during LR and HR.

	ID	$\delta^{13}C$		$\delta^{15}N$		$\Delta^{14}C$ (‰)	Age (BP)	$\Delta^{14}C$ (‰)	Age (BP)
		LR	HR	LR	HR	LR		HR	
P.macrosturtensis	B	-23±0.19	-23.51±0.81	14.66±0.27	14.62±0.49	32.9±2.3	modern	17.6±1.6	modern
P.mesosturtensis	M	-23.37±0.19	-23.72±0.14	15.43±0.53	14.01±0.05	37.0±2.2	modern	27.0±1.6	modern
P.microsturtensis	S	-23.6	-24±0.43	14.40	13.07±0.42	22.9±3.1	modern	18.3±1.6	modern

Table S6.6. Low rainfall (LR) amino acids spectrum for stygofauna, hemipterans, ants and roots, separated by non-essentials (NEAA: aspartic acid (Asx), serine (Ser), glutamine (Glx), glycine (Gly), alanine (ala), proline (Pro) and glutamic acid (Glu)), essentials (EAA: threonine (Thr), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), lysine (Lys) and tyrosine (Tyr)) and conditionals (COND: histidine (His) and tyrosine (Tyr)).

Taxon	ID	Replicates	NEAA							EAA								COND	
			Asx	Ser	Glx	Gly	Ala	Pro	Glu	Thr	Val	Met	Ile	Leu	Phe	Lys	Arg	His	Tyr
Paroster macrosturtensis	B	1	-18.054	-10.353	-19.108	-9.933	-20.093	-17.478	-19.021	-14.307	-24.776	-23.566	-24.302		-24.348		-19.952	-18.784	-23.712
		2	-17.225	-10.501	-18.365	-10.096	-19.925	-18.224		-15.094	-23.994	-23.832		-26.019	-24.527	-21.476	-19.171		-23.237
Paroster mesosturtensis	M	1	-19.27	-11.204	-20.295	-12.688	-20.861	-15.548	-19.696	-15.52	-24.735	-24.602	-22.202		-26.077		-20.374	-17.924	-24.543
		2	-18.422	-11.437		-11.836	-21.599	-14.66	-20.546		-24.36	-25.382	-23.044		-26.407		-20.471	-18.496	-24.59
Paroster microsturtensis	S	1	-22.552	-12.827	-22.423	-14.388	-24.486	-19.105	-23.426	-17.153	-28.182	-28.025	-26.063		-29.023		-23.567	-20.753	-27.734
		2	-22.297	-11.957		-15.287	-23.987	-18.229	-23.452		-27.279	-27.192	-25.234		-28.928		-22.803	-19.841	-26.828
Paroster macrosturtensis larvae	Blv	1	-20.634	-9.09	-20.202	-14.589	-21.6	-19.735	-18.671	-19.747	-26.092		-25.414		-26.563		-20.759	-19.144	-25.165
		2	-20.481	-9.343		-13.744	-22.423	-18.88	-18.26		-25.852		-24.655		-26.388		-20.413	-19.717	-24.966
Paroster mesosturtensisl arvae	Mlv	1	-19.12	-6.571	-20.356	-16.576	-21.387	-16.585	-18.977	-21.515	-24.983	-27.548	-25.352		-27.154	-20.835	-19.808	-20.133	-25.892
		2	-19.058	-6.822	-19.749	-15.659	-20.862	-16.165	-18.985	-21.824	-24.862	-26.775	-24.579		-27.006	-21.28	-19.378	-20.12	-25.918
Paroster microsturtensisl arvae	Slv	1	-19.452	-8.375	-17.775	-13.756	-21.683	-20.519	-20.9	-17.21	-25.389	-26.574		-26.472	-26.752	-19.718	-18.742		-26.009
		2	-18.746	-8.443	-17.732	-14.643	-21.465	-20.234	-20.157	-17.127	-25.284	-26.554		-27.31	-27.292	-20.046	-18.439		-26.053
Scutachiltonia axfordi	AM1	1	-17.019	-5.299	-15.082	-6.436	-18.377	-16.816		-16.062	-25.975		-19.86	-21.449	-22.141	-17.837	-15.828	-8.917	-20.467
		2	-15.175	-6.431	-13.098	-5.01	-22.456	-16.536		-13.911	-23.128		-20.18	-23.723	-21.747	-18.282	-14.787	-20.91	-9.59
Yilgarniella sturtensis	AM2	1	-19.036	-8.605	-18.681	-6.513	-20.352	-16.714		-14.227	-25.359		-22.055	-24.342	-24.684	-21.813	-18.575	-18.095	-23.525
		2	-19.151	-8.019	-24.013	-7.103	-20.23	-16.546		-1.516	-25.597		-22.843	-23.961	-26.508	-20.482	-20.543	-12.576	-21.951
Stygochiltonia bradfordae	AM3	1	-21.633	-8.7	-21.546	-8.767	-24.212	-22.308		-14.806	-28.659		-24.796	-27.248	-28.192	-21.873	-23.273	-18.847	-25.092
		2	-21.774	-9.601	-27.747	-9.228	-23.948	-23.214		-3.535	-29.014		-26.567	-27.387	-28.34	-22.622	-23.816	-18.945	-24.868
Tubificidae sp.	OL	1	-23.262	-15.692	-24.114		-26.425	-25.526	-27.138	-22.507	-31.532	-29.026	-31.604		-31.68	-27.568	-27.604	-26.316	-30.681
		2	-23.597	-16.318	-24.551	-20.926	-26.287	-26.041	-26.225	-22.398	-31.384	-28.851	-31.694		-31.352	-28.022	-27.554	-26.367	-30.927
Oribatida sp.	OR	1	-20.885	-11.825		-14.452	-21.224	-18.871	-19.218		-26.379		-21.452		-24.721		-18.986	-20.299	-24.149
		2	-19.994	-12.145	-17.773	-13.785	-21.13	-19.739		-15.068	-26.341			-24.621	-23.941	-20.019	-18.706		-24.509
Hempitera sp.	HM	1	-19.604	-16.878	-21.844	-20.566	-25.866	-20.298		-13.273	-30.654		-26.008	-27.331	-29.119	-24.759	-24.294	-24.375	-26.804
Linepithema humile	Ants	1	-19.248	-16.881	-23.302	-19.598	-24.536	-19.714	-24.261	-14.622	-27.328	-26.621	-23.236		-25.56		-19.029	-22.568	-25.282
		2	-19.082	-16.708	-22.76	-19.742	-23.686	-20.46		-14.889	-26.729			-27.406	-24.778	-19.518	-18.291		
Acacia Aneura	Roots	1	-19.397	-11.733	-22.077	-17.433	-22.445	-21.256		-18.725	-26.535		-21.651	-24.737	-23.127		-20.591		-23.249
		2	-19.061	-12.572	-21.582	-17.924	-21.933	-20.748		-18.62	-26.806				-23.53	-21.814	-19.945	-19.834	-23.476

Table S6.7. High rainfall (HR) amino acids spectrum for stygofauna, hemipterans, ants and roots, separated by non essentials (NEAA: aspartic acid (Asx), serine (Ser), glutamine (Glx), glycine (Gly), alanine (ala), proline (Pro) and glutamic acid (Glu)), essentials (EAA: threonine (Thr), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), lysine (Lys) and tyrosine (Tyr)) and conditionals (COND: histidine (His) and tyrosine (Tyr)).

Taxon	ID	Replicates	NEAA							EAA								COND	
			Asx	Ser	Glx	Gly	Ala	Pro	Glu	Thr	Val	Met	Ile	Leu	Phe	Lys	Arg	His	Tyr
Paroster macrosturtensis	B	1	-18.981	-11.825	-18.609	-11.385	-19.968	-16.435	-12.624	-15.99	-25.892		-25.381	-26.035	-26.728	-21.94	-20.826		
		2	-18.35	-11.415	-17.774	-11.232	-19.328	-17.232		-16.725	-24.984				-25.868		-19.981	-18.349	-23.381
Paroster mesosturtensis	M	1	-23.876	-18.06	-23.557	-16.643	-25.964	-21.987	-23.065	-21.67	-29.768	-32.187	-28.902	-29.865	-31.075	-24.674	-26.052		
Paroster microsturtensis	S	1	-20.865	-11.435	-20.798	-13.937	-22.623	-17.816	-18.898	-14.569	-26.773	-30.766	-25.661	-26.806	-29.037	-22.886	-22.632		
Paroster macrosturtensis larvae	Blv	1	-22.032	-12.583	-20.627	-13.557	-24.294	-19.428	-16.875	-18.706	-28.488	-29.773	-26.385	-27.94	-28.623	-22.888	-22.761		
		2	-21.253				-24.15				-27.76				-27.818		-21.939		-26.1
Paroster mesosturtensisl arvae	Mlv	1	-23.876	-18.06	-23.557	-16.643	-25.964	-21.987	-23.065	-21.67	-29.768	-32.187	-28.902	-29.865	-31.075	-24.674	-26.052		
		2				-17.238				-20.873	-28.997							-21.78	-27.678
Paroster microsturtensisl arvae	Slv	1	-24.391	-17.615	-24.241	-18.735	-26.035	-22.186	-23.133	-19.036	-30.074	-32.127	-28.827	-29.946	-31.545	-25.108	-26.446		
		2	-23.9	-18.454	-24.237		-26.188		-22.384								-26.728	-22.586	-28.808
Scutachiltonia axfordi	AM1	1	-24.151	-12.209	-23.8	-13.066	-24.458	-22.004	-19.787	-19.089	-29.886	-29.858	-27.014	-29.218	-29.862	-25.057	-24.182	-21.926	-27.292
		2	-23.303	-12.501	-23.308	-12.704	-23.798	-21.357	-19.729	-19.071	-29.15	-29.157	-26.118	-28.459	-29.25	-24.967	-23.416	-21.239	-26.281
Yilgarniella sturtensis	AM2	1	-24.005	-13.496	-23.898	-15.148	-26.737	-23.713	-26.256	-22.018	-30.956	-27.096	-28.127	-29.494	-30.747	-24.607	-25.401	-22.119	-27.88
		2	-23.987	-13.85	-23.31	-14.701	-25.818	-24.344	-25.651	-21.258	-30.777	-26.435	-27.485	-28.69	-29.863	-23.935	-24.58	-22.361	-26.988
Stygochiltonia bradfordae	AM3	1	-23.246	-11.931	-22.681	-13.183	-23.938	-21.778	-21.818	-21.876	-28.858	-30.87	-23.334	-24.557	-28.65	-19.548	-22.867	-20.429	
		2	-23.532	-12.805	-22.688	-13.2	-23.735	-21.05	-22.511	-21.906	-28.54		-24.252	-25.425	-27.913	-20.432	-23.016	-19.753	-24.501
Tubificidae sp.	OL	1	-20.246	-14.108	-20.249	-15.601	-23.938	-20.468	-23.209	-17.579	-27.927	-23.131	-22.983	-26.679	-28.788	-21.984	-21.623		
Oribatida sp.	OR	1	-20.9		-22.5	-6.0	-22.1	-19.7		-20.0	-27.6		-27.8		-27.4	-23.7	-20.7	-23.0	-26.4
		2	-19.97	-4.92	-21.682	-5.821	-22.258	-19.678		-20.389	-27.446		-27.819			-24.338	-21.291	-22.323	-25.809
Hempitera sp.	HM	1	-17.063	-13.928	-19.642	-18.187	-18.778	-17.191		-13.276	-23.053	-25.424			-24.304		-18.207		-24.166
		2	-17.355	-13.431	-19.757	-19.031	-18.044	-17.883	-19.964	-14.052	-22.696	-26.539			-24.093		-17.382	-18.92	-23.299
Linepithema humile	Ants	1	-18.638	-17.83	-21.075	-17.378	-22.405	-21.459		-14.819	-25.557				-23.848		-17.043	-15.863	-23.024
		2					-22.125	-21.507							-23.319		-17.845	-16.413	-23.175
Acacia Aneura	Roots	1	-17.44	-9.399	-19.053	-17.491	-20.073	-19.149	-18.868	-14.122	-23.015	-22.693	-23.716		-20.023	-18.652	-14.937	-16.923	-20.488
		2	-16.762	-8.691	-18.47		-19.861	-19.035			-22.776	-22.382	-23.561		-19.789	-17.885	-15.187	-16.812	-19.435

Table S6.8. Dietary contributions (in %) of copepods (cyclopoida and harpacticoida) and amphipods AM1 and AM2 under LR and HR.

	Contributions (%)									
	LR					HR				
	DIC	DOC	POC	Roots	Sediment	DIC	DOC	POC	Roots	Sediment
Cyclopoida	10±10.1	25.9±14.1	19.1±22.2	12.7±10.5	32.3±26.7	12.6±10.6	41.1±16.6	17.8±14.3	9.6±8.6	18.9±22.3
Harpacticoida	10.7±11	26.1±14.7	20.3±23.7	11±9.8	31.9±26.2	8.9±7.5	51±17.1	19.6±17	6.6±6.2	13.9±15.1
AM1	6.3±5.4	51.7±8.3	14.4±13.9	8.3±5.2	19.3±11.7	3±2.1	77.5±11	12.9±11.1	2.2±1.3	4.4±3.3
AM2	4.7±5.6	55.7±9.4	13.5±14.9	7.8±6.5	18.3±14.7	1.4±1.4	84.9±11.9	10±12.1	1.2±1.1	2.5±2.7

Table S6.9. Bacterial abundances in groundwater samples (GW) and samples of amphipods AM1, AM2 and AM3 during LR and HR. Information extracted from the abundances of ZOTUs per each class.

Class	LR				HR			
	GW	AM1	AM2	AM3	GW	AM1	AM2	AM3
Alphaproteobacteria	26.50	8.00	27.00	20.50	151.00	710.50	161.50	381.00
Betaproteobacteria	0.00	0.00	6.00	130.50	0.00	2.50	4.00	2.00
Deltaproteobacteria	0.00	0.00	0.00	0.00	0.00	0.50	19.00	0.00
Actinobacteria	2.00	207.00	47.50	12.50	0.00	261.50	147.50	19.50
Bacilli	0.00	5.00	168.00	323.00	0.00	37.00	50.00	72.50
Clostridia	0.00	0.00	11.00	0.00	0.00	103.50	60.50	1506.00
Deinococci	0.00	0.00	0.00	0.00	0.00	0.00	8.00	0.00

Table S6.10. Abundance data (from Saccò et al., 2019b) of the different stygofaunal taxa at Sturt Meadows detected during the sampling campaigns LR1, LR2 and HR (first day of sample collecting).

RAINFALL PERIOD		LR1																														
GEOLOGICAL ZONES		A1						A2							A3					CD								A4				
TAXON	ID	A8	C5	F4	H5	J7	N4	L4	S2	U2	W2	Q2	W3	W4	C1	D1	E1	G1	H2	A11	D13	E10	E11	Q3	Q4	S8	B13	C13	E12	E13	F13	
Tubificidae	TU														2																	
Oribatida	OR			2			2					1	1									1		1		3		1	1		2	
Harpacticoida	H	1	3	9	17	18	5	7	13	17	12		8	27	5	15	12	3	72	21	8	19		2	8	27	7	1		6	11		
Cyclopoida	C	18	6	1	11	16	36	8	9	14	2	5	3	3	2	6	1	5	131	92	1	33	11	19	14	55	2	269	3	26	1	
Chiltoniidae (juveniles)	AMJ				15	1		3	15	4	5			1					4								3			6	11	
Scutachiltonia axfordi	AM1		19	1	14		13		1	7		1		1		1		1	9	4	5		2					6		7	
Yilgarniella sturtensis	AM2		1	1	23		13		21		2		2	2				3	2	2	7	2			2			3				
Stygochiltonia bradfordae	AM3			1	3				6	3			1					1	4			2							2		
Paroster macrosturtensis	B		1		1	1							1				1		2								1		11	1	6	
Paroster mesosturtensis	M			3	1		3		9	1	1		1	1	4	1	4	1	3	9	1				1				1	9		
Paroster microsturtensis	S		1		2		1		6							3	2	1	1		6			1					1	5	1	
Paroster macrosturtensis larvae	Blv		1										1				1	1	2	1	1									1		
Paroster mesosturtensis larvae	Mlv								1																		1					
Paroster microsturtensis larvae	Slv		1						1	1	1		3			1	1	1	1		2		2	1	1				1	2		
		LR2																														
Tubificidae	TU																								1							
Oribatida	OR			1			2		1	1	8	123		3		1		1		1		2			2		1		1	1		
Harpacticoida	H	82	26	5	12	2	18	2	8	2	17	7	4	29	259	7	22		11	6	1	2	3	34	3	3	12		1	2	1	
Cyclopoida	C	86	29	22	24	4	16	2	16	129	7	97	87	19	68	16	31	4	22	35	31	9	29	66	1		6	33	7	81	38	
Chiltoniidae (juvelines)	AMJ	32	6		1	1													6	1		1							2	5		
Scutachiltonia axfordi	AM1	3	1		3	1	1		1	6	1		12	4			2	2		2				1			8	1	9	1	1	
Yilgarniella sturtensis	AM2	28	11		1	2				6		2	11	2		2	6		4	6	1	5		1	1		4		6	4	2	
Stygochiltonia bradfordae	AM3	2	2		1	1	3			1			1				1	1	2	2	1	5	2	1				2	2		1	
Paroster macrosturtensis	B	1				2			2	1	2		7					3		3	1						4		7	3	9	
Paroster mesosturtensis	M	3				1	1			7		3	6	2	2		3			5	22						1			2		
Paroster microsturtensis	S	1				1	3	1			1		7	1		1				1	17						7		2	3		
Paroster macrosturtensis larvae	Blv		1				1						3							6							1					
Paroster mesosturtensis larvae	Mlv																				2											
Paroster microsturtensis larvae	Slv	1	2			3						2	1			2	1			5	6			4			2			1		
		HR																														
Tubificidae	TU	14	1			1													3			1										1
Oribatida	OR											29						2	3											3		
Harpacticoida	H	64	44	13	1	32	17	8	4	83	6		5	4	3	7	154	11	19	13	12	2	26	9	2	13	8	3	7	14	4	
Cyclopoida	C	54	91	23	17	22	12	4	8	19	4	2	23	13	15	2	87	1	29	148	77	66	76	15	11	16	137	41	39	36	47	
Chiltoniidae (juveniles)	AMJ	7	8		14	2				3	1			1			8		2	6	2		1			3	2		2	7	4	
Scutachiltonia axfordi	AM1				2		4			13			2	2			2	1	1	1			1				4	2	8		4	
Yilgarniella sturtensis	AM2	2	3		9	4	2			12			7	1			13		2	4		3	4				2	8		5	7	
Stygochiltonia bradfordae	AM3		1							1										1		8						7		1		
Paroster macrosturtensis	B	4			3					3	9		28			3				6			1	3			1		2	5	8	
Paroster mesosturtensis	M	7	2		6	1	3	1	1	1		6	4	2	1		1		1	11		1	6	1	1		4	1		11		
Paroster microsturtensis	S	22	3	3	6	1	3			14	1	3	9	7				1		8			2				7		2	6	5	
Paroster macrosturtensis larvae	Blv									1				1			1		1		2									1		
Paroster mesosturtensis larvae	Mlv																			2		1						1				
Paroster microsturtensis larvae	Slv		1																	2	1						2			4		

Chapter 7 | Critical evaluation

The research presented here provides a comprehensive ecological analysis of the dynamics sustaining biota in Western Australian calcretes. However, as with any project operating within a limited time and budget, a number of aspects had to lie outside the scope of the work, and so several research gaps remain.

- Only one natural system was studied. Given the specificity of subterranean environments, where each aquifer potentially forms isolated "islands" with endemic fauna, the conclusions about biogeochemical mechanisms cannot be directly extrapolated to all groundwater ecosystems, meaning that substantial uncertainty remains in defining the specific driving ecological forces shaping subterranean ecosystems globally.

- Stygofaunal opportunism provides challenges in interpreting subterranean food web interactions. Despite fifteen years of taxonomic and ecological studies, Sturt Meadows stygofauna still lack detailed empirical data on species interactions and ethology at community level. Indeed, major obstacles such as limited accessibility to aquifers, and groundwater environments in general, provide critical challenges to these investigations under natural conditions. Alternatives to field experiments will be necessary for exhaustive interpretation of the ecological patterns in groundwaters.

- In chapter 2, we proposed creating stygofaunal diet estimations through the integration of data from genetics and biogeochemistry into multifactorial Bayesian mixing models, as refining dietary analysis in this way has been proved to improve the accuracy of food web interpretations in other research fileds. However, although initial modelling attempts were made, they are not, at this stage, robust (see section 7.3 below). I am undertaking work to address these issues, but completing the modelling now sits outside the scope of this PhD thesis. Given the technical challenges that conventional groundwater trophic ecology studies face, application of these models will considerably advance our understanding of stygofaunal feeding patterns.

- This thesis presents an original and significant reconstruction of ecological baseline information and trophic habits from a calcrete aquifer. However, to expand this to

groundwater management studies, monitoring of hydrological and biological dynamics will be required across a longer time period.

To address these gaps, I suggest here four main research directions that will provide guidelines for future investigations.

7.1 Alternative environments

Further studies involving higher numbers of samples from more biodiverse systems or complex trophic assemblages (i.e. alluvial aquifers, karsts, etc.) would help test the applicability of my findings and ultimately refine the accuracy of my research design. This might involve also extending this research design to surficial groundwater fed ecosystems to understand the widely accepted but rarely bio-geochemically assessed linkage between aquatic surface and subterranean environments. Other poorly studied and hard to access ecosystems such as thermal springs or anchialine caves could also benefit from this interdisciplinary approach.

7.2 Laboratory experiments

Mesocosm experiments would allow detailed investigation of stygofaunal trophic behaviors and refinment of the interpretation of species-specific dynamics from cryptic environments such as groundwaters. Furthermore, application of sophisticated isotopic techniques on laboratory monitored communities, such as CSIA 'carbon fingerprinting' (an organic compound-based technique for identifying plant, fungal or bacterial origins of amino acids) would also improve our understanding of the functional linkages between microbes and stygofauna, helping untangle groundwater energy flows. The use of bio-tracers (together with artificially enriched compounds) would help pinpoint the key biochemical pathways and bring a better understanding on the time framework of organic matter assimilations within subterranean biota.

7.3 Modeling tools

Significant refinement of diet analysis can be achieved by incorporating further information beyond the conventional $\delta^{13}C$ and $\delta^{15}N$ proxies, such as radiocarbon data ($\Delta^{14}C$), a key

tracer in untangling carbon incorporation and trophic pathways. Concurrently, metagenomics data can provide crucial semi-quantitative information on dietary preferences that can refine statistical modelling once this data are integrated as prior information.

At the start of my PhD research, I hypothesized that the application of multifactorial designs combined with the more accurate isotopic data provided by CSIA (i.e. carbon essential amino acids, nitrogen trophic amino acids) had the potential to significantly improve the estimation of diet proportions when compared to conventional bulk approaches. This assumption was tested in a manuscript, not included in this thesis that was recently submitted for review. Constructive comments generated through the review process have inspired me to revisit the assumptions around methodological aspects often made in these modelling processes.

In particular, emerging from the review, it became clear that groundwater multifactorial modelling based on CSIA data faces a number of challenges. For instance, given the scarce availability of stygofaunal species-specific isotopic data, the definition of offsets (trophic discrimination factors) from specific amino acids (and $\Delta^{14}C$ data) is potentially exposed to bias. The incorporation of all potential prey sources is key to estimate their importance within diets of consumers/predators, which means that modeling of representative stygofaunal diet proportions is often limited by the scarce accessibility and intrinsic cryptic nature of groundwater systems.

However, by incorporating advances from other research fields such as oceanography or archaeology, I hope to overcome these obstacles. For example, remarkable progress in characterizing offsets of invertebrate taxa has been made recently for aquatic surface environments. In addition, technological advancements are quickly improving the detectability ranges of machines such as LC-iRMS, GC-iRMS or AMS and allowing reduced costs and automatisation of sample pre-treatments, reducing the sample size, and therefore the number of individual organisms required. Concurrently, novel biomonitoring tools such as eDNA provide crucial information on stygofaunal community composition, enabling thorough testing of the accuracy and representativeness of the conventional sampling techiques.

The question arises of to what extent we need to apply CSIA in groundwater studies. The isotopic data acquired is undoubtedly more detailed. Nonetheless, at present there are significant cost differences in bulk and compound specific studies that, depending on the lab, can mount to as high as hundreds or even thousands of dollars per sample. Where analyses are sought on large numbers of samples within a screening context (for example, monitoring changes in groundwater trophic ecology as part of large scale environmental impact assessment), a balance between cost and precision of outcome must be struck.

In my view, studies involving extensive sampling from multiple groundwater systems, or those otherwise constrained by operational costs, should consider using bulk tissue SIA, at least in the first instance. As shown in earlier chapters, despite its averaging of biochemical fractionation pathways, SIA still allows elucidation of the main flows shaping their biochemical functioning and enables comparison between systems or environmental conditions. However, where studies are seeking maximum precision with respect to detailed taxonomic changes in feeding, a case can be made for the use of CSIA. In either case, robust whole-system research designs should include maximum prior data as well as multiple isotopic proxies.

7.4 Developing ecological management in groundwaters

Long-term experiments at Sturt Meadows or similar aquifers expanding the current research design to i.e. five years would allow development of specific conservation plans. This would help delineate strategic designs for the management of calcrete ecosystems, together with guidance for other similar groundwater environments, in Australia and around the world. The field of ecotoxicology could also benefit from the application of novel approaches such as $\delta^{13}C$ and $\delta^{15}N$ CSIA, since such biochemical studies would lead to considerable improvements in the accuracy of environmental assessments both in pristine and contaminated groundwaters. The characterization of the carbon and nitrogen amino acid spectrum would allow detection and characterization of anomalous biochemical patterns, e.g. fertilizer-related, within the microbial and stygofaunal communities. Moreover, the presented multidisciplinary approach, if linked to the study of increasingly abundant exotic species in groundwaters, such as the crayfish *Procambarus clarkii* (Girard, 1852) (see Mazza et al. (2014) discussed in chapter 6), also has the potential to unveil the functional impact of invasive species in subterranean ecosystems.

Chapter 8 | Conclusions

This thesis provides original and significant insights into the ecological functioning of groundwater ecosystems in the shallow calcretes that characterize the subterranean framework of Australia's immense Yilgarn region. Research based on conventional multivariate techniques allowed elucidation of the niche interactions under contrasting rainfall periods, enabling broader comprehension of the subterranean ecology at Sturt Meadows. Moreover, the innovative application of a research design combining isotopic chemistry, radiocarbon analysis and molecular genetics (chapter 2) improved the understanding of arid zone subterranean biogeochemical patterns and untangled the ecological mechanisms sustaining groundwater biodiversity. These outcomes allowed to address the aim I of the thesis, 'refine groundwater functional ecology studies through the application of novel techniques widely employed in other fields', by bringing together usually disconnected analytical approaches within a sophisticated holistic design.

Groundwater environments play a crucial role within the carbon cycle and as water reservoirs, meaning that improved understanding of their ecosystem dynamics is crucial. This work contributes in providing essential baseline knowledge that, once integrated within long term projects, will be key in groundwater conservation and water resource management. Given that natural ecosystems progressively face increased climate change effects, tools and knowledge developed through this research will aid rapid assessment of rapidly changing conditions and enable modeling of future scenarios linked with natural and anthropogenic impacts in calcrete aquifers. Crucially, the research in this thesis links the "who" of ecosystem taxonomy to the "how" of functional ecology, significantly broadening the potential of groundwater impact assessments and monitoring.

This research project was carried out during one of the most severe droughts ever recorded in the Australian continent, and this resulted in a substantial hydrological deficit of aquifer recharge *sensu stricto*. Nonetheless, the research on ecological niche occupations (chapter 3) indicated that varied rainfall regimes triggered shifts in stygofaunal ecological niche occupations driven by increased nutrient availability and dissolved oxygen concentrations. Given the usual scattered and unpredictable rainfall events on site, our results indicated that stygofaunal community at Sturt Meadows calcrete presents high levels of ecological tolerance as a response to evolutionary and adaptative forces. These findings fulfilled the overarching aim II of the thesis, which involved the objective to 'understand the role played

by rainfall conditions in shaping ecological niche occupations amongst the stygofaunal community from an arid zone aquifer'.

However, elucidation of ecological dynamics solely *via* conventional multivariate approaches was also demonstrated to be incomplete, stressing the need to incorporate multidisciplinary studies for a robust whole-system understanding. Novel investigation based on incorporation of isotopic techniques (CSIA and SIA, chapter 4) allowed great insight into the food web dynamics and the key biogeochemical driving forces that forge them. Analysis of species-specific isotopic data enabled elucidation of rainfall-driven shifts in carbon flows and pinpointed two trophic levels (amphipod consumers and predatory beetles). In addition, stygofaunal trophic behaviours, dominated by opportunism and omnivory, shaped bottom-up (differential OM assimilations within consumers) and top-down (increased pressures from predators after high rainfall) controls linked with rainfall regimes. The outcomes of this study, the first incorporating advanced isotopic approaches in subterranean environments, cast new light on trophic dynamics in groundwaters and comprehensively addressed the aim III of the thesis: 'elucidate calcrete energy flows and stygofaunal food web interactions under contrasting rainfall periods'.

Other aspects explored in the research are subterranean rainfall-driven carbon inputs and their cascade effects within the microbial (chapter 5) and stygofaunal (chapter 6) communities. The overarching aims of the thesis number IV ('unravel the biogeochemical mechanisms shaping changes in local organic source inputs and microbial metabolic shifts') and V ('investigate the rainfall-driven ecological dynamics characterizing potential trophic cascades across the subterranean biota of the calcrete') were addressed through the implementation of multi-technique assessment - comprising geochemistry and genetics - of the ecological and biogeochemical flows. Our findings illustrated that rainfall acts as a driver in regulating input of old carbon sources into the calcrete that is ultimately available and exploited by the microbial community. Concurrently, subterranean invertebrate population dynamics coupled with shifts in microbially-derived organic matter incorporations, indicated a tendency towards more deterministic driving forces under the high rainfall regime.

The current thesis widens perspectives within the small but quickly growing research area of groundwater ecology, providing explanation of the biogeochemical dynamics sustaining

biota in shallow calcretes. This investigation demonstrates once again that, within an increasingly complex world, the present and future of science can immensely benefit from the integration of techniques and designs from usually disconnected areas of research. Indeed, given the rapidly changing global environmental scenarios we are currently experiencing, how we will assess and preserve our ecosystems will depend on our ability to share advanced knowledge and strengthen networks across disciplines.

May the scientist reflect

Ecologists are homologous with criminologists.

Both professions are driven by passion, curiosity and audacity.

The two explore obscure, hidden, and prodigiously complex dynamics.

The counterparts need to accurately characterise the background before diving in.

They depend on the most advanced techniques to unravel patterns.

Together, they require holistic approaches based on refined evidence.

But most importantly, they similarly save lives:

Criminal investigators by shedding light on the offenders' culpability and standing up for justice,

Ecologists by elucidating concealed paths for a better appreciation of nature, and a brighter and more sustainable tomorrow for future generations.

APPENDIX

Presentations to conferences

What's going on down (under) there? Unravelling biochemical flows under differential rainfall periods in a Western Australian calcrete (oral presentation) - **Mattia Saccò**, Alison J. Blyth, Karina Meredith, Colin Smith, Quan Hua, Debashish Mazumder, William F. Humphreys, Nicole White, Kliti Grice - Australasian Groundwater Conference (AGC), 24th - 27th November 2019, Brisbane (Queensland).

Can metabarcoding provide insights into trophic web interactions underground? (oral presentation) - Nicole White, Mahsa Mousavi-Mousaviderazmahalleh, Matthew Campbell, William Humphreys, Alison Blyth, **Mattia Saccò** - Australasian Groundwater Conference (AGC), 24th - 27th November 2019, Brisbane (Queensland).

Stable isotope analysis in understanding functional responses of groundwater ecosystems to climatic and anthropogenic change (oral presentation) - **Mattia Saccò**, Alison J. Blyth, William F. Humphreys, Alison Khul, Debashish Mazumder, Karina Meredith, Quan Hua, Colin Smith, Kliti Grice - Stable Isotope Mass Spectrometry Users Group meeting (SIMSUG), 26th - 28th June 2019, Bristol (UK).

Elucidating the food web structure of the subterranean invertebrate communities of arid zone Western Australia (oral presentation) - **Mattia Saccò**, Alison J. Blyth, William F. Humphreys, Alex Laini, Quan Hua, Kliti Grice - 24th International Conference on Subterranean Biology (ICSB), 20th - 24th August 2018, Aveiro (Portugal).

Food-web dynamics through compound-specific isotope analysis (CSIA): a study of western australian (wa) stygofauna (poster presentation) - **Mattia Saccò**, Alison J. Blyth, William Humphreys, Bill Bateman, Colin Smith, Kliti Grice - 28th International Meeting on Organic Geochemistry (IMOG), 17th - 22nd September 2017, Florence (Italy).

Geosphere-biosphere transitions in groundwaters: radiocarbon dating as a tool to unravel stygofaunaltrophic relationships (poster presentation) - **Mattia Saccò**, Alison J. Blyth, Quan Hua, William Humphreys, Bill Bateman, Kliti Grice - 2017 TIGeR (The Institute for GEoscience Research) Conference 13th - 15th September 2017, Perth (WA).

Biogeochemical trends within calcrete aquifers recharge processes: preliminary results based on historical data (oral presentation) - **Mattia Saccò**, Alison J. Blyth, William Humphrey, Steven Cooper, Andrew Austin, Alex Laini, Bill Bateman, Kliti Grice - Australasian Groundwater Conference (AGC), 11th - 13th July 2017, Sydney (NSW).

The biogeochemical processes in groundwater environments of arid mining areas Western Australia: a novel isotopic based approach focused on the macroinvertebrates trophic relationships (poster presentation) - **Mattia Saccò**, Alison J. Blyth, Bill Humphrey, Bill Bateman, Kliti Grice - 2016 TIGeR (The Institute for GEoscience Research) Conference, 26th - 28th September 2016, Perth (WA).

Unravelling the food-web structure of the subterranean invertebrate communities of arid zone Western Australia (poster presentation) - **Mattia Saccò**, Alison J. Blyth, William Humphrey, Steven Cooper, Andrew Austin, Alex Laini, Bill Bateman, Kliti Grice - 19th Australian Organic Geochemistry Conference (AOGC), 4th-7th December 2016, Freemantle (WA).

Seminars

Functional ecology of calcrete aquifers in arid zone Western Australia - **Mattia Saccò,** University of Vienna (Vienna, Austria), 9th December 2019; Institute of Ecosystem Study (ISE) (Verbania, Italy, 17th December 2019; University of Parma (Parma, Italy), 18th December 2019).

Working book chapters

'Groundwater food webs' in Groundwater ecology and Evolution, 2nd edition (Elsevier), 2020 - Michael Venarsky, Kevin Simon, Clementine François, Laurent Simon, **Mattia Saccò**, Christian Griebler.

'Trophic interactions in groundwater environments' in Encyclopedia of Inland Waters, 2nd edition (Elsevier) (2020) - **Mattia Saccò**, Alison J. Blyth, William F. Humphreys.

Other research outcomes

Refining trophic dynamics through multi-factor Bayesian mixing models: a case study of subterranean beetles - **Mattia Saccò**, Alison J. Blyth, William F. Humphreys, Steven J. B. Cooper, Andrew D. Austin, Josephine Hyde, Alison Kuhl, Debashish Mazumder, Quan Hua, Colin Smith, Nicole E. White, Kliti Grice – *In press* in *Ecology and Evolution* (2020).

Communities in high definition: spatial and environmental factors affecting small scale distribution of aquatic invertebrates (oral presentation) - Gemma Burgazzi, Alex Laini, **Mattia Saccò**, Pierluigi Viaroli - 2nd International Conference on Community Ecology, 4th - 6th June 2019, Bologna (Italy).

Communities in high definition: spatial and environmental factors shaping small-scale distributions of aquatic invertebrates - Gemma Burgazzi, Alex Laini, Otso Ovaskainen, **Mattia Saccò**, Pierluigi Viaroli – *In press* in *Freshwater Biology* (2020).

Integrated management of three artificial wetlands to improve water quality and biodiversity (oral presentation) - Miguel Martin, Carmen Hernández-Crespo, Pablo Vera, **Mattia Saccò**, Sara Gargallo, Maria Antonia Rodrigo - 16th IWA International Conference on Organisation, 30th September - 4th October 2018, Valencia (Spain)

Study along two years of the macroinvertebrate community in three constructed wetlands in the Albufera Natural Park (Valencia, Spain) (poster presentation) - **Mattia Saccò**, Juan Rueda, Carmen Hernández-Crespo, Roberta Callicó, Vicent Benedito - 16th IWA International Conference on Organisation, 30th September - 4th October 2018, Valencia (Spain).

Exploratory study on the optimisation of sampling effort in a non-vegetated lagoon within a Mediterranean wetland (Albufera Natural Park, Valencia, Spain) - **Mattia Saccò**, Juan Rueda Sevilla, Vicente Genovés Gómez, Roberta Callicó Fortunato, M. Eugenia Rodrigo Santamalia, Vicent Benedito Durà – *In press* in *Ecological Indicators* (2020).

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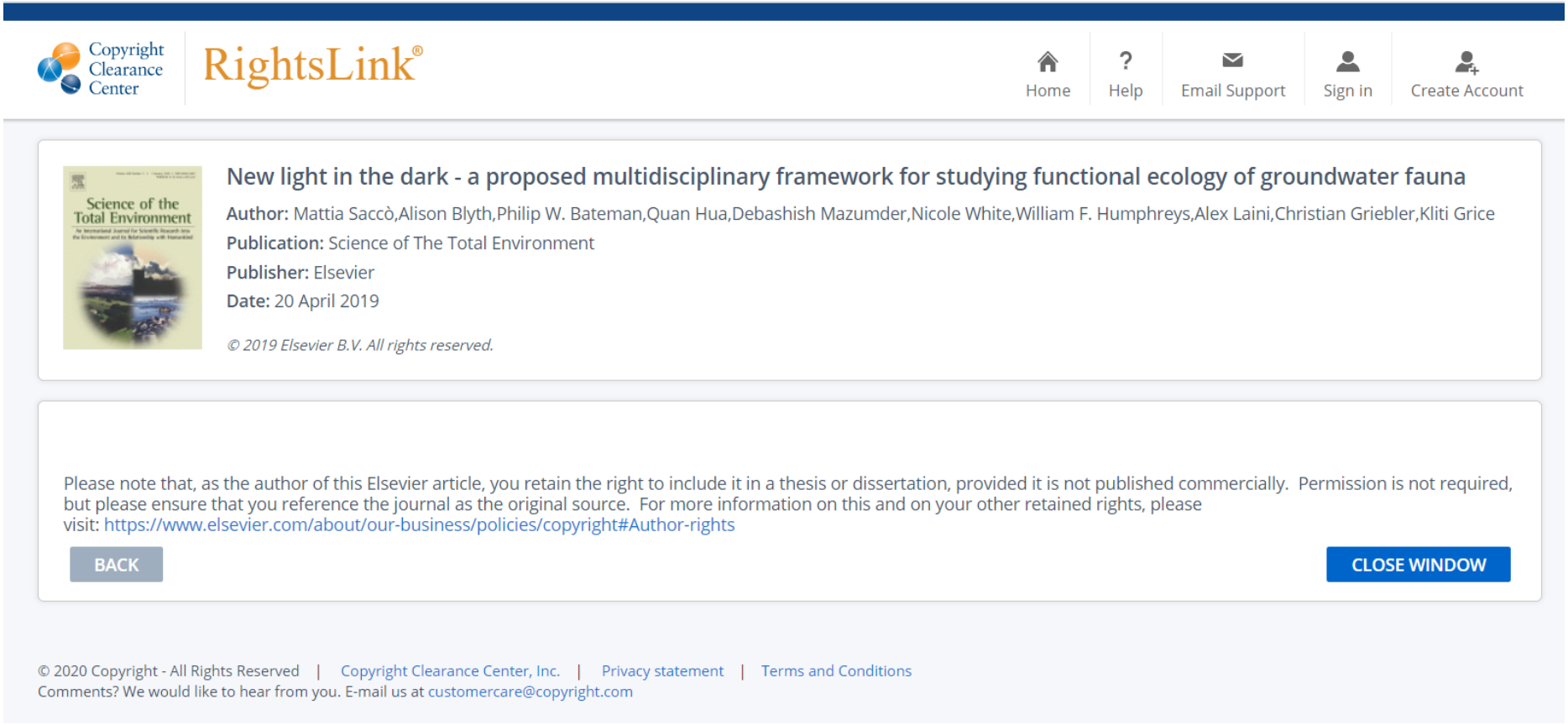

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Authorship statements

Chapter 2 | New light in the dark - a proposed multidisciplinary framework for studying functional ecology of groundwater fauna

Saccò designed the review, wrote all sections other than those drafted by Hua and Griebler, and recruited and coordinated the author team for specialist input. Blyth contributed to the original research idea and reviewed and edited the manuscript. Bateman provided guidance on the review design and edited the manuscript. Hua drafted the radiocarbon section and edited the manuscript. Mazumder provided advice for the sections on stable isotope analysis, while White provided help in shaping the genetic section. Humphreys contributed to the original research idea and reviewed the manuscript. Laini provided specialist input for the statistical section on Bayesian mixing models. Griebler drafted the ecotoxicological section and edited the manuscript. Grice reviewed and edited the manuscript.

Name	**Contribution percentage**	**Signature**	**Date**
Mattia Saccò	60%		27/05/2020
Alison J. Blyth	7%		27/05/2020
Philip W. Bateman	3%		27/05/2020
Quan Hua	8%		27/05/2020
Debashish Mazumder	2%		27/05/2020

Nicole White	3%		27/05/2020
William F. Humphreys	3%		27/05/2020
Alex Laini	4%		27/05/2020
Christian Griebler	8%		27/05/2020
Kliti Grice	2%		27/05/2020

Chapter 3 | Stygofaunal community trends along varied rainfall conditions: deciphering ecological niche dynamics of a shallow calcrete in Western Australia

Saccò undertook all primary research, fieldwork, and analyses, designed the statistical processes, led the interpretation and wrote the manuscript. Blyth provided funding, contributed to the analytical design and data interpretation and edited the manuscript. Humphreys contributed to the original research idea and funding, and assisted in fieldwork and interpretation. Karasiewicz provided assistance with the statistical analysis and edited the analytical sections of the manuscript. Meredith helped with the interpretation of the hydrological patterns and edited the manuscript. Laini helped with multivariate statistical tests and edited the manuscript. Cooper contributed to the original research idea and provided funding and editorial input for the manuscript. Bateman reviewed the manuscript and edited the biological sections. Grice reviewed and edited the manuscript.

Name	**Contribution percentage**	**Signature**	**Date**
Mattia Saccò	70%		27/05/2020
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Stéphane Karasiewicz	7%		27/05/2020
Karina Meredith	3%		27/05/2020
Alex Laini	4%		27/05/2020

Steven J. B. Cooper	3%		27/05/2020
Philip W. Bateman	2%		27/05/2020
Kliti Grice	1%		27/05/2020

Chapter 4 | Elucidating stygofaunal trophic web interactions *via* isotopic ecology

Saccò undertook the primary research, fieldwork and analyses, designed the statistical processes, and led the writing of the manuscript. Blyth conceived the original research idea and provided funding, contributed to the analytical design, data interpretation, and writing the manuscript. Humphreys contributed to the original research idea and funding, fieldwork and interpretation of groundwater dynamics. Khul (N CSIA), Mazumder (SIA) and Smith (C CSIA) analysed samples and processed the data. Grice contributed to the analytical design and data interpretation.

Name	**Contribution percentage**	**Signature**	**Date**
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Alison Kuhl	5%		27/05/2020
Debashish Mazumder	5%		27/05/2020
Colin Smith	4%		27/05/2020
Kliti Grice	2%		27/05/2020

Chapter 5 | Tracking down carbon inputs underground from an arid zone Australian calcrete

Saccò undertook the primary research, fieldwork and analyses, designed the statistical processes, and led the writing of the manuscript. Blyth provided funding, contributed to the data interpretation and edited the manuscript. Humphreys contributed to the original research idea and funding, fieldwork and interpretation of groundwater dynamics. Middleton helped with fluorescence analysis and edited the manuscript. White carried out genetic analyses and helped with the metabarcoding data interpretation. Campbell provided assistance with bioinformatics and editing the genetic sections of the manuscript. Mousavi-Derazmahalleh helped with statistical and qualitative analysis of the genetic data and edited the manuscript. Laini provided assistance with the statistical analysis of the isotopic data. Hua helped with the interpretation of radiocarbon data and edited the manuscript. Meredith helped with the interpretation of the hydrological patterns and edited the manuscript. Cooper provided funding and editorial input for the manuscript. Griebler reviewed the interpretation of the microbiological patterns and edited the manuscript. Grierson and Grice reviewed and edited the manuscript.

Name	**Contribution percentage**	**Signature**	**Date**
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William F. Humphreys	2%		27/05/2020
Jen A. Middleton	5%		27/05/2020

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Steven J. B. Cooper	1%		27/05/2020
Christian Griebler	2%		27/05/2020
Pauline Grierson	2%		27/05/2020
Kliti Grice	2%		27/05/2020

Chapter 6 | What's going on down (under) there? Unravelling biochemical flows under differential rainfall periods in a Western Australian calcrete

Saccò undertook the primary research, fieldwork and analyses, designed the statistical processes, and led the writing of the manuscript. Blyth provided funding, contributed to the data interpretation and edited the manuscript. Humphreys contributed to the original research idea and funding, field work and interpretation of groundwater dynamics. Cooper provided funding and editorial input for the manuscript. White carried out genetic analyses and helped with the metabarcoding data interpretation. Campbell provided assistance with bioinformatics and edited the genetic sections of the manuscript. Mousavi-Derazmahalleh helped with statistical and qualitative analysis of the genetic data and edited the manuscript. Hua helped with the interpretation of radiocarbon data and edited the manuscript. Mazumder (SIA) and Smith (C CSIA) analysed samples and processed the data. Grice reviewed and edited the manuscript.

Name	**Contribution percentage**	**Signature**	**Date**
Mattia Saccò	70%		27/05/2020
Alison J. Blyth	8%		27/05/2020
William F. Humphreys	2%		27/05/2020
Steven J. B. Cooper	1%		27/05/2020
Nicole E. White	5%		27/05/2020

Matthew Campbell	5%		27/05/2020
Mahsa Mousavi-Derazmahalleh	3%		27/05/2020
Quan Hua	3%		27/05/2020
Debashish Mazumder	1%		27/05/2020
Colin Smith	1%		27/05/2020
Kliti Grice	1%		27/05/2020