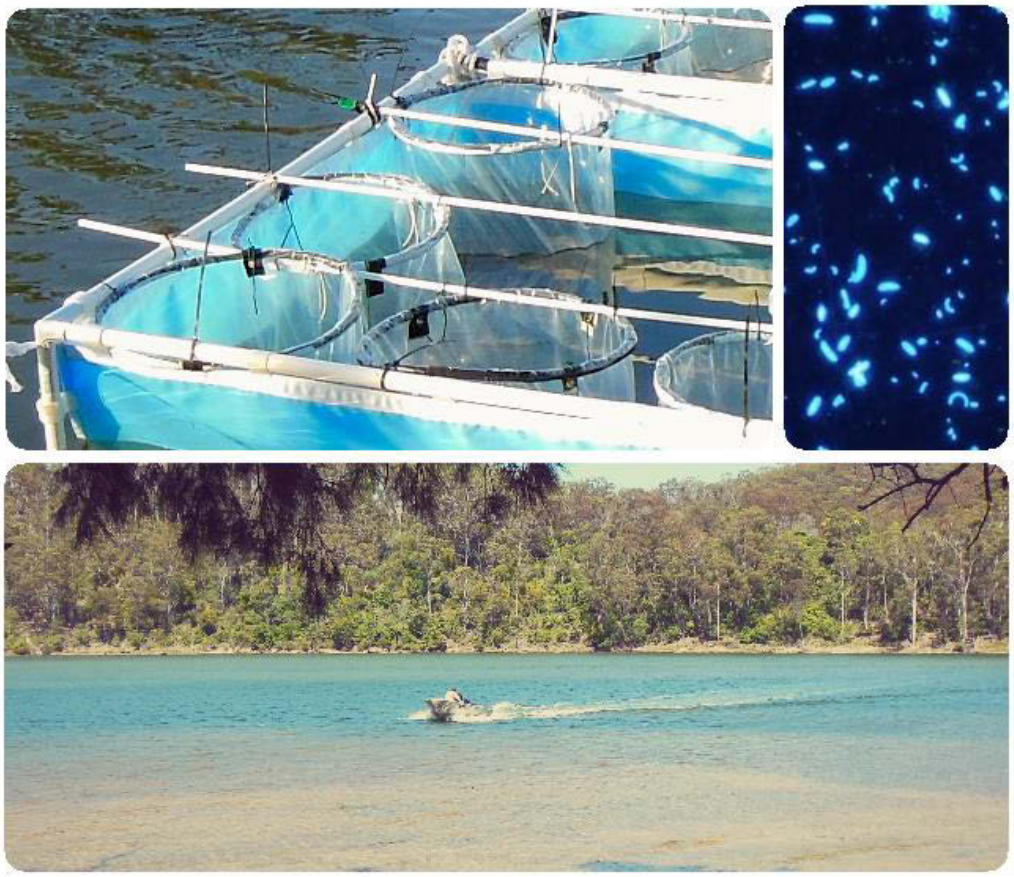
Freshwater inflows to estuaries: Organic carbon and microbial food webs in south-east Australia



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A thesis in fulfilment of the requirements
for the degree of Doctor of Philosophy

March 2015

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Certificate of original authorship

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text. I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signature Date

Acknowledgements

I have had an amazing time completing this research. I am immensely appreciative for having the privilege to devote a period of my life to this intellectual pursuit.

I am deeply grateful for the guidance and support from my supervisor Dr Simon Mitrovic. Since I started working with Simon during my honours he has provided a great combination of intellectual rigor, technical expertise and academic freedom. Whether sitting in a boat in the rain, meeting over coffee, on long drives to the south coast or the sampling beers after field work, Simon made this work not only possible, but incredibly enjoyable.

I would like to acknowledge the support provided through the Peter Cullen Scholarship funded by NSW Office of Water, Sydney Water, State Water, Sydney Catchment Authority and Hunter Water. Without the financial support of this scholarship much of this research would not have been possible. I would like thank scholarship coordinator Simon Williams for his support and advice throughout my candidature. I would also like to thank the Wentworth Group of Concerned Scientists and other Cullen scholars for their support, guidance and luncheons. I acknowledge the financial support received via the Australian Postgraduate Award. I also acknowledge the financial support received from the Association for the Sciences of Limnology and Oceanography and Australian Society of Limnology in helping disseminate my findings.

Much thanks goes to the staff from the NSW Office of Water. Particular thanks goes to Dr John Brayan, Adam, Ellie, Yunis, John and the other staff of the Wolli Creek laboratory for assistance with sample analysis. Many thanks to Ivor Growns for advice and guidance. Thanks to Dave Ryan for field assistance and advice. Thanks to Doug Westhorpe for the advice and the pina coladas. Thanks to Tsuyoshi Kobayashi for advice on bacteria and zooplankton. Thanks to Dan Roelke for helping our mesocosms not disappear to the depths of the estuary.

Special thanks must go Ann-Marie Rohlfs. Ann-Marie rode shotgun in the boat and car for most of the last year four years, even when we weren't quite sure how to operate either. Sharing our time between the ice of the snowy mountains and sand of the south coast has been one of the best experiences of this PhD.

Many thanks to Richard Lim for advice, support, and being great company in Taiwan. Thanks to my office buddies Steff and Bec for their support and good times. Thanks to the staff at UTS including Peter, Gemma, Sue, Jane, Rod and Jason. Thank you Darren Baldwin, Joel Hoffman and other anonymous reviewers for their helpful comments.

I would like to extend a massive thank you to all my friends that have helped out. Huge thanks to Carla Thomas for help in the field and for all the microscope hours. Big thanks to Hannah Walters for her help on the microscope, boat and salad rolls. Thanks to Patch Sinclair for the plankton pictures. Thanks to Jordan Iles for his help during field work. Thanks to Dayna Williams, Alice Blackwood, Chris Moore and Emma Kefford for their help. Much thanks to my number one champs Tessa Rex and Jess Minshall.

Thanks to my family who have always been supportive in my endeavours doing "…something about the water". Finally the biggest thanks and love to my best buddies Beck and Charlie. I could totally have done this without you, but what would have been the point?

Preface

This thesis consists of seven chapters. Chapters two to six are self-contained and written as journal articles and have either been published, are under revision or soon to be submitted. I have presented them here similar to their published or submitted form and as a result some repetition occurs. These papers are presented in a logical theoretical order. Where these papers cross reference each other I have included chapter numbers. To prevent unnecessary duplication, a single reference list is provided at the end of the thesis. In addition because Chapters two to six are co-authored papers there is a shift from singular, I, to the plural we, within the text.

This thesis is a compilation of my own work with guidance from my supervisor and others. I conceptualized my research, conducted all data collection and analysis, and wrote the manuscripts. My supervisors and co-authors proof-read and edited the final manuscript versions. Publication details and Contributions of co-authors are detailed below:

Chapter 2: Hitchcock, J. N and Mitrovic, S. M. 2014. Highs and Lows: the effect of differently sized freshwater inflows on estuarine carbon, nitrogen, phosphorus, bacteria and chlorophyll *a* dynamics. *Estuarine, Coastal and Shelf Science*. In press, doi:10.1016/j.ecss.2014.12.002

S. M. Mitrovic gave conceptual advice, guidance and field assistance.

Chapter 3: Hitchcock J. N. and Mitrovic, S. M. 2015. After the flood: Changing dissolved organic carbon bioavailability and bacterial growth following inflows to estuaries. *Biogeochemistry*. In press, doi:10.1007/s10533-015-0094-3

S. M. Mitrovic gave conceptual advice, guidance and field assistance.

Chapter 4: Hitchcock, J.N. & Mitrovic, S.M. 2013, 'Different resource limitation by carbon, nitrogen and phosphorus between base flow and high flow conditions for estuarine bacteria and phytoplankton', *Estuarine, Coastal and Shelf Science*, vol. 135, pp. 106-15.

S. M. Mitrovic gave conceptual advice, guidance and field assistance.

Chapter 5: Hitchcock, J. N., Mitrovic, S. M., Hadwen, W. L., Growns, I. O. and Rohlfs, A. 2014. Zooplankton responses to freshwater inflows and organic matter pulses in a wave-dominated estuary. *Marine and Freshwater Research*. In prep.

S. M. Mitrovic gave conceptual advice, guidance and field assistance.

W. L. Hadwen provided conceptual advice, guidance and sample analysis relating to stable isotopes.

I. O. Growns provided conceptual advice and guidance.

A. Rohlfs provided field assistance.

Chapter 6: Hitchcock, J. N., Mitrovic, S. M., Hadwen, W. L., Roelke, D. L, Growns, I. O. and Rohlfs, A. 2014. Chapter 6 Terrestrial dissolved organic carbon subsidises estuarine zooplankton: an *in-situ* mesocosm study. *Limnology and Oceanography*, In prep.

S. M. Mitrovic gave conceptual advice, guidance and field assistance.

W. L. Hadwen provided conceptual advice, guidance and sample analysis relating to stable isotopes.

D. L. Roelke provided conceptual advice, guidance and field assistance.

I. O. Growns provided conceptual advice and guidance.

A. Rohlfs provided field assistance.

Other papers published during my candidature but not forming part of this thesis:

Mitrovic, S.M., Westhorpe, D.P., Kobayashi, T., Baldwin, D.S., Ryan, D., Hitchcock, J.N., 2014. Short term changes in zooplankton density and community structure in response to different sources of dissolved organic carbon in an unconstrained lowland river: evidence for food web support. Journal of Plankton Research, vol. 36, pp 1488-1500

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List of Abbreviations

BB	Bacterial biomass
BDOC	Bioavailable dissolved organic carbon
BGE	Bacterial growth efficiency
BGR	Bacterial growth rate
C	Carbon
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
DIN	Dissolved inorganic nitrogen
DIP	Dissolved inorganic phosphorus
DON	Dissolved organic nitrogen
DNP	Dissolved non-reactive phosphorus
FWI	Freshwater inflows
LTB%	Long-term percent carbon bioavailability
LTC	Long-term bioavailable dissolved organic carbon
N	Nitrogen
P	Phosphorus
POC	Particulate organic carbon
Q	Mean daily discharge
$Q10$	Mean 10 day antecedent discharge
STB%	Short-term percent carbon bioavailability
STC	Short term bioavailable dissolved organic carbon
SUVA	Specific ultra-violet light absorption
TN	Total nitrogen
TP	Total phosphorus
WSP	Water sharing plan

Abstract

Freshwater inflows (FWI) play a crucial role in maintaining estuarine processes and productivity. River regulation and extraction have greatly reduced FWI to estuaries. Little attention has been paid to the role FWI has in delivering organic carbon to estuaries. The aim of this thesis was to define the relationship between freshwater inflows, organic carbon, bacteria and zooplankton dynamics. To do this, I performed a series of monitoring and experimental studies on the Bega and Clyde River estuaries, Australia.

Discharge on both rivers was highly episodic during the study. On the Bega and Clyde Rivers, increasing dissolved organic carbon (DOC) concentrations were closely coupled with increasing discharge. The bioavailability of DOC increased during FWI events, and in turn bacterial growth rates were also higher during and immediately following inflow events. Bacterial growth was carbon limited most of the time, though during high flows, bacteria often became phosphorus limited. Changing availability of DOC and phosphorus during inflow events was the main reason for shifting resource limitation. On both rivers bacterial biomass was positively related to increasing DOC and phosphorus concentrations. Highly episodic discharge during this study had a major structuring role over carbon and bacteria dynamics.

On the Bega River I found strong evidence that allochthonous carbon and bacteria can subsidise zooplankton production following the input of DOC during FWI events. Zooplankton density increased following a flooding event on the Bega River and stable isotope analysis indicated allochthonous terrestrial carbon was the dominant source of carbon utilised by zooplankton. Experimental mesocosms confirmed that allochthonous carbon and bacteria can support increased zooplankton in the presence of high subsidies.

The individual studies forming this thesis all contribute new insights to their respective sub-disciplines within aquatic ecology. Viewed together, they present a novel conceptualisation of hydrology and freshwater inflows in the coastal carbon cycle and microbial food webs in south-east Australian estuaries. The results provide a strong case to protect freshwater inflows to estuaries.

Chapter 1 Introduction

1.1 Scope and need for this study

Estuaries are highly dynamic and productive ecosystems. They are sites of great ecological importance and have high social, economic and cultural value. In a broad sense, the ecological character of estuaries is a reflection of their flow regime and the interplay between fresh and saline water (Arthington, 2012). Freshwater inflows (FWI) play a key role in regulating and maintaining estuarine processes and functions (Gillanders and Kingsford, 2002). Much of the ecological variability of estuaries occurs through variability in FWI.

In Australia, as globally, river regulation and extraction has greatly reduced FWI to many estuaries (Alber, 2002; Gillanders and Kingsford, 2002). These changes have caused significant harm to many estuaries (Estevez, 2002). Reduced inflows can affect a range of estuarine processes including salinity regimes, habitat connectivity and dispersion, mixing, and the delivery of materials from the catchment. Demand for freshwater resources in coastal catchments is high and growing. Further, climate change is leading to changes in precipitation and flow patterns in many catchments (Murphy and Timbal, 2008). There is a strong need to develop a greater understanding of the effects of reduced inflows and develop appropriate management strategies to minimise ecological impacts.

Environmental flows are increasingly being implemented to mitigate the impacts of alterations to natural flow regimes. The Australian government's *National Water Initiative* was agreed to in 2004 and includes requirements for states and territories to consider the impact of flow regulation *"...on the environment downstream (including the estuary)"* (NWC, 2004). In NSW, water reform agreements resulted in the Water Management Act 2000 which recognised "*the fundamental health of our rivers and groundwater systems and associated wetlands, floodplains and estuaries has to be protected*" (NOW, 2010b). The main management tool used in NSW are Water Sharing Plans (WSP), that are used to define how much and when water can be extracted and stored in a catchment. Provisions for environmental flows vary between catchments but include rules such as extraction limits, end-of-system flows, dam

releases, low and high flow pump restrictions or thresh-holds and environmental allowances and releases (NOW, 2010a). These rules are based on a set of guiding principles, the '*River Flow Objectives*', which include goals of protecting natural low flows, protecting or restoring medium and high flows, maintaining natural flow variability and maintaining or rehabilitating estuarine processes and habitats.

A major impediment to setting effective environmental flow rules are knowledge gaps regarding what facets of the hydrological regime are most important to maintain and restore estuarine processes. Scheltinga et al. (2006) assessed information needs required for environmental flows to estuaries, outlining high priority areas where research is most needed. These include: what flow regime conditions are needed to maintain estuarine health; developing a conceptual understanding of the major ecological processes and linkages in rivers and estuaries; how estuarine productivity is changed by flows; and understanding the relationship between flow and biogeochemistry in estuaries. At the centre of these priorities is the role that FWI play in the delivery of organic carbon, and its subsequent ecological effects on estuaries. This process is particularly important because, as Pierson et al. (2002) wrote in in their report on the flow requirements of estuaries *"...the input of this material "drives" estuarine food webs and is responsible for the high productivity of estuaries*".

Elucidating the role of FWI and organic carbon in estuaries is more than a management question. Organic carbon is currently at the centre of a number of debates at the forefront aquatic sciences relating to cross system subsidies, ecosystem metabolism, the microbial role in the carbon cycle and the energetic sources supporting higher trophic organisms. These debates, their importance and research gaps are outlined in the following sections.

1.2 Freshwater inflows and allochthonous organic carbon inputs to estuaries

The organic carbon pool in estuaries can be conceptualised as autochthonous, coming from primary production within the estuary, or allochthonous, coming from outside the estuary. Allochthonous organic carbon is primarily derived from soils and plant material (Thurman, 1985). FWI are the main source of allochthonous organic carbon

in estuaries; other minor sources include surface flow from adjacent land and precipitation (Aitkenhead-Peterson et al., 2003; Thurman, 1985).

The majority of allochthonous organic carbon enters rivers, and in turn estuaries, during episodic freshwater inflow events such as floods (Hinton et al., 1997; Raymond and Saiers, 2010; Webster et al., 1987). During these events, rainfall travels across catchment surfaces and through soil horizons, entering rivers and is then transported downstream. Organic carbon concentrations can increase significantly during high inflows (Aitkenhead-Peterson et al., 2003; Hinton et al., 1997). At the catchment scale, there are a variety of factors that influence carbon dynamics including; precipitation, land use, temperature, water regulation and dams, soil and vegetation types and geomorphology (Findlay and Sinsabaugh, 2003). When riverine carbon enters the estuary there are another set of influencing factors including; biological transformation, production of autochthonous carbon, retention time, mixing and dilution, flocculation, adsorbtion and aggregation (Day et al., 2013).

The biogeochemical forces that influence organic matter and energy transfer are at the core of the major theoretical frameworks for rivers and catchments. The '*River Continuum Concept'* (Vannote et al., 1980) emphasizes inputs from the upper catchment and riparian zone. The '*Flood Pulse Concept'* (Junk et al., 1989) highlights the importance of floodplain connectivity in organic matter loading. The '*Riverine Productivity model'* (Thorp and Delong, 1994) underlines the importance of in channel production from primary producers. Junk and Wantzen's (2004) updated 'Flood Pulse Concept' provides the most comprehensive framework, integrating all three theories to describe the delivery of organic matter to estuaries. It suggests that in the upper catchment, the majority of organic carbon entering the system is derived from terrestrial sources. At the middle reaches of a system, primary production becomes more important. At the lower floodplain zone, biological activity on floodplains can become a major contributor. Inherently linked to these theories is the role of hydrological events in facilitating cross-system subsidies between the terrestrial and aquatic environments.

Variability in hydrology both temporally within catchments and between catchments is often high. This is particularly true in Australia, where hydrological variability is much greater than other global regions (Finlayson and McMahon, 1988). Climate

change is predicted to lead to greater variability in precipitation and increased incidents of extreme weather events in the region (Murphy and Timbal, 2008). This makes it hard to generalize about patterns of FWI and associated carbon inputs. There is a strong need to develop a conceptual understanding of how FWI structure organic carbon dynamics in estuaries. This is of particular importance in systems that are regulated and the natural flow regime is interrupted (Poff et al., 1997).

The majority of the total organic carbon pool is comprised of dissolved organic carbon (DOC), the fraction of organic carbon <0.45 μm. The DOC pool is comprised of a complex variety of compounds, often bound to other nutrients such as nitrogen and phosphorus. Around 50% of the DOC entering aquatic systems has been suggested to comprise of humic substances; high-molecular-weight (HMW) compounds formed by secondary synthesis (Aitkenhead-Peterson et al., 2003). Around 30% of the DOC pool is estimated to be in the form of macromolecular hydrophilic acids, and around 20% are low-molecular-weight (LMW) organics such as carbohydrates, carboxylic acids and amino acids. The chemical composition of the DOC pool is highly dynamic and may be influenced by a range of factors including land use (Asmala et al., 2013), presence of wetlands (Fellman et al., 2008), pollution (Wiegner and Seitzinger, 2004), microbial and photochemical transformation (Moran et al., 2000), and hydrological flow paths (Sobczak and Findlay, 2002).

The chemical composition of DOC defines its biological reactivity (Berggren et al., 2010). One of the simplest measures of DOC quality is bioavailability (Guillemette and Del Giorgio, 2011). Bioavailable DOC (BDOC) refers to the proportion of DOC removed by bacteria over a period of time. BDOC determinations are most commonly determined via bioassay incubations where changes in DOC concentration are measured over time, usually 4 weeks, and have been widely studied in aquatic environments (see del Giorgio and Davis, 2003 and references therein). Estimates of BDOC in estuaries vary from 2% to >25% of the total DOC pool (Cole et al., 2011; Hanisch et al., 2010; Sanderson et al., 2009).

Allochthonous DOC entering estuaries via rivers has historically been considered refractory due to its age and high concentrations of humic substances; thus largely unimportant biologically (Steinberg, 2003). However, work over the last decades has indicated the important role of allochthonous DOC in biogeochemical cycling and

estuarine metabolism (Howarth et al., 1996; Wikner et al., 1999). Changes in hydrological flow paths during FWI events lead to changes in the chemical composition of the DOC pool (Aitkenhead-Peterson et al., 2003). During base flow conditions, water typically travels through deeper mineral dominated soils that are low in DOC. During storm events, water travels across catchment surfaces and through carbon rich upper soil horizons. In freshwater systems a handful of studies have found conflicting results on how DOC bioavailability varies between high and low flow periods (Fellman et al., 2009; Gremm and Kaplan, 1998; Leff and Meyer, 1991; McLaughlin and Kaplan, 2013; Wiegner et al., 2009). How DOC bioavailability may vary during and after FWI is still largely unknown. To my knowledge no previous studies have examined how the bioavailability of DOC entering estuaries changes during FWI events.

1.3 Bacteria and organic carbon

Heterotrophic bacteria are the main consumers of DOC in aquatic environments (Azam et al., 1983). Bacteria may utilise both DOC and POC as a potential carbon source. In coasts and oceans, bacterial production is most commonly linked to chlorophyll *a* and primary production, due to bacteria being reliant on autotrophic DOC (Gasol and Duarte, 2000). In estuaries however, inputs of allochthonous DOC lead to bacterial production often being uncoupled from primary production (Moran and Covert, 2003). Bacterial production and growth rates have been shown to be consistently higher in estuarine systems compared to freshwater and marine systems (del Giorgio and Cole, 1998; White et al., 1991). Many estuaries are net heterotrophic, that is, respiration is greater than primary production, highlighting the importance of allochthonous carbon in estuarine metabolism (Battin et al., 2008). The sources of DOC utilized by bacteria may vary temporally (Fouilland and Mostajir, 2010). For example, bacterial production may be coupled to discharge following a period of high flows, and linked to primary production during low flow periods (Ochs et al., 2010). Bacterial carbon sources may also vary spatially, with bacteria closely coupled to discharge in the upper freshwater sections of estuaries, and more closely related to chlorophyll *a* and primary production in the lower, more marine parts of estuaries (Hoch and Kirchman, 1993).

Understanding and predicting how bacteria may respond to FWI remains a complex task. There are a range of factors that influence bacterial responses to FWI including: the quantity and quality of DOC substrates, nitrogen and phosphorus concentrations, temperature, salinity, extracellular enzymes, dissolved oxygen, synergistic effects of autotrophic carbon, residence times, grazing pressure and viral lysis (Bianchi and Argyrou, 1997; Hoffman and Bronk, 2006; Pollard and Ducklow, 2011).

One key way to conceive the relationship between bacteria (and phytoplankton) and carbon and nutrients is by testing what resources are limiting growth (Costa et al., 2009; Elser et al., 1995). This is commonly performed using carbon and nutrient addition bioassays. Bacterial production is most commonly limited by carbon (Findlay et al., 1992; Hoikkala et al., 2009). When DOC is in excess, nitrogen and phosphorus may become primary limiting factors (Cunha and Almeida, 2009; Pinhassi et al., 2006). Shifts in bacterial limitation from carbon to nitrogen or phosphorus can lead to changes in their relationship with phytoplankton; from bacterial reliance on phytoplankton for autochthonous carbon, to competition with phytoplankton for nitrogen or phosphorus which may potentially limit primary production (Currie and Kalff, 1984; Jansson, 1993; Thingstad et al., 1993). How bacterial resource limitation may change during FWI events in estuaries is largely unknown and needs greater understanding.

1.4 Zooplankton, inflows and the microbial loop

Estuaries support high zooplankton biomass compared to adjacent oceans (Benfield, 2012). Zooplankton have high secondary production and are important prey for higher trophic levels. Fish recruitment and survival is in many cases linked to the availability of zooplankton (Castonguay et al., 2008; Murphy et al., 2012). The most important of these are generally copepods, cladocerans, rotifers and other crustaceans and their nauplii (Benfield, 2012). Some zooplankton may also feed directly on POC or detritus, though it is generally considered a poor resource (Heinle et al., 1977).

The traditional view of estuarine and coastal food webs held that large phytoplankton, such as diatoms and dinoflagellates, were the main source of food consumed by zooplankton, predominantly copepods, which were in turn consumed by small fish (Steele, 1974). Whilst bacteria had long been conceptualised as playing important

roles in decomposition and remineralisation, they were generally not considered an important food resource (Lindeman, 1942; Odum, 1968; Pomeroy, 1974; Waksman, 1934). In 1983 a number of microbial ecologists from around the world came together to formulate a concept they described as the 'microbial loop' (Azam et al., 1983). Their paper formulates an alternative pathway of energy transfer to high trophic levels in food webs. They state bacterial biomass is supported by DOC, including a large portion of that fixed by phytoplankton; bacteria then serve as food for phagotrophic protists such as nanoflagellates and ciliates, which in turn serve as food for zooplankton.

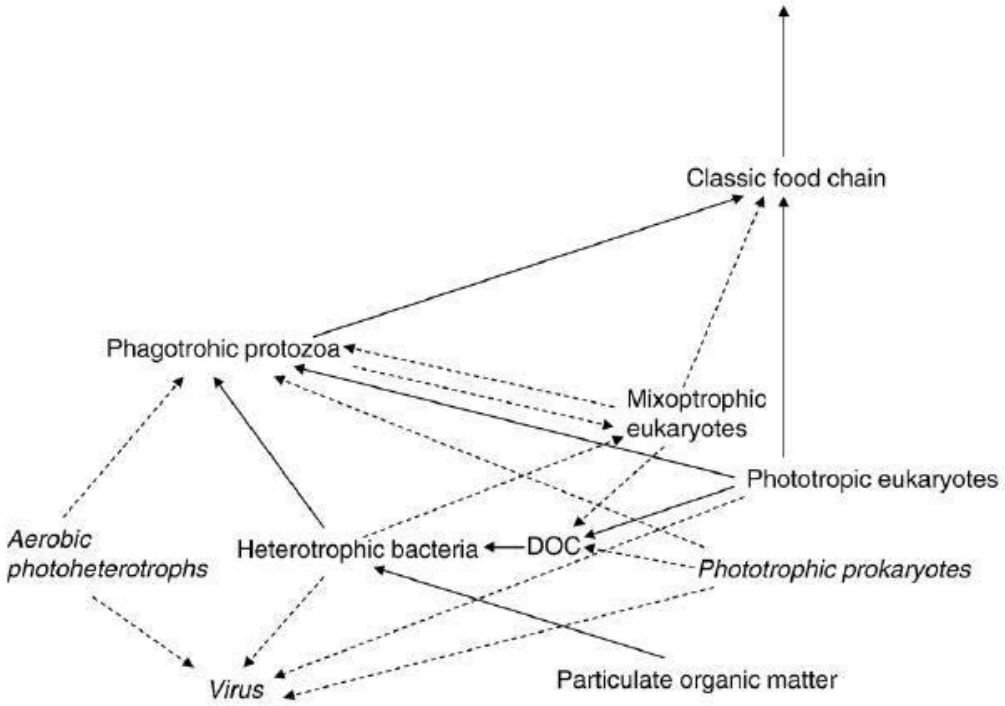

Figure 1.1 The microbial loop. This is an updated version reprinted from Fenchel (2008). Broken lines represent relationships added since the original paper of Azam et al. 1983. Reprinted with permission from Elsevier.

Since the publication of this paper there has been a great deal of debate over whether the microbial loop acts as a 'sink', where organic carbon is primarily lost through respiration, or 'link', where carbon is transferred to higher trophic levels (Ducklow et al., 1986; Sherr et al., 1987). The dominant paradigm is that the microbial loop acts primarily as a sink, as the multiple trophic levels between bacteria and zooplankton results in the majority of organic carbon being lost as respiration (Fenchel, 2008).

There is however, growing evidence that bacteria can provide significant support for zooplankton production in systems that receive large inputs of allochthonous DOC (Faithfull et al., 2012; Mitrovic et al., 2014).

There is a tight coupling between zooplankton and biogeochemical changes (Sugimoto and Tadokoro, 1997). FWI events and other disturbances can alter planktonic communities at fast time scales (Allan and Froneman, 2008; Hoover et al., 2006). There have been relatively few studies investigating zooplankton responses to FWI events and organic carbon loading in estuaries. Those that have been conducted have found evidence both in support and against the hypothesis that inflow derived allochthonous carbon can support zooplankton production (Hoover et al., 2006; Sobczak et al., 2005; Vaque et al., 1992). Studying zooplankton responses to FWI events is difficult due to the complex biogeochemical dynamics present in estuaries during these times, such as changes in salinity and advective forcing. (Kobayashi et al., 1998; Primo et al., 2009).

Studies utilising stable isotopes are being increasingly used to trace the sources of organic matter supporting zooplankton. The isotopic ratio of consumers generally resembles that of their diet. Sources of organic carbon such as terrestrial plants, soils and phytoplankton often have unique $\delta^{13}C$ signatures (ratio of ^{13}C:^{12}C). Analysis of $\delta^{13}C$ in consumers provides a strong indication of organic matter in their diet, whilst analysis of $\delta^{15}N$ (ratio of ^{15}N:^{14}N) can be used to identify the trophic status of an organism as trophic fractionation of $\delta^{15}N$ occurs between consumer and diet (Mill et al., 2007). Stable isotope studies have continued to fuel debate around the relative importance of autochthonous versus allochthonous carbon supporting zooplankton (Karlsson et al., 2014). However, there is growing evidence that after FWI events, or during years with high rainfall, zooplankton, may receive increased support from allochthonous DOC sources (Atwood et al., 2011; Hoffman et al., 2008). Understanding how the sources of carbon supporting zooplankton change following inflows, and if the microbial loop can as a link for energy to higher trophic levels, remain frontiers in aquatic ecology.

1.5 Study sites

The main focus for this study is the Bega River Estuary (Fig. 1.2). This site was selected as it had been identified by the NSW Office of Water (the state government body responsible for implementing water sharing plans) as a priority for research. This was based on communities which had a high economic dependence on the estuaries that were most at risk to adverse effects of over extraction. The Bega River Estuary (-36° 42' 43.64", +149° 54' 8.76") is a shallow wave-dominated estuary (Roy et al., 2001). The estuarine volume, 6371 ML, is relatively small compared to its catchment of 1941km^2. The catchment is split into two sub-catchments, the Brogo River, regulated by Brogo Dam and the Bega River which has two smaller dams, Cochrane and Candello. The upper sections of the catchment consist of mainly forested areas, whilst the middle catchment is mostly cleared for agriculture, dominated by the dairy industry (Brierley et al., 1999; Tozer et al., 2010). The freshwater tidal section has been mostly cleared for agriculture and irrigated farmland, however the rest of the estuary is largely unmodified. Broad scale land clearing since colonisation has drastically altered the river and estuary via the transportation of sandy top soils into the river channel (Brooks and Brierley, 1997).

The Bega estuary is highly valued for its recreational fishing, cultural significance and contribution to tourism. The Bega River catchment water sharing plan (WSP) commenced in 2011. The only environmental flow rules specific to the estuary are cease to pump rules, where pumping from the tidal pool must stop when no inflow is present. Rules are present in other sections of the catchment, the most relevant to the estuary is a 'first flush' rule in the riverine section below Brogo dam, where after a 30 day period of low flow, the first 24 hours of a flow event must not be extracted. Because the estuary is at the very end of the river, it is influenced by the sum of all water extraction throughout the catchment.

The Clyde River Estuary NSW, Australia, (-35° 33' 11.44", +150° 11' 12.11") was the second location selected for this study, and features in chapters 2-4 (Fig 1.2). The Clyde River estuary, is a tidally dominated estuary. Its estuary volume, 50737 ML, is relatively large compared to its catchment size of 1791 km^2 (Roy et al., 2001). The river is unregulated, with no significant structures or water extraction. The catchment is almost entirely forested, with some forestry activities in the upper catchment. The

lower sections of the estuary have a small urban settlement as well as oyster and other fisheries activities (Tozer et al., 2010). It was selected as a study site in chapters 2-4 of this study, which focus on inflows and organic carbon dynamics, to provide both a contrast between catchments with unmodified flow regimes and low anthropogenic nutrient pollution, as well as a broader perspective of estuaries in the region. The Clyde River catchment has a WSP due to commence in 2014. As there is little extraction and no structures on the river, it essentially has a natural flow regime and no specific environmental flow rules.

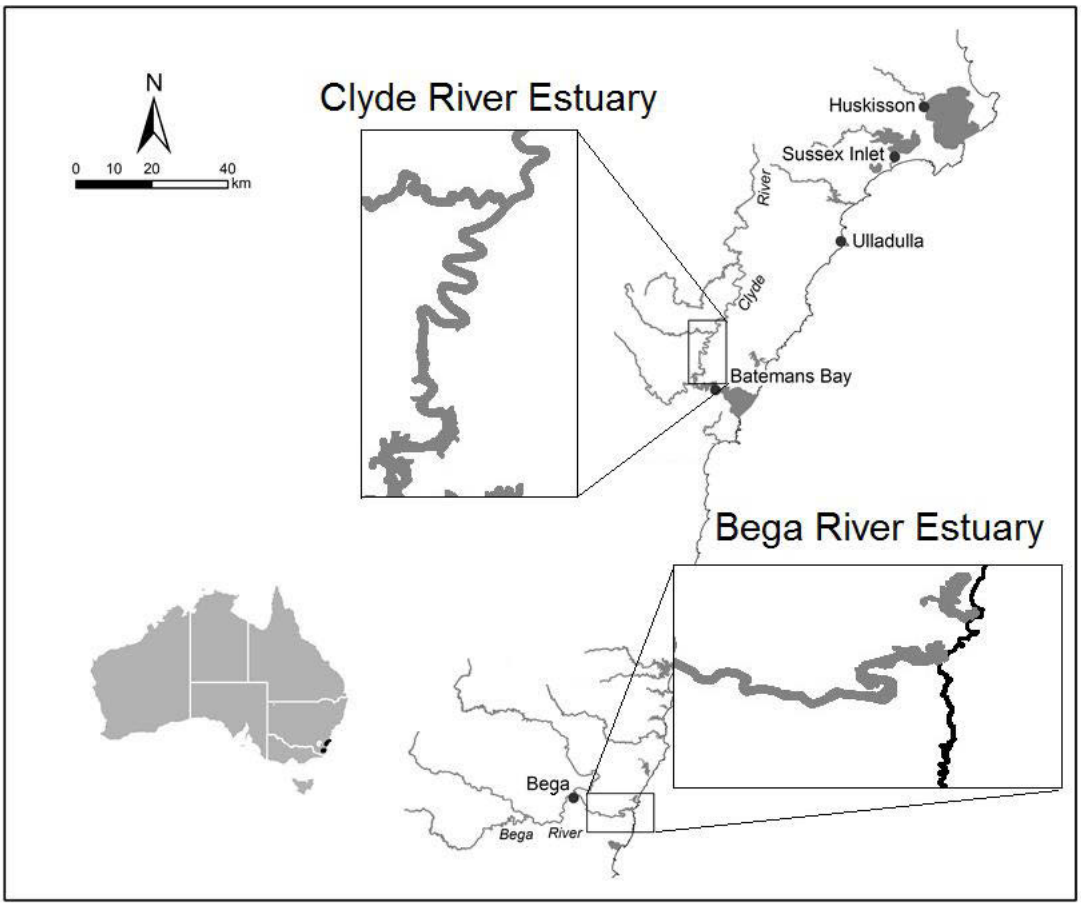

Figure 1.2 Bega and Clyde Rivers. Boxes represent the estuary and main study areas. Different stations were examined in each chapter; locations of these sites are listed in each chapter respectively.

1.6 Aims and overview

The broad aim of this thesis was to define the role that freshwater inflows have over organic carbon, bacteria and zooplankton dynamics. In each chapter of this thesis I address key scientific questions within sub-disciplines relating to organic carbon and microbial food webs. I approach each question with the novel conceptual framework that freshwater inflows plays an important structuring force in the carbon and food web dynamics of each estuary. The research approach adopted here utilised monitoring studies which then informed manipulative experiments (Fig 1.3). As a whole this thesis is novel, not only in its framing of hydrology, but in integrating what are generally sub-disciplines into a coherent research agenda.

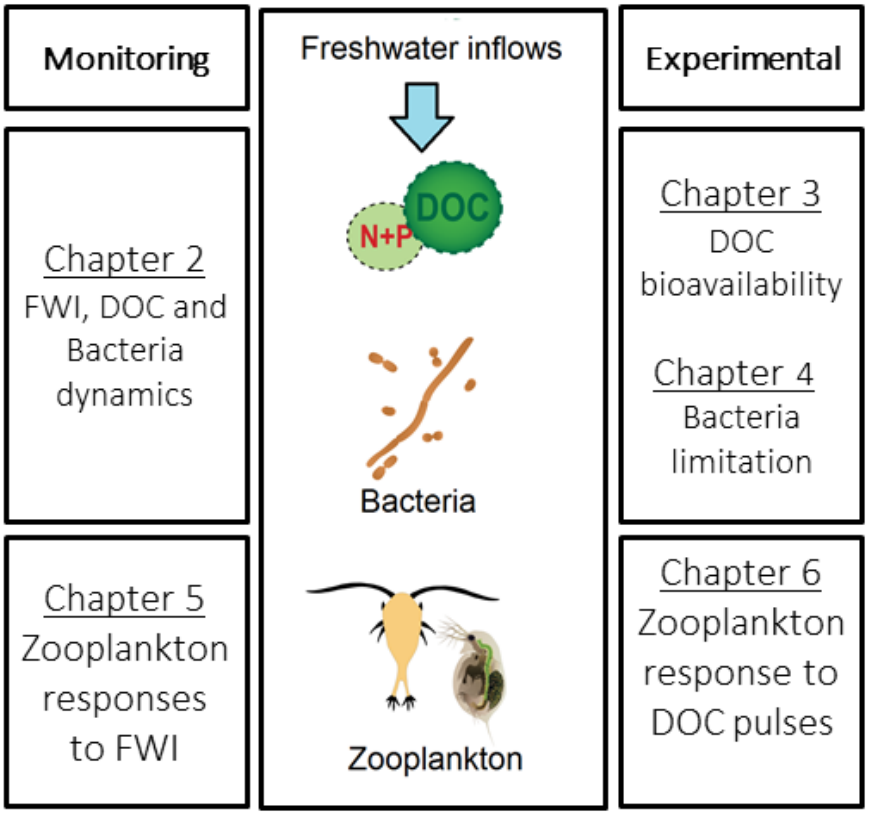

Fig 1.3 Conceptual approach to research. Chapters 2 and 5 used a monitoring approach, which helped inform the *in-situ* and *in-vitro* experiments of chapters 3, 4 and 6. Chapters 2 through 4 focus on DOC and bacteria, whilst chapters 5 and 6 focus on zooplankton.

A core part of this thesis is to provide feedback and information on the FWI requirements needed to maintain and restore estuarine functions and productivity in the Bega River. The Bega River WSP is reviewed every 5 to 10 years and rules adjusted through an adaptive management processes. More broadly the knowledge gained through this work will also be useful in determining flow requirements and research priorities in other NSW estuaries.

Chapters 2-4 of this thesis focus on both the Bega and Clyde River estuaries. Due to time and labour constraints, chapters 5 and 6 focus solely on the Bega River, which is a high priority for the state government.

Chapter 2: In this chapter I investigate the influence that freshwater inflows have on organic carbon, nutrient, bacteria and chlorophyll *a* dynamics in the Bega and Clyde estuaries. This was done through a 30 month monitoring study. This study is novel in that I conceptualise freshwater inflow conditions as large 'floods', medium sized 'freshes', and low 'base' flows. The specific aims were to:

- Define the factors regulating organic carbon, nitrogen and phosphorus concentrations in each estuary
- Define the factors structuring bacterial biomass and chlorophyll *a* dynamics
- Describe how these factors differ between flood, fresh and base flow conditions

Chapter 3: Building on the work from chapter 2 that linked DOC concentrations to freshwater inflows, in chapter 3 I aim to understand how DOC bioavailability changes during freshwater inflow events. I did this by performing a series of *in-vitro* bioassay experiments. This is the first study that I am aware of that has attempted to do this in estuaries. The specific aims were to:

- Understand how the bioavailability of DOC changes during and following freshwater inflows
- Describe changes in bacterial growth rates during and following freshwater inflows

Chapter 4: In chapter 4, I follow up on the results of chapter 3 that found bioavailable DOC is high following freshwater inflow, but bacterial growth rates appear to be limited by nutrients. The aim of this chapter is to understand the resources that limit bacterial and primary production in estuaries and how this varies with FWI. I did this by performing a series of *in-situ* bioassay experiments on the Bega and Clyde River estuaries. This is the first study that I am aware of that has tested how bacterial resource limitation changes in estuaries between high and low inflow periods. The specific aims were to:

- Define what resources limit bacterial growth during high and low freshwater inflows?
- Define what resources limit phytoplankton growth during high and low freshwater inflows?
- Describe how the relationship between bacteria and phytoplankton change between high and low freshwater inflows?

Chapter 5: In this chapter I build upon the knowledge gained from chapters 2-4 that freshwater inflows result in pulses of organic carbon and increased bacterial production. The aim in chapter 5 is to understand how zooplankton respond to inflows on the Bega River. I did this by monitoring zooplankton responses during a short but intensive period of FWI events. The specific aims were to:

- Describe how zooplankton assemblage structure changes during freshwater inflow events
- Understand if increased organic carbon and bacterial biomass results in increased zooplankton density
- Define the relative importance of allochthonous versus autochthonous carbon sources to zooplankton and how they change during freshwater inflows

Chapter 6: In this chapter I build on the result of chapter 5 that found zooplankton density may increase after inflows, and that zooplankton are supported by allochthonous carbon. The aim of this chapter was to test experimentally how zooplankton respond to DOC additions. I performed an *in-situ* mesocosm experiment on the Bega River, simulating DOC pulses of different sizes. This study is novel in its

attempt to mimic both the quality and gradient of DOC concentrations that are received to estuaries during inflows. The specific aims were to:

- Understand if DOC and bacteria can support zooplankton production
- Define how zooplankton density and support from DOC change under different concentrations

Chapter 7: In this chapter, the findings of chapters 2 to 6 are discussed and overall conclusions are drawn. Recommendations for management of environmental flows to the Bega River and estuaries more broadly as discussed, as well as suggested future research directions.

Chapter 2 Highs and Lows: the effect of differently sized freshwater inflows on estuarine carbon, nitrogen, phosphorus, bacteria and chlorophyll *a* dynamics

2.1 Abstract

Freshwater inflows play a key role in the delivery of organic carbon to estuaries. However, our understanding of the dynamics between discharge and carbon globally is limited. In this study we performed a 30-month monitoring study on the Bega and Clyde River estuaries, Australia, to understand the influence that discharge had on carbon, nitrogen, phosphorus, bacteria and chlorophyll *a* dynamics. We hypothesised that 1) discharge would be the most important factor influencing carbon and nutrient concentrations, though during low flows chlorophyll *a* would also be positively related to carbon, 2) bacteria would be related to dissolved organic carbon (DOC), and chlorophyll *a* to temperature, nitrogen and phosphorus, and 3) that concentrations of carbon, nitrogen, phosphorus, bacterial biomass and chlorophyll *a* would be significantly different between large 'flood flows', smaller 'fresh flows' and base flow conditions. We found that discharge was always the most important factor influencing carbon and nutrient concentrations, and that primary production appeared to have little influence on the variation in DOC concentration even during base flow conditions. We suggest this relationship is likely due to highly episodic discharge that occurred during the study period. Bacteria were related to DOC in the lower estuary sites, but phosphorus in the upper estuary. We suggest this is likely due to the input of bioavailable carbon in the upper estuary leading bacteria to be P limited, which changes downstream to carbon limitation as DOC becomes more refractory. Chlorophyll *a* was positively related to temperature but not nutrients, which we suggest may be due to competition with bacteria for phosphorus in the upper estuary. Carbon, nitrogen and phosphorus concentrations were different under flood, fresh and base flow conditions, though these differences sometimes varied between estuary locations for different resources. Overall, the results demonstrate that discharge plays an important structuring role for carbon, nutrient and bacteria dynamics on the Bega and Clyde Rivers, and that the differences observed between flood and fresh inflows

suggest that further study into the influence of differently sized inflow events is important.

2.2 Introduction

Freshwater inflows play a critical role in defining estuarine functioning. Each year rivers transport, transform and store up to 1.9 pg C y^{-1}, of which around half is transported to estuaries and coasts (Cole et al., 2007). Biogeochemists have a long history of studying organic matter in aquatic ecosystems (Findlay, 2003). Despite this, as suggested by Cauwet (2002), having a precise understanding of the nature of organic carbon delivered by rivers is difficult. Part of this stems from the overwhelming complexity and dynamism of the processes involved. At the catchment scale, there are a variety of factors that influence carbon dynamics including; precipitation, land use, temperature, water regulation and dams, soil and vegetation types and geomorphology (Findlay and Sinsabaugh, 2003). When riverine carbon enters the estuary there are another set of influencing factors including; biological transformation, production of autochthonous carbon (primary production within the estuary), retention time, mixing and dilution, flocculation, adsorbtion and aggregation (Day et al., 2012). Essentially this means that predicting the relationship between discharge and organic carbon in a particular system is extremely difficult without long-term data that captures both temporal and spatial variability.

The majority of allochthonous carbon enters rivers, and in turn estuaries, during episodic flow events (Hinton et al., 1997; Raymond and Saiers, 2010; Webster et al., 1987; Westhorpe and Mitrovic, 2012). A number of studies have conceptualised hydrologic conditions in the rivers as ‘storm flow’ and ‘base flow’ which has been useful in demonstrating that whilst storm flow conditions only occur for short periods of time, they disproportionally account for a large portion of organic carbon exported from the catchment (Buffam et al., 2001; Hinton et al., 1997; Raymond and Saiers, 2010). Puckridge et al. (1998), whilst not specifically talking about carbon dynamics, went further separating storm flow conditions into ‘flood pulses’ and ‘flow pulses’. Flood pulses are large flows of water that inundate floodplains and may variably occur every year or two, whereas flow pulses are smaller flow events where water does not break the banks of a channel and occur more frequently. Few studies that have approached the topic of discharge and carbon in estuaries have utilised these types of

frameworks to describe conditions. Such a framework may prove a useful way of discerning the different ways that discharge influences the movement of carbon in estuaries.

Phytoplankton and bacteria both play keys roles in the carbon, nitrogen and phosphorus cycles (Lin et al., 2006). A great deal of attention has been paid to the influence of autochthonous vs. allochthonous influences on estuarine metabolism (McDowell and Asbury, 1994). It is common that carbon is more related to discharge during wet or high flow periods and related to primary production during dry or low flow periods (Ochs et al., 2010). In many systems where allochthonous inputs are small, primary production is the main source of dissolved organic carbon and in turn bacterial production is closely coupled with phytoplankton biomass or chlorophyll *a* (Cole et al., 1988). In estuaries, phytoplankton are predominantly limited by temperature and the availability of nitrogen and phosphorus (Eppley, 1972). Nutrient limitation may be relieved with freshwater inflows that bring available nitrogen and phosphorus, but conversely if inflows bring high loads of suspended sediment or coloured organic matter then primary production may be light limited (Cloern, 1987).

When allochthonous inputs are high, bacteria may become uncoupled from phytoplankton or chlorophyll *a* (Hoch and Kirchman, 1993; Ning et al., 2005). Bacteria are most regularly limited by DOC (Malone, 1977) but may also be limited by nitrogen and phosphorus and may compete with phytoplankton for nutrients (Thingstad et al., 1998). Bacteria can quickly metabolise available DOC acting as a significant carbon sink, as well as a potential link for organic matter subsidising food webs (Findlay et al., 1992). Estuaries are commonly net heterotrophic, indicating the importance of allochthonous carbon to bacterial metabolism (Fouilland and Mostajir, 2010). Understanding how discharge affects these processes has far reaching ecological implications.

Much of our knowledge about estuarine carbon, nitrogen and phosphorus dynamics derives from North American and European systems (Tank et al., 2010 and references therein). The last decade has seen increasing studies occurring outside of these regions (for example Thottathil et al., 2008; Vargas et al., 2011). In Australia the study of organic carbon in estuaries has been fairly limited (Abrantes and Sheaves, 2010b; Eyre and Twigg, 1997; Ford et al., 2005; Petrone, 2010; Petrone et al., 2011; Petrone et al.,

2009). Thus, there is a need to pay greater attention to Australian catchments, in particular as temperate and sub-tropical systems in Australia exhibit particularly episodic discharge and precipitation (Finlayson and McMahon, 1988). The purpose of our study was to investigate the influence that freshwater inflows have on organic carbon and nutrient dynamics and how these dynamics change between flood, fresh and base flow conditions. Through this we explore 3 particular assertions: 1) carbon, nitrogen and phosphorus concentrations can be explained through a combination of discharge, and for DOC, chlorophyll *a* during base flows; 2) that bacteria are related to DOC concentrations and phytoplankton to temperature as well as nitrogen and phosphorus and 3) that carbon, nutrient and bacteria concentrations are highest during flood flows and chlorophyll *a* concentrations highest during fresh flows. This work was carried out on the Bega and Clyde River estuaries, which were chosen as they are situated in a region of Australia where carbon dynamics have not previously been reported. Our study took place over a 30 month period, encompassing a range of monitoring stations spanning the length of each estuary. Sampling occurred on a monthly basis, with increased frequency during and after high flow events in order to capture the full range of flow variability.

2.3 Methods

2.3.1 Study sites

The Bega River estuary, NSW, Australia, (-36° 42' 43.64", +149° 54' 8.76") is a shallow, wave-dominated estuary (Fig. 2.1). The estuarine volume, 6371 ML, is relatively small compared to its catchment of 1941 km^2 (Roy et al., 2001). The catchment is split into two sub-catchments, the Brogo River, regulated by Brogo Dam and Bega River which has two smaller dams, Cochrane and Candello. The upper sections of the catchment consist of mainly forested areas, whilst the middle catchment is mostly cleared for agriculture, dominated by the dairy industry (Brierley et al., 1999; Tozer et al., 2010). The freshwater tidal section has been mostly cleared for agriculture and irrigated farmland, however the rest of the estuary is largely unmodified. Broad scale land clearing since colonisation has drastically altered the river and estuary via the transportation of sandy top soils into the river channel (Brooks and Brierley, 1997).

Mean annual rainfall is 605 mm (1994-2014) and was above average between 2010-2012 with a mean of 862 mm per year.

The Clyde River estuary, NSW, Australia, (-35° 33' 11.44", +150° 11' 12.11") is a tidally dominated estuary (Fig. 2.1). Its estuary volume, 50737 ML is relatively large compared to its catchment size, 1791 km^2 (Roy et al., 2001). The river is unregulated, with no significant structures or water extraction. The catchment is almost entirely forested, with some forestry activities in the upper catchment. The lower sections of the estuary have a small urban settlement as well as oyster and other fisheries activities (Tozer et al., 2010). Average rainfall is 889 mm (1991-2014) and was above average between 2010-2012 with a mean of 1027 mm.

On each estuary five monitoring stations were sampled encompassing the length of the estuary (Fig. 2.1). Salinity ranged from 0 (at station 1) to 35 (at station 5) on the practical salinity scale.

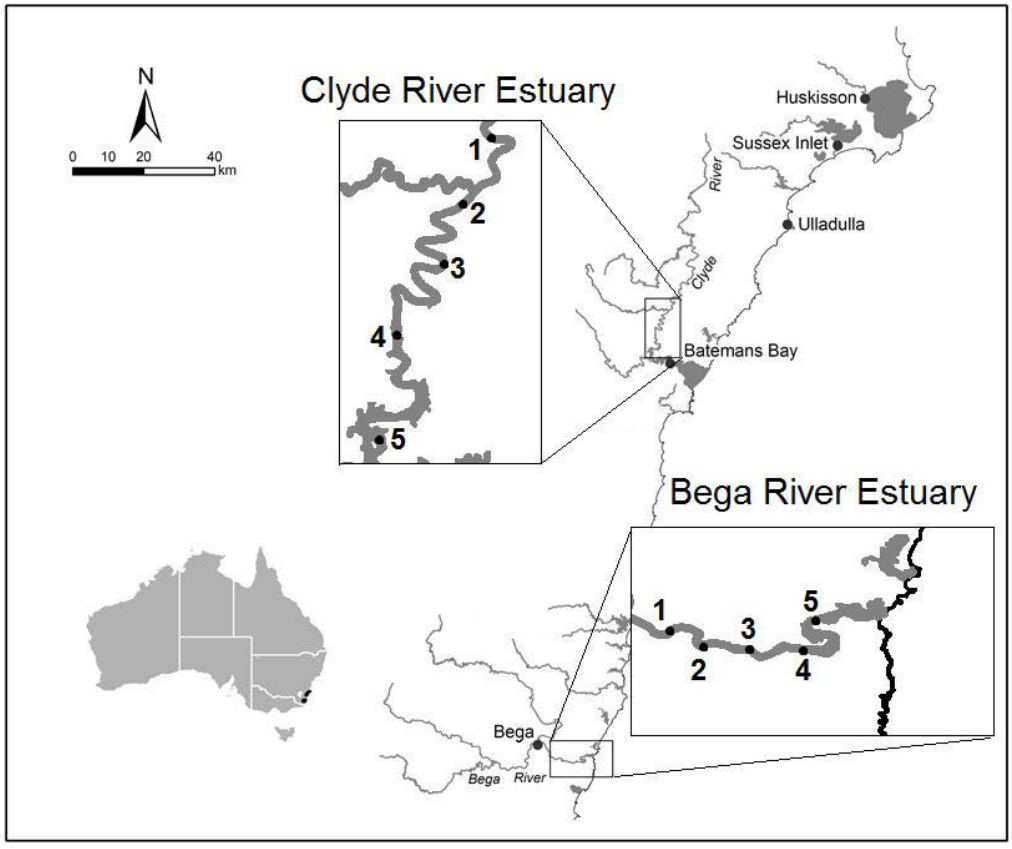

Figure 2.1 Map of the Bega and Clyde River catchments, NSW, Australia. Numbers indicate sampling stations. In each river, station 1 is located in the freshwater tidal zone, whilst station 5 is the most marine.

2.3.2 Discharge and Flow Classification

Discharge information was obtained from one gauging station on the Clyde River and two gauging stations on the Bega and Brogo Rivers, which were combined to give a single value for the Bega River. Discharge (m^3s^{-1}) data presented here was calculated as a daily average flow rate. On the Bega River these gauging stations were located approximately 5 km upstream of the tidal zone and on the Clyde River the gauging station is located approximately 11 km upstream of the tidal zone. There are no tributaries located between the gauging stations and tidal zone. In order to distinguish base flow from storm flow conditions hydrograph separation was performed via WHAT system recursive digital filter method (Lim et al., 2005), with a filter parameter of 0.98 and BFI_{max} of 0.80. Sampling that occurred during storm flow conditions were further conceptualised as either flood flow >150 m^3s^{-1} or fresh flow <150 m^3s^{-1}. This threshold was chosen as it differentiated the flood and fresh flows based on our observations of over the bank flooding in the freshwater tidal reaches of both rivers.

2.3.3 Sampling procedures and analyses

Sampling was performed between June 2010 and November 2012. On the Bega River sampling occurred on five occasions during flood flows, eight occasions during fresh flows and 26 occasions during base flow conditions. The Clyde River was sampled on seven occasions during flood flows, seven occasions during fresh flows and 23 occasions during base flow conditions (Fig. 2). Sampling was performed moving upstream on the ebb tide within 0-2 hours of high tide at the mouth of each estuary. Temperature, conductivity, dissolved oxygen and pH were measured with a Hydrolab Surveyor and MS5 Sonde probe. Turbidity samples were measured using a Hach 2100 turbidimeter.

Triplicate samples for dissolved organic carbon (DOC), particulate organic carbon (POC), total nitrogen (TN), total phosphorus (TP) and chlorophyll *a* were taken by hand 20cm below the water surface. DOC and POC samples were collected in pre-combusted and sample rinsed 100 mL glass bottles, acidified with 2N hydrochloric acid and refrigerated at 4°C. Samples were analysed in the laboratory by the High Temperature Combustion Method (APHA, 2005) with DOC samples being pre-filtered to 0.45µm using PTFE syringe filters. TN and TP samples were collected in

pre-washed and sample rinsed 50 mL PET bottles, frozen and later analysed using a segmented flow analyser (OI Analytical Model FS3100) according to standard methods (APHA, 2005).

Samples for chlorophyll *a* were determined by filtering 500 mL of water onto GF/C filters. Filters were frozen until subsequent determination by Standard Methods (APHA, 2005) using the grinding technique and acetone as a solute with correction for phaeophytin. A detection limit of 1µg L^{-1} was used for chlorophyll *a* analysis. Bacterial biomass samples (10 mL) were collected in sterile centrifuge tubes and fixed with 0.4 mL of concentrated 0.2 µm filtered formalin (37% Formaldehyde) and stored at 4ºC. In the laboratory, subsamples (2 mL) were stained with DAPI (4'6-diamindion-2-phenylindole) at a final concentration of 1 mg mL^{-1} for 15 minutes, and filtered through a polycarbonate black 0.2 µm pore-sized filter (Porter and Feig, 1980). Polycarbonate filters were mounted onto microscope slides and non-fluorescence immersion oil used. Slides were examined at ×100 using a fluorescence-equipped Olympus BX41 compound microscope. For each slide ≥500 total cells were captured using an Olympus DP72 camera and cellSens Standard software (version 1.3). Images were analysed for cell abundance and volume using CellC software (Selinummi et al., 2005). Bacterial biomass was calculated using the formula given by (Romanova and Sazhin, 2010).

2.3.4 Data analysis

Multiple stepwise linear regression analysis was performed using SPSS Ver. 21 as an exploratory tool to understand what variables were important in influencing water quality and biological outputs. All models were validated using bootstrapping (1000 samples, 95% confidence interval) to ensure significant regressions were not the result of chance (significance values <0.05). We created models for DOC, POC, turbidity, TN, TP, bacterial biomass and chlorophyll *a.* Independent variables used in all analysis were discharge, temperature and salinity. In addition, for testing chlorophyll a*,* DOC, POC, TN, TP and turbidity were used. For testing bacteria, DOC, POC, TN, TP and Chlorophyll *a* were also employed. Data was first tested for normality via a Shapiro-Wilk test and non-normally distributed data was transformed (Ln [x+1]), after which all variables passed (P>0.05). Dependent variables were first tested against discharge as either mean daily discharge on the day of sampling (Q1) or mean 10 day

antecedent discharge (*Q*10) to find the best fit value for discharge. *Q*10 is the measure of the average discharge from the preceding 10 days and is used as a way to integrate the effect of previous flow events (e.g. McDowell and Asbury, 1994; Raymond and Saiers, 2010). We first tested 5, 10, 15 and 20 day antecedent discharge against DOC concentrations, *Q*10 was chosen as it most commonly returned the highest R^2 values. For chlorophyll a, values below the detection limit were excluded and chlorophyll *a* was not used for analysis at stations 1 and 2 on the Clyde River due to the high number of data points below detection limits. We also performed additional multiple-regression analysis for DOC using only data recorded during base flow periods to test the assertion that chlorophyll *a* would be related to DOC only during base flow periods.

Differences in DOC, POC, TN, TP, turbidity, bacterial biomass and chlorophyll *a* between flow categories and sampling stations were analysed using permutational multivariate analysis of variance (PERMANOVA) with PRIMER + PERMANOVA software ver. 6 (Anderson, 2001). Homogeneity of variance was tested using PERMDISP (Anderson et al., 2008). Data that was skewed was log transformed. Where unequal dispersion was still present after transformation the chances of a type-I error were reduced by rejecting the null hypotheses at a probability of 0.01. Pair-wise t-tests were employed with PERMANOVA to test which treatments and time periods were significantly different. Monte Carlo asymptotic *P*-values were generated to increase the number of permutations tested during t-tests.

2.4 Results

2.4.1 Organic carbon concentrations

Dissolved organic carbon (DOC) and particulate organic carbon (POC) concentrations varied greatly over time on both the Bega and Clyde Rivers (Fig. 2.2). Organic carbon concentrations were significantly different between flow categories on both rivers (Table 2.1). Concentrations were generally highest during flood flows, followed by fresh and then base flow (Fig. 2.4A,B). The exception was on the Clyde River at the lower estuary stations 4 and 5, where both DOC and POC were similar under fresh and base flow conditions. DOC was the dominant fraction of carbon present under all conditions in the estuary.

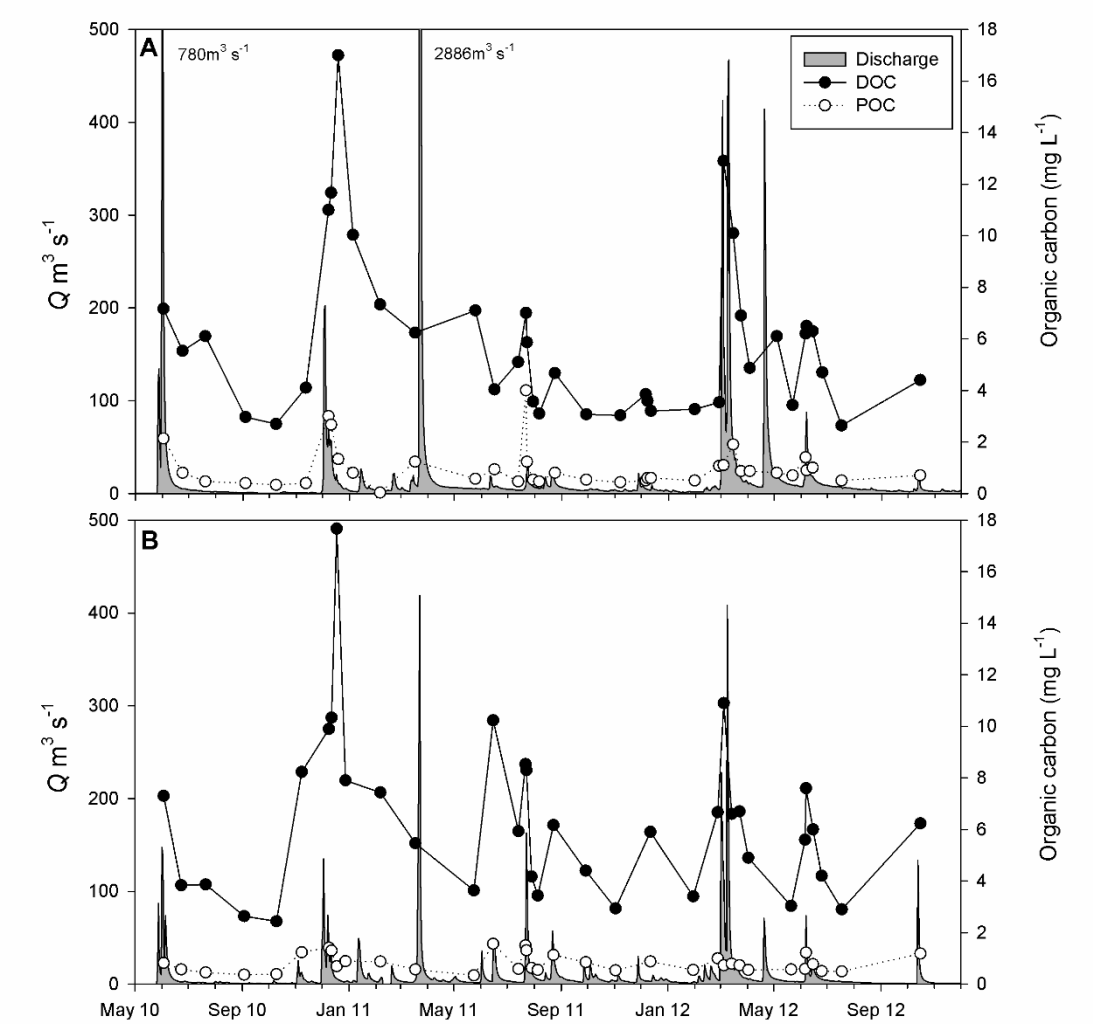

Figure 2.2. Time series of discharge and organic carbon values at sampling station 1 on the A) Bega and B) Clyde Rivers, May 2010 to October 2012. Closed circles indicate dissolved organic carbon and open circles particulate organic carbon. NB: sampling was carried out before but not directly after the largest flood in April 2011.

Multiple-regression analysis showed that discharge was the most important factor of those tested in explaining organic carbon variation (Table 2.2, Fig. 2.5A,B,C,D). *Q*1 (mean daily discharge on the day of sampling) was the most useful factor in explaining POC variance, except at station 5 on the Clyde River where *Q*10 (mean 10 day antecedent discharge) provided a better measure. *Q*1 was also the factor that best described variance in DOC at the upper stations on both the Bega and Clyde Rivers. At the middle and lower stations, *Q*10 provided a better measure for explaining DOC concentrations (Table 2.2). Multiple-regression analysis during only base flow periods showed discharge still explained more variation in carbon concentrations than other factors including chlorophyll *a* (Fig. 2.5C,D).

2.4.2 Nitrogen and Phosphorus concentrations

Both total nitrogen (TN) and total phosphorus (TP) also varied greatly during the study period (Fig. 2.3A,B). TN was significantly different between flow categories (Table 2.1). On the Bega River TN was highest under flood flows followed by fresh and then base flow conditions (Fig. 2.4E). On the Clyde River TN was significantly higher during flood flows compared to fresh flows at stations 2 and 4, but not at stations 1, 3, 5 (Fig. 2.4F). TN was significantly higher during fresh flow compared to base flow at stations 1 and 3. Multiple-regression analysis showed *Q*10 as the best explanatory factor to explain variance in TN on the Clyde River (Table 2.2, Fig. 2.6D). On the Bega River variance in TN was generally better explained by *Q*1 (Fig. 2.6C). Overall nitrogen concentrations were much lower on the Clyde River in comparison to the Bega River.

On the Bega River mean concentrations of TP were not significantly different between flood and fresh flow at the upper stations 1-3, but were significantly higher during flood flow at stations 4 and 5 (Table 2.1, Fig. 2.4G)). Multiple-regression analysis showed *Q1* explained the most variance in TP concentrations (Table 2.2, Fig. 2.6E). TP concentrations were very low on the Clyde River under all flow conditions (Fig. 2.4H). At the lower estuary stations we could find no factor from those tested that explained TP variance (Fig. 2.6F), however at the station 1 and 2 TP was positively related to *Q*1 (Table 2.2).

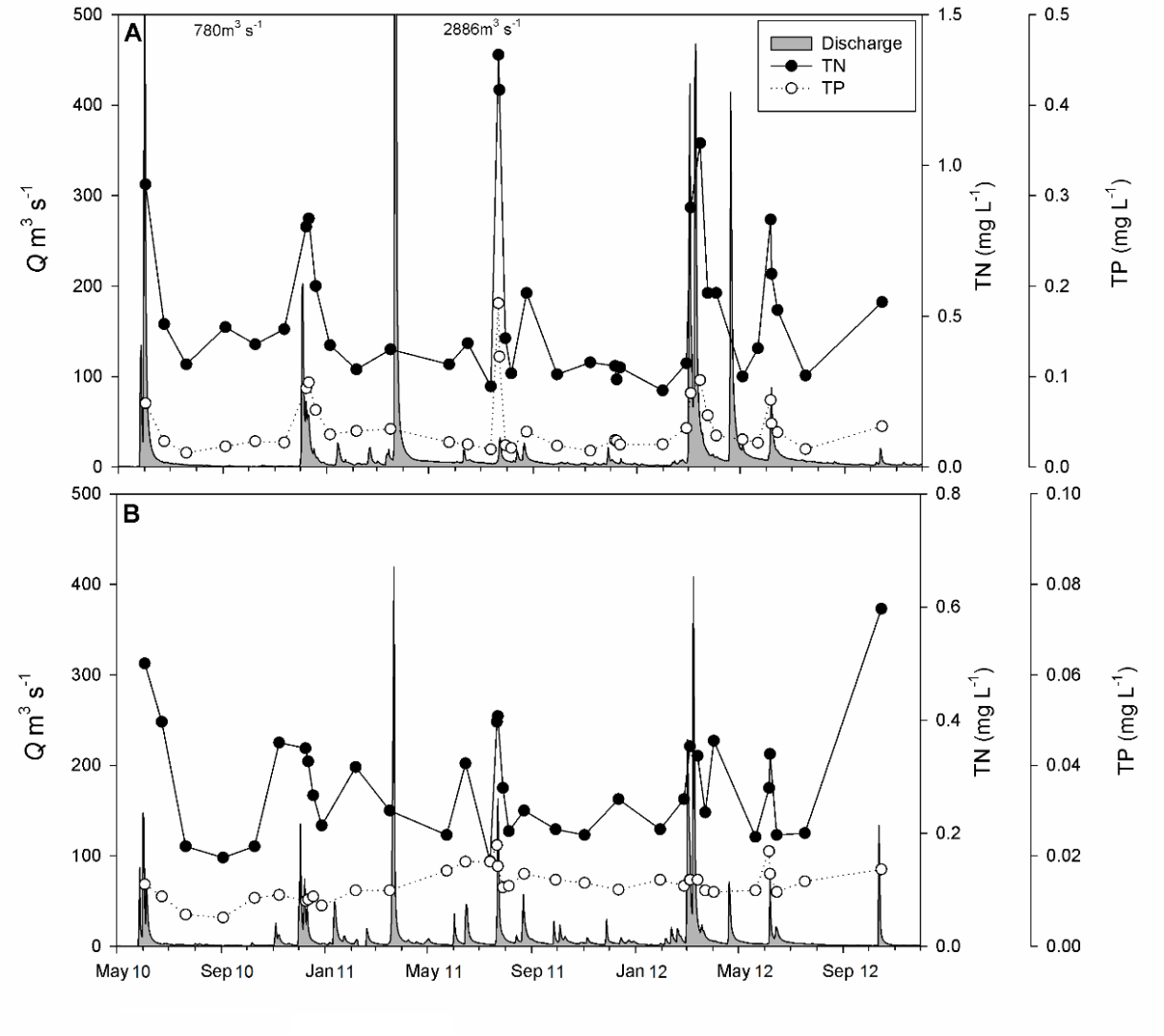

Figure 2.3. Time series of discharge and nutrients at sampling station 1, May 2010 to October 2012. A) TN and TP on the Bega River, B) TN and TP on the Clyde River.

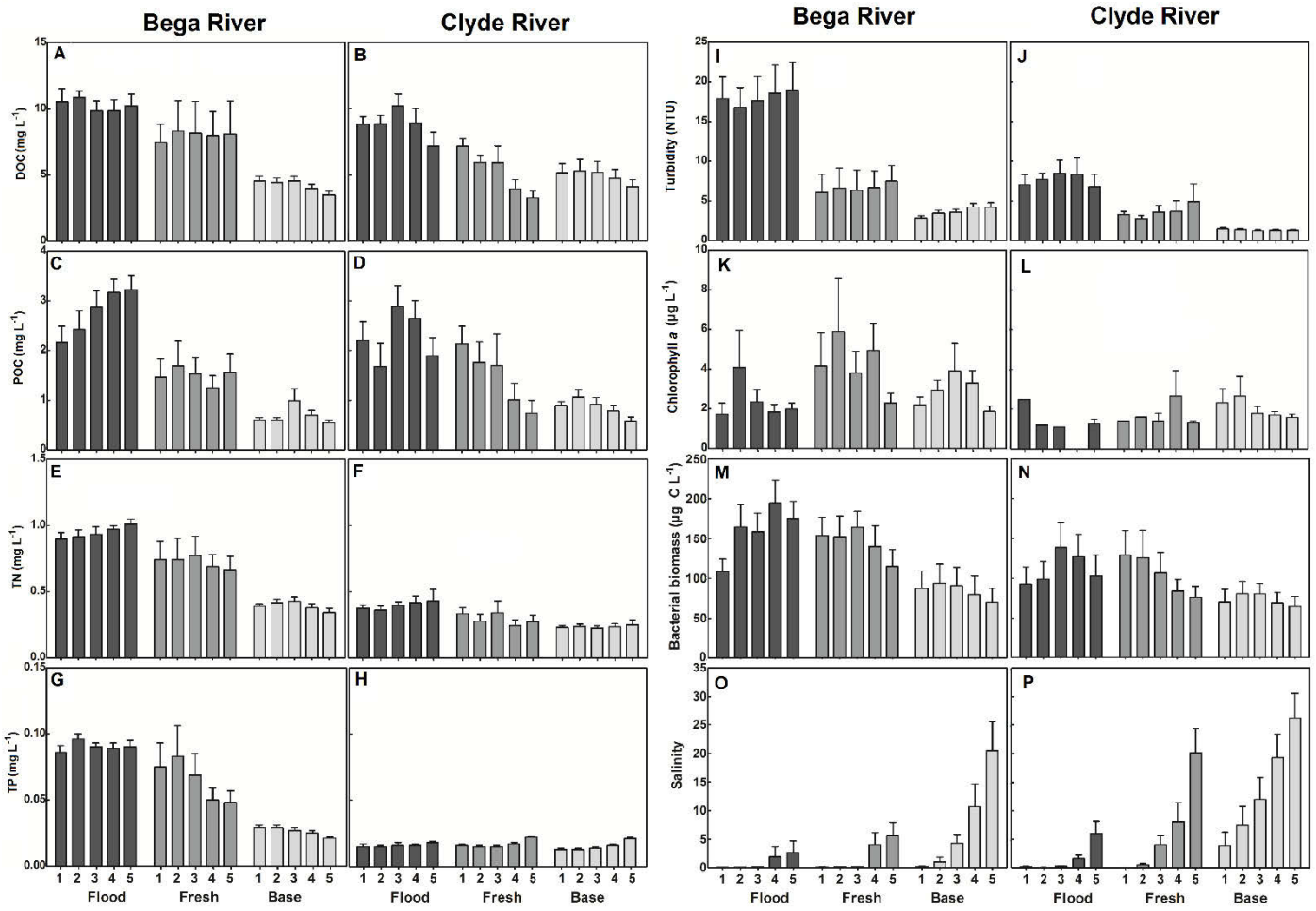

Figure 2.4. Mean concentrations of various constituents at stations 1 to 5 under flood, fresh and base flow categories. A) DOC Bega River, B) DOC Clyde River, C) POC Bega River, D) POC Clyde River, E) TN Bega River, F) TN Clyde River, G) TP Bega River, H) TP Clyde River, I) Turbidity Bega River, J) Turbidity Clyde River, K) Chlorophyll *a* Bega River, L) Chlorophyll *a* Clyde River, M) Bacterial biomass Bega River, N) Bacterial biomass Clyde River, O) Salinity Bega River, P) Salinity Clyde River. Error bars are ± standard error.

Table 2.1. Results of PERMANOVA analysis, Bega and Clyde Rivers. Pseudo *F*-values, degrees of freedom (df) and *P*-values on the Bega and Clyde River.

	Between flow categories				Between sampling stations				Flow category/sampling station interaction			
	Pseudo *f*	df	*P*	Perms	Pseudo *f*	df	*P*	Perms	Pseudo *f*	df	*P*	Perms
Bega River												
DOC	104.4	2	**0.001**	999	0.539	4	0.697	998	0.400	8	0.914	998
POC	146.5	2	**0.001**	999	1.255	4	0.264	999	1.290	8	0.253	997
TN	126.82	2	**0.001**	998	0.199	4	0.924	999	0.426	8	0.896	999
TP	171.32	2	**0.001**	998	3.478	4	**0.020**	999	1.751	8	0.090	999
Turbidity	101.66	2	**0.001**	999	0.676	4	0.590	997	0.182	8	0.994	994
Bacterial biomass	23.63	2	**0.001**	999	0.504	4	0.736	998	0.72797	8	0.728	999
Chlorophyll *a*	8.2278	2	**0.001**	999	2.411	4	0.057	998	0.052	8	0.838	999
Clyde River												
DOC	36.837	2	**0.001**	998	5.239	4	**0.001**	999	0.977	8	0.459	997
POC	50.991	2	**0.001**	999	5.242	4	**0.001**	998	2.659	8	**0.004**	999
TN	29.961	2	**0.001**	998	0.341	4	0.843	998	0.631	8	0.736	998
TP	2.402	2	0.088	997	10.306	4	**0.001**	999	0.932	8	0.502	999
Turbidity	186.63	2	**0.001**	999	0.056	4	0.996	999	0.561	8	0.806	999
Bacterial biomass	10.348	2	**0.001**	999	0.857	4	0.478	999	0.42839	8	0.895	997
Chlorophyll *a*	nt	-	-	-	-	-	-	-	-	-	-	-

nt- not tested due to a number of values below detection limit

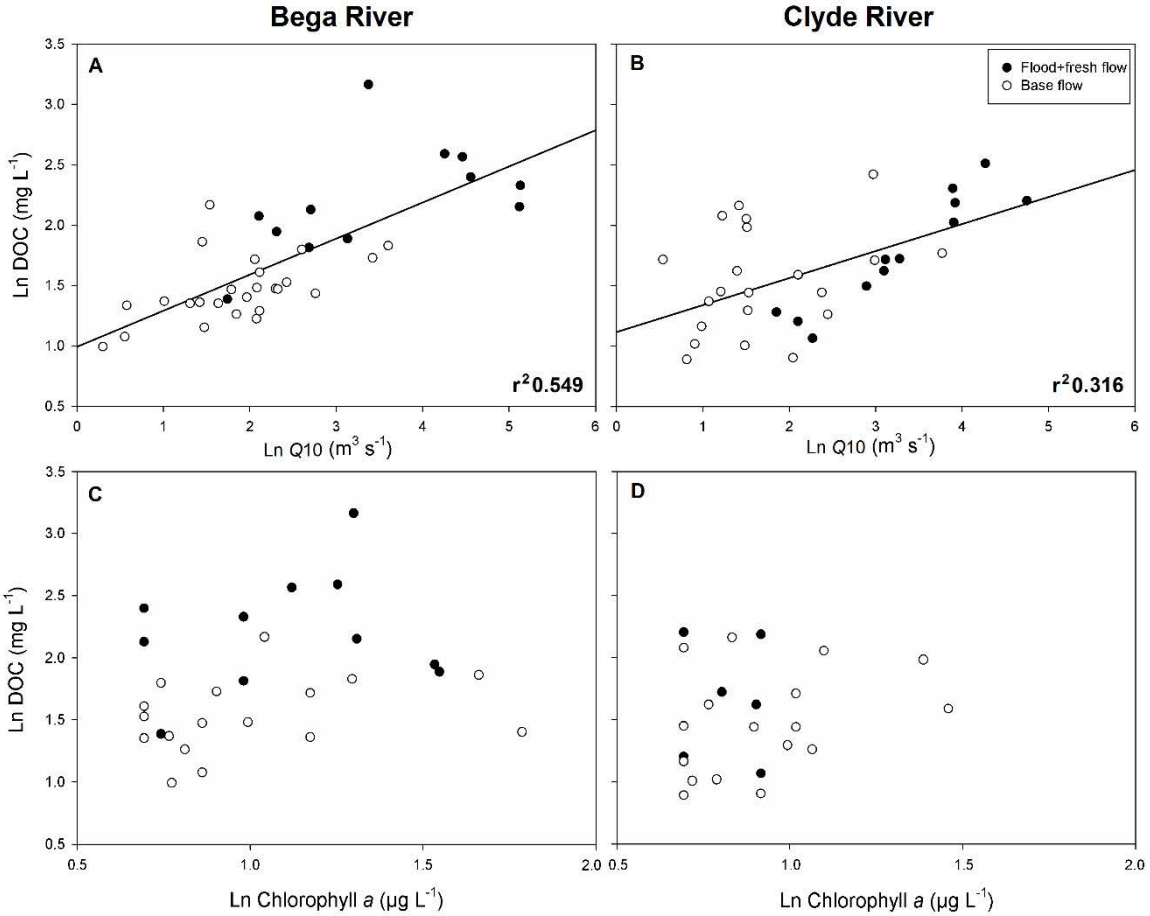

Figure 2.5. Results of the multiple stepwise linear regression analysis for DOC on the Bega and Clyde Rivers at sampling station 5. A) Ln DOC vs Ln 10 day antecedent discharge on the Bega River, B) Ln DOC vs Ln 10 day antecedent discharge on the Clyde River, C) Ln DOC vs Ln Chlorophyll *a* on the Bega River, D) Ln DOC vs Ln Chlorophyll *a* on the Clyde River. Closed circles are samples taken during flood and flow conditions, open circles are samples taken during base flow conditions. The overlap between flood+fresh and base flow points indicate dates when conditions had returned to base flow however mean daily discharge was still high, predominantly in April and May 2012.

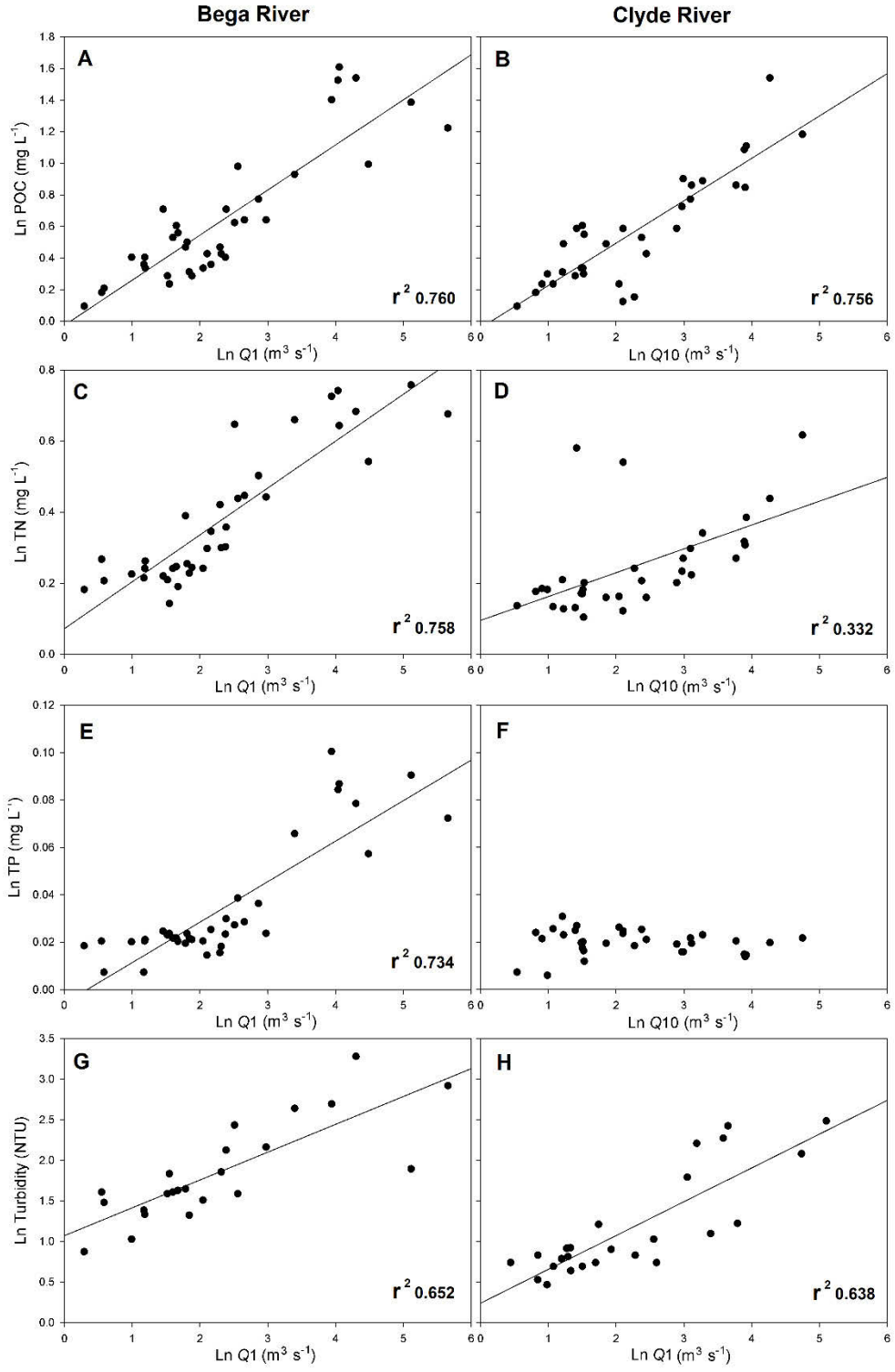

Figure 2.6. Results of the multiple stepwise linear regression analysis for POC, TN, TP and turbidity on the Bega and Clyde Rivers at sampling station 5. A) Ln POC vs Ln discharge, Bega River, B)) Ln POC vs Ln 10 day antecedent discharge, Clyde River, C) Ln TN vs Ln discharge Bega River, D) Ln TN vs Ln 10 day antecedent discharge, Clyde River, E) Ln TP vs Ln discharge, Bega River, F) Ln TP vs Ln 10 day antecedent discharge, Clyde River, G) Ln turbidity vs Ln discharge, Bega River, H) Ln turbidity vs Ln 1 day antecedent discharge, Clyde River.

2.4.3 Turbidity and Salinity

Mean turbidity varied greatly during the study and was significantly different between flow categories (Table 2.1). Turbidity was higher during flood flows compared to fresh flows on both rivers (Table 2.1, Fig. 2.4I, J). The exception was sampling station 5 on the Clyde River where there was no significant difference between flood and fresh flows. Turbidity was not significantly different between fresh and base flow conditions, except sampling station 1 on the Bega River where turbidity was higher during fresh flows. Turbidity was approximately 2-fold higher on the Bega River compared to the Clyde River. Multiple-regression analysis showed *Q*1 provided the best factor in predicating variance in turbidity concentrations (Table 2.2, Fig. 2.6G,H).

Differences in salinity ranges were clear between flood, fresh and base flow conditions (Fig. 2.4O, P). On the Bega River salinity peaked at station 5 with a mean of 3 under flood flow conditions, 6 during fresh flows and 20 during base flow. On the Clyde River salinity at station 5 was 6 during flood flow, 20 under fresh flow conditions and 26 during base flow. On the Bega River, freshwater dominated at stations 1-3 under flood and fresh flow conditions, but only extended to station 1 during base flow. On the Clyde River freshwater dominated at station 1-3 under flood conditions and station 1 during fresh flows.

2.4.4 Bacterial Biomass

Bacterial biomass significantly varied between flow categories (Table 2.1). During flood flows bacterial biomass was lower at the upstream station 1 on the Bega River and on the Clyde River stations 1 and 2 compared to fresh flows (Fig. 2.4M, N). At the downstream stations 4 and 5 the reverse was true with bacterial biomass higher during floods compared to fresh flows. Bacterial biomass was lowest during base flow conditions, except at the lower station on the Clyde River were it was similarly low during fresh flow conditions. Multiple-regression showed variation in bacterial biomass at stations 1-3 on both rivers could be best explained by TP concentrations (Table 2.2, Fig. 2.7A,B). On the Bega at station 4 a combination of DOC and TP proved the best predictors of bacterial biomass and station 5 DOC was the best variable (Fig. 2.7C). On the Clyde River DOC was the best variable at station 4 and *Q*10 the best variable at station 5 (Fig. 2.7D).

2.4.5 Chlorophyll *a*

Chlorophyll *a* concentrations were significantly different between flow categories on the Bega River with the high concentrations during fresh flows at station 4 accounting for most of the difference (Table 2.1, Fig. 2.4K). On the Clyde River chlorophyll *a* concentrations were low and often below the methodological detection limit of <1 µg L^{-1}. All values below detection limits were excluded from the calculation of the means (Fig. 2.4L) and PERMANOVA analysis was not conducted on the Clyde River due to too many missing values. At most stations we were unable to create models to explain variation in chlorophyll *a* concentrations, the exceptions were stations 4 and 5 on the Clyde River which were positively related to temperature (Table 2.2).

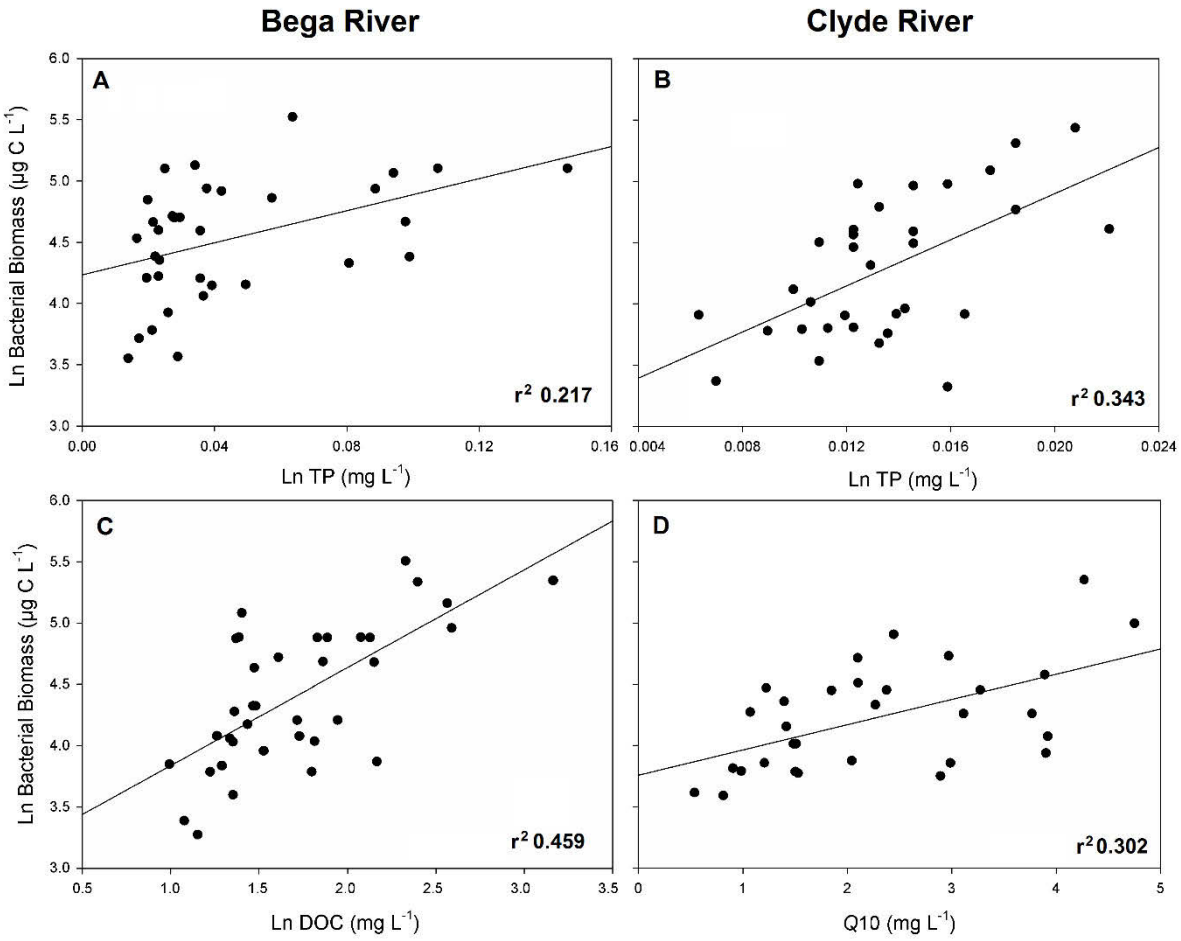

Figure 2.7. Results of the multiple stepwise linear regression analysis for bacterial biomass on the Bega and Clyde Rivers. A) Ln bacterial biomass vs Ln TP, Bega River, station 1, B) Ln bacterial biomass vs Ln TP Clyde River, station 1, C) Ln bacterial biomass vs Ln DOC, Bega River, station 5, D) Ln bacterial biomass vs Ln 10 day antecedent discharge, Clyde River, station 5.

Table 2.2. Multiple stepwise regression analysis for DOC, POC, TN, TP, turbidity and chlorophyll *a* on the Bega and Clyde Rivers. The best fit models were created with a single variable in most cases. Where no equation is listed, no significant relationship could be found between the dependent and any independent variable. All models were validated using the bootstrapping procedure in SPSS Ver. 21 and had significance values <0.05.

		Bega River					Clyde River				
	Station	Model	r^2	N	F	P	Model	r^2	N	F	P
DOC	1	Ln (DOC+1) = 1.39 + 0.193 Ln (*Q*1 +1)	0.369	39	21.9	<0.001	Ln (DOC+1) = 1.42 + 0.213 Ln (*Q*1 +1)	0.419	36	24.5	<0.001
	2	Ln (DOC+1) = 1.323 + 0.231 Ln (*Q*1 +1)	0.46	35	28.01	<0.001	Ln (DOC+1) = 1.33 + 0.247 Ln (*Q*10 +1)	0.467	31	25.46	<0.001
	3	Ln (DOC+1) = 1.34 + 0.212 Ln (*Q*10 +1)	0.427	36	25.31	<0.001	Ln (DOC+1) = 1.30 + 0.264 Ln (*Q*10 +1)	0.403	31	19356	<0.001
	4	Ln (DOC+1) = 1.14 + 0.262 Ln (*Q*10 +1)	0.53	36	38.4	<0.001	Ln (DOC+1) = 1.09 + 0.298 Ln (*Q*10 +1)	0.474	36	30.61	<0.001
	5	Ln (DOC+1) = 0.997 + 0.298 Ln (*Q*10 +1)	0.549	37	42.62	<0.001	Ln (DOC+1) = 1.11 + 0.225 Ln (*Q*10 +1)	0.316	33	14.33	<0.001
POC	1	Ln (POC+1) = 0.222 + 0.172 Ln (*Q*1 +1)	0.453	38	29.81	<0.001	Ln (POC+1) = 0.340 + 0.206 Ln (*Q*1 +1)	0.52	35	35.78	<0.001
	2	Ln (POC+1) = 0.143+ 0.219 Ln (*Q*1 +1)	0.593	35	48.15	<0.001	Ln (POC+1) = 0.372 + 0.180 Ln (*Q*1 +1)	0.386	31	18.27	<0.001
	3	Ln (POC+1) = 0.182 + 0.230 Ln (*Q*1 +1)	0.622	36	56.06	<0.001	Ln (POC+1) = 0.239 + 0.256 Ln (*Q*1 +1)	0.545	31	34.79	<0.001
	4	Ln (POC+1) = 0.0917 + 0.257 Ln (*Q*1 +1)	0.661	36	66.25	<0.001	Ln (POC+1) = 0.0331 + 0.304 Ln (*Q*1 +1)	0.651	36	63.31	<0.001
	5	Ln (POC+1) = -0.0244 + 0.285 Ln (*Q*1 +1)	0.76	37	111	<0.001	Ln (POC+1) = -0.0418 + 0.268 Ln (*Q*10 +1)	0.756	33	92.22	<0.001
TN	1	Ln (TN+1) = 0.204 + 0.0875 Ln (*Q*1 +1)	0.457	38	30.25	<0.001	Ln (TN+1) = 0.156 + 0.0390 Ln (*Q*1 +1)	0.384	35	20.54	<0.001
	2	Ln (TN+1) = 0.197 + 0.0974 Ln (*Q*1 +1)	0.569	35	43.54	<0.001	Ln (TN+1) = 0.130 + 0.0487 Ln (*Q*10 +1)	0.577	31	39.61	<0.001
	3	Ln (TN+1) = 0.199 + 0.101 Ln (*Q*1 +1)	0.542	36	40.32	<0.001	Ln (TN+1) = 0.110 + 0.0580 Ln (*Q*10 +1)	0.595	30	41.12	<0.001
	4	Ln (TN+1) = 0.132 + 0.117 Ln (*Q*1 +1)	0.681	36	72.65	<0.001	Ln (TN+1) = 0.0756 + 0.0731 Ln (*Q*10 +1)	0.587	36	48.31	<0.001
	5	Ln (TN+1) = 0.0723 + 0.132 Ln (*Q*1 +1)	0.758	37	130.1	<0.001	Ln (TN+1) = 0.0955 + 0.0671 Ln (*Q*10 +1)	0.332	33	15.41	<0.001
TP	1	Ln (TP+1) = 0.0110 + 0.0142 Ln (*Q*1 +1)	0.325	38	17.34	<0.001	Ln (TP+1) = 0.0110 + 0.00111 Ln (*Q*1 +1)	0.148	35	5.72	0.023
	2	Ln (TP+1) = 0.00282 + 0.0186 Ln (*Q*1 +1)	0.49	35	31.71	<0.001	Ln (TP+1) = 0.0114 + 0.000928 Ln (*Q*1 +1)	0.167	31	5.81	0.022
	3	Ln (TP+1) = 0.00213 + 0.0173 Ln (*Q*1 +1)	0.537	36	39.4	<0.001	-				
	4	Ln (TP+1) = - 0.00116 + 0.0168 Ln (*Q*1 +1)	0.686	36	74.42	<0.001	-				
	5	Ln (TP+1) = - 0.00572 + 0.0171 Ln (*Q*1 +1)	0.734	37	96.49	<0.001	-				
Turbidity	1	Ln (Turbidity +1) = 0.753 + 0.374 Ln (*Q*1 +1)	0.68	23	44.64	<0.001	Ln (Turbidity +1) = 0.364 + 0.0367 Ln (*Q*1 +1)	0.713	25	57.05	<0.001
	2	Ln (Turbidity +1) = 0.919 + 0.347 Ln (*Q*1 +1)	0.675	23	43.63	<0.001	Ln (Turbidity +1) = 0.289 + 0.394 Ln (*Q*1 +1)	0.744	25	66.71	<0.001
	3	Ln (Turbidity +1) = 0.951 + 0.341 Ln (*Q*1 +1)	0.643	23	37.78	<0.001	Ln (Turbidity +1) = 0.184 + 0.431 Ln (*Q*1 +1)	0.719	25	58.95	<0.001
	4	Ln (Turbidity +1) = 1.13 + 0.313 Ln (*Q*1 +1)	0.638	23	36.94	<0.001	Ln (Turbidity +1) = 0.218 + 0.432 Ln (*Q*1 +1)	0.731	25	62.41	<0.001
	5	Ln (Turbidity +1) = 1.07 + 0.343 Ln (*Q*1 +1)	0.652	23	39.33	<0.001	Ln (Turbidity +1) = 0.290 + 0.387 Ln (*Q*1 +1)	0.638	25	40.53	<0.001
Bacterial biomass	1	Ln (BB +1) = 4.186 + 6.983 Ln (TP +1)	0.217	38	9.986	<0.001	Ln (BB +1) = 3.017 + 94.172 Ln (TP +1)	0.343	32	15.63	<0.001
	2	Ln (BB +1) = 4.157 + 10.260 Ln (TP +1)	0.387	35	20.83	<0.001	Ln (BB +1) = 3.468 + 66.404 Ln (TP +1)	0.201	29	6.77	<0.001
	3	Ln (BB +1) = 4.174 + 11.113 Ln (TP +1)	0.337	36	12.28	<0.001	Ln (BB +1) = 3.951 + 1.988 Ln (TP +1)	0.400	30	18.67	<0.001
	4	Ln (BB +1) = 3.311 + 0.405 Ln (DOC +1) + 11.800 x Ln (TP +1)	0.444	36	14.98	<0.001	Ln (BB +1) = 3.480 + 0.490 Ln (DOC +1)	0.221	34	9.07	0.005
	5	Ln (BB +1) = 3.041 + (0.798 Ln (DOC +1)	0.459	37	29.71	<0.01	Ln (BB +1) = 3.041 + 0.798 Ln (*Q*10 +1)	0.302	31	12.54	0.001
Chlorophyll *a*	1	-					-				
	2	-					-				
	3	-					-				
	4	-					Ln (Chlorophyll *a* + 1) = -0.224 + 0.379 Ln (Temp +1)	0.218	21	8.06	0.008
	5	-					Ln (Chlorophyll *a* + 1) = -0.261 + 0.389 Ln (Temp +1)	0.219	21	5.34	0.032

2.5 Discussion

2.5.1 Discharge and organic carbon dynamics

Discharge plays an overwhelming role in structuring organic carbon dynamics on the Bega and Clyde Rivers. We predicted that organic carbon would be positively related to discharge, except during base flow periods, when it would be related to chlorophyll a. Discharge was the only factor that could explain the changes in the variation of organic carbon concentrations for each estuary, including during base flows (Table 2.2, Fig. 2.5). This is somewhat different to many systems where autochthonous carbon and other factors such as temperature are often implicated in estuarine organic carbon dynamics (e.g. Fisher et al., 1998; Minor et al., 2006; Peterson et al., 1994). Jassby et al. (1993) found that the influence of discharge on DOC dynamics in San Francisco Bay varied both spatially within the estuary and temporally between years. The dominance of discharge may in part be explained by its highly episodic nature in South-East Australia. During this study, flood flows occurred two or three times a year and fresh flows around every month, delivering allochthonous carbon to the estuary. At the upper stations $Q1$ was the best predictor for DOC concentrations; at downstream stations $Q10$ was usually a better predictor. The likely main reason for this distinction in discharge variables is that retention times in the upper estuary are short; DOC concentrations there likely reflect riverine inputs on the day of sampling. At the downstream sites retention times are longer and reflect riverine DOC inputs from both the day of sampling and days prior. This study also took place during a period of above average precipitation; it is possible that over a longer period of time covering drier years that primary production may have a stronger control over organic carbon.

Previous studies that have examined the relationship between discharge and carbon have used a dichotomy of storm and base flow to characterise different discharge conditions. Buffam et al. (2001) for example showed in freshwater streams that storm flow, whilst only occurring for a fraction of the time compared to base flow, accounted for over half the annual DOC load. Wiegner et al. (2009) and Leff and Meyer (1991) have shown that the bioavailability of DOC to heterotrophic bacteria can change between storm and base flow conditions. Our separation of storm flows into flood,

fresh and base flow categories here has proven useful as a simple way of understanding how variable hydrological conditions may structure carbon dynamics.

The highest organic carbon concentrations generally coincided with flood flows as we predicted (Fig. 2.4A-D). This is not surprising as it is well established that storm flows play a key role in mobilising organic carbon in catchments (Buffam et al., 2001; Hinton et al., 1997). Similar results were found in another Australian estuary, the Fitzroy River, where Ford et al. (2005) showed DOC concentrations were higher following high flows compared to base flow conditions. The role of floods in facilitating cross-boundary carbon subsidisation are emphasised in the flood pulse concept (Junk et al., 1989). The interface of terrestrial-aquatic environments, particularly during flood events are recognised as important 'hot spots and moments' in biogeochemical cycling (McClain et al., 2003).

Under fresh flow conditions DOC concentrations were only moderately lower than during flood flow conditions (Fig. 2.4A-D). During fresh flows connectivity to the flood plain is likely to be minimal and from our observations did not occur in upper reaches of either river. This suggests an increased importance of organic matter from other sources such as the upper catchment, river banks and benches, in-stream production and direct riparian inputs during fresh flows. The relative contribution of POC to the total carbon pool also significantly reduced under fresh flow conditions. This is likely due to the lack of connectivity to the flood plain during fresh flows, as well as decreased erosive force compared to larger floods (Depetris and Kempe, 1993).

Our flow categories presented a different relationship between inflows and DOC on the lower stations of the Clyde River. Here DOC and POC concentrations during fresh flows were similar to base flow conditions. This is most likely due to dilution as the estuarine volume becomes significantly larger in the lower estuary and tidal exchange with the mouth increases. McKee et al. (2000) showed on the Richmond estuary, Australia, that of the total loads of TN and TP delivered to the estuary during floods, a much larger proportion was exported to the ocean during large floods compared to smaller floods. Longer retention times of materials also influences biological factors. For example long retention times of DOC in estuaries may lead to increased bacterial degradation (Hopkinson et al., 1998). We did not study retention times or mixing dynamics during this study however salinity data gives at least some insight into the

likely differences in advective force and the penetration of freshwater downstream during fresh and flood flows (Fig. 2.4O, P).

Though the size of inflow is clearly important, the frequency and timing between flow events can also exert an influence on organic carbon concentrations. Schäfer et al. (2002) showed the period of time between flooding and inundation will affect organic matter and suspended solids export. We observed that during two similar sized floods that occurred on the Bega and Clyde Rivers during March 2012, DOC concentrations at the upper most station were 13 and 11 mg L^{-1} respectively during the first flood, and 11 and 7 mg L^{-1} respectively during a flood ten days later (Fig. 2). This pattern was also demonstrated by Buffam et al. (2001) who observed two floods in quick succession, finding DOC and nitrogen concentrations being considerably lower during the second flood. This occurrence is also regularly seen in tropical catchments where the first flush at the start of the wet season will deliver much higher loads of organic carbon before it declines, despite discharge remaining high (Brinson, 1976). This suggests that the first flow event after an extended period of base flow may be more important for delivering organic carbon to the estuary. Others have used additional hydrological variables to develop a more nuanced understanding of the influence of discharge. Examples of these include the amplitude of the rising limb of a flood, which accounts for the pre-flood river level (Puckridge et al., 1998), and discounted flow, which reflects flow history (Kuhnert et al., 2012). We did not have enough data points in our dataset to accurately employ such techniques. Integrating these into further conceptual work regarding differently sized inflow may be useful.

2.5.2 Nutrient and turbidity dynamics

Nitrogen and phosphorus dynamics were similar to that of carbon on the Bega River and were correlated with discharge (Fig. 2.6C-F). At the upper stations TN and TP concentrations were not significantly different between flood and fresh flow. At the lower stations TN and TP were significantly lower during fresh flows in comparison to flood flows (Fig. 2.4E-H). These results are consistent with reporting in the literature that discharge is regularly the dominant source of nitrogen and phosphorus (Bouwman et al., 2005; Boynton and Kemp, 2000; Conley et al., 1995; Froelich et al., 1982; Seitzinger et al., 2002). TN and TP and have been shown to significantly increase in concentration following flooding events (Eyre and Twigg, 1997).

Some clear distinctions between the Bega and Clyde catchments are highlighted by the TN and TP concentrations which were always 2-3 fold higher on the Bega River (Fig. 2.4). Whilst TN was positively related to discharge on the Clyde River, TP, which remained low at all times, was not or showed only a weak relationship (Fig. 2.3D). These results can be mostly explained through differences in catchment land use. The Clyde River catchment is mostly undisturbed with riparian vegetation intact (Tozer et al., 2010); TN and TP concentrations here always fell within the range expected in an unpolluted estuary (ANZECC, 2000). The Bega River has been heavily cleared for agriculture (Brierley et al., 1999; Tozer et al., 2010), in particular dairy, which is widely associated with increased nutrient loading to catchments and estuaries (Drewry et al., 2006; Harris, 2001; Jones et al., 2009).

Turbidity also showed some clear distinctions between flow categories. On both estuaries turbidity was significantly higher during flood flows compared to fresh flows. Turbidity was in turn significantly higher during fresh flows than base flows (Fig. 2.4I, J). This is an important distinction between these flow categorises as whilst mean turbidity remains low at 5-6 NTU in the Bega River during fresh flows, during flood flows it is around 18 NTU, enough to potentially negatively impact on primary production as high turbidity is linked with reduced light availability for autotrophs (Cloern, 1987; Lloyd et al., 1987). The turbidity results also indicate differences between catchments due to land use. Turbidity was always much higher on the Bega River, probably due to clearing of vegetation for agriculture which has led to enhanced erosion and sediment transfer to the river channel in the catchment (Fryirs and Brierley, 2001).

2.5.3 Biological responses to discharge and changing DOC and nutrient dynamics

Bacterial biomass and production are commonly correlated to primary production or chlorophyll *a* due to bacterial reliance on autochthonous DOC (Cole et al., 1988). However, this may not be the case in systems receiving large inputs of allochthonous DOC such as estuaries (Almeida et al., 2005; Ameryk et al., 2005). We predicted that bacteria would be primarily related to DOC. This assertion was only partly confirmed as bacteria were correlated to both DOC and discharge in the lower estuary, and TP in the upper estuary (Fig. 2.7). Bacterial biomass was never related to chlorophyll *a*. One

potential hypothesis to explain these results may be that the size and variation of inputs of allochthonous DOC was much greater than dissolved autochthonous carbon production. These results support findings in other studies that bacteria may be more closely related to DOC and discharge when allochthonous inputs are high (Hoch and Kirchman, 1993; Ochs et al., 2010).

Previous work conducted on these rivers has shown that bacteria are mostly carbon limited but this may switch to nitrogen or phosphorus limitation under high flow conditions when available DOC concentrations are high (Chapter 4; Hitchcock and Mitrovic, 2013). One possible explanation for a switch in factors explaining bacterial biomass variation from TP in the upper estuary to DOC and discharge in the lower estuary, may be related to the biological transformation of DOC. Bioavailability of allochthonous DOC in both estuaries to vary between 10-30% (Chapter 3). As this DOC enters the estuary at station 1, the DOC pool may still be largely available and bacteria become phosphorus limited. As the DOC pool makes its way to the lower estuary it becomes more refractory and bacteria become carbon limited. Bacteria have been linked to both DOC and phosphorus in other systems (Cotner and Biddanda, 2002; Pinhassi et al., 2006). This hypothesis is supported by mean bacterial biomass at each station which, with the exception of the upstream sites during flood flows, decreased in concentration from upstream to downstream (Fig. 2.4 M,N). Another possible explanation for increases in bacteria may be due to transport from the catchment during floods. Crump et al. (2004) has previously shown that estuarine bacteria may come from a variety of sources including the terrestrial environment. Whilst some of the bacteria in the these estuaries may have originated from allochthonous sources, the variance in biomass is more likely related to *in-situ* growth as we previously found bacterial growth rates to be significantly higher during inflow events (Chapter 3). Another important consideration is that we used a fix station design. This means that in most cases the plankton communities present at the lower marine stations during base flows, are likely different to those under flood flows. Understanding how plankton community assemblages change during inflows is beyond the scope of this chapter, though is discussed in more detail in Chapter 5.

In estuaries, phytoplankton are commonly correlated to both temperature or season as well as available nutrients (Malone, 1977). We have previously shown that

phytoplankton are primarily nitrogen limited on the Bega River and phosphorus limited on the Clyde River (Hitchcock and Mitrovic, 2013). Thus we predicted chlorophyll *a* would be related to these nutrients; as well as have a broader relationship to temperature. During the study period the only factor that helped to explain chlorophyll *a* concentrations from those tested was temperature, which is regularly found in estuaries (Eppley, 1972). However, it is relatively common for chlorophyll *a* to be also correlated to nutrient availability (Rudek et al., 1991). One possible explanation for why chlorophyll *a* was not related to nitrogen or phosphorus may be that bacteria regularly outcompeted phytoplankton for available nutrients. It's previously been shown that bacteria can outcompete phytoplankton for nutrients due to having greater surface area to volume ratios (Currie and Kalff, 1984; Thingstad et al., 1993). We have previously seen evidence of competition for nutrients under high flow conditions on both estuaries in previous work (Hitchcock and Mitrovic, 2013). Another possible reason it was hard to explain chlorophyll *a* variance may be limitation by micronutrients (Hecky and Kilham, 1988). There is a range of other factors not measured here that may explain chlorophyll *a* was not related to changing nitrogen and phosphorous concentrations such as grazing pressure and the presence of other nutrients such as Si and Fe (Paerl and Justic, 2013).

Bacterial biomass and chlorophyll *a* both showed significant differences between flow categories. We predicted that concentrations of both would be highest under fresh flow conditions, as although flood flows may bring more resources, they may also lead to the washout of plankton from the estuary. Our results partly confirmed this assertion, showing that bacterial biomass was higher at the upstream stations under fresh flows compared to flood flows, though not at the middle and lower stations (Fig. 2.4M, N). The chlorophyll *a* results showed concentrations highest during fresh flows on the Bega River, but no pattern on the Clyde River (Fig. 2.4K, L). The role of advective forcing appears to play a role during flood flows at the upper stations, likely washing bacteria and phytoplankton downstream. These results may also be related to increased residence times of organic carbon and nutrients during smaller inflows, allowing greater utilisation of materials (Battin et al., 2008). The utility of separating storm flows into flood and fresh flows appears to be useful in understanding what responses in different sections of the estuary we might expect from bacteria, but much less useful in trying to understand chlorophyll *a* dynamics.

Freshwater inflows play a key role in many aspects of estuarine and aquatic ecosystem functioning (Roelke et al., 2012). These results support our prediction that discharge would be an important factor influencing carbon and nutrient dynamics on the Bega and Clyde Rivers. However, we also predicted that chlorophyll *a* would be important during low flow periods; this was not the case during the study period. Chlorophyll *a* during this study generally remained low and was most related to temperature, but not to nutrient concentrations. Bacterial biomass was related to DOC and discharge in the lower estuary which supported our assertion that carbon would be the main determining factor. At the upper stations phosphorus was the factor that best explained bacterial variation. These results have important implications for future studies of biogeochemical cycling in estuaries, supporting the idea that freshwater inflow events can be considered 'hot moments' of intense transformation (McClain et al., 2003). The results also indicate that freshwater inflow events are important in facilitating a cross systems resource subsidy. Whilst there has been significant debate about the importance of allochthonous carbon in aquatic food webs (Brett et al., 2009; Hoffman et al., 2008), these results suggest that inflow events constitute key moments where significant amounts of allochthonous carbon may become available to the microbial loop and potentially available to higher trophic organisms (Fenchel, 2008).

The separation of discharge into different flow categories of flood, fresh and base flow appeared useful for understanding some factors, but less for others. Whilst carbon and nutrients were significantly lower during base flow as predicted, the differences between flood and fresh flows were mostly only seen in the lower estuary. The exception was for POC which was always much higher during flood flows in comparison to fresh flows. Similarly turbidity was also much higher during flood flows compared to fresh and base flows. Bacterial biomass and chlorophyll *a* results also partially supported our prediction that concentrations would be higher under fresh flow conditions, as they were for bacteria in the upper estuary and for chlorophyll *a* on the Bega River. Overall concentrations for all factors were generally lowest during base flow conditions. The distinction between flood and fresh flow conditions is partially limited; effecting individual factors and estuary locations differently, and is likely influenced by a range of interrelated factors beyond the scope of this study. The significant differences that were witnessed between flood and fresh flows however suggest that future research into understanding the influence of differently sized

inflows will be important in estuarine systems. This will be particularly important as many coastal catchments have had their flow regimes radically altered through extraction and the presence of dams.

Chapter 3 After the flood: Changing dissolved organic carbon bioavailability and bacterial growth following inflows to estuaries

3.1 Abstract

Freshwater inflows play an important role in delivering dissolved organic carbon (DOC) to estuaries. Although considerable DOC can be delivered to estuaries during episodic inflow events, such as floods, little information exists on how the bioavailability of DOC may change during these periods. In this study we used *in-vitro* bioassay incubation experiments to examine how bioavailability changed following inflow events in two temperate south east Australian estuaries. We measured short-term (2 days) and long-term (28 days) bioavailable DOC and determined percentage bioavailability, bacterial growth rates and bacterial growth efficiencies, all with and without excess nitrogen and phosphorus to control for nutrient limitation. Our results showed bioavailable DOC (BDOC) varied between 0.13-3.62 mg C L^{-1}, equivalent to 2.5-31% of initial concentrations. Bacterial growth rates were highest at the peak of flow and reduced steadily as discharge returned to base flow conditions. Multiple-regression analysis showed discharge and specific ultraviolet light absorbance were the best factors for predicting variance in BDOC whilst discharge and BDOC were the best factors for predicting bacterial growth rates. The addition of nutrients often led to significantly higher measurements of BDOC and bacterial growth rates on both rivers. Bacterial growth efficiency varied between 3.46-68.18% and was highest when DOC bioavailability was lowest. At other times bacterial growth efficiency was highly variable and could not be predicted from environmental variables. These results highlight the importance of freshwater inflow events as intense moments of biogeochemical transformation in estuaries.

3.2 Introduction

Each year rivers globally transport, transform or store almost 2 Pg of terrestrial carbon (Battin et al., 2008). Much of this carbon enters estuaries before reaching coastal systems. Dissolved organic carbon (DOC) has long been conceptualised as playing a key role in biogeochemical cycles and microbial food webs (Azam et al., 1983;

Pomeroy, 1974). However, allochthonous DOC entering estuaries via rivers has historically been considered refractory and largely non-labile due to its age and high concentrations of humic substances, however this view is changing (Marín-Spiotta et al., 2014). Studies over the last two decades have indicated the important role of allochthonous DOC in biogeochemical cycling in estuaries (Howarth et al., 1996; Wikner et al., 1999). Many estuaries have been shown to be net heterotrophic, that is, respiration is greater than primary production, highlighting the importance of allochthonous carbon in estuarine metabolism (Battin et al., 2008) The contribution that DOC makes to estuarine metabolism is largely dependent on its bioavailability.

Bioavailable DOC (BDOC) refers to the proportion of DOC removed by bacteria over a period of time. BDOC determinations are most commonly determined via bioassay incubations where changes in DOC concentration are measured over time (Guillemette and Del Giorgio, 2011) and have been widely studied in aquatic environments (see del Giorgio and Davis, 2003 and references therein). Estimates of BDOC in estuaries vary from 2% to >25% (Cole et al., 2011; Hanisch et al., 2010; Sanderson et al., 2009). The bioavailability of DOC is primarily determined by its chemical composition (Berggren et al., 2010). The chemical composition of DOC entering estuaries may be influenced by a range of factors including land use (Asmala et al., 2013), presence of wetlands (Fellman et al., 2008), pollution and anthropogenic impacts (Wiegner and Seitzinger, 2004), microbial and photochemical transformation (Moran et al., 2000), and hydrological flow paths (Sobczak and Findlay, 2002). The other important environmental factor related to bioavailability is solubility, which is of particular importance has hydrologic flow paths change during inflow events (Schmidt et al., 2011).

Changes in hydrological flow path occur during precipitation and inflow events (Aitkenhead-Peterson et al., 2003; Sanderman et al., 2008). During base flow conditions, water typically travels through deeper mineral dominated soils that are low in DOC. During storm events, water travels across catchment surfaces and through carbon rich upper soil horizons. This shift in flow paths causes changes in the chemical composition of the DOC pool. Increases in discharge have been correalted to increases in humic substances (Hood et al., 2006; Nguyen et al., 2010), which are in turn associated with decreases in BDOC (Berggren et al., 2009). However, at the same time

changes in flow paths during storms may also bring DOC that is 'fresher' or less biogenetically altered (Peter et al., 2012), and diagenetical alteration has been found to be inversely related to bioavailability (Chapelle et al., 2009).

Measurements of how BDOC may change during freshwater inflow events are rare. There are a few studies from streams and rivers, however these have found contrasting results. Some authors have found that DOC bioavailability may increase or show mixed results following flow events (Fellman et al., 2009; Gremm and Kaplan, 1998; McLaughlin and Kaplan, 2013). These authors cite shifts in DOC composition and source material responsible for changes in bioavailability. Other authors have found that DOC bioavailability decreases during storms (Leff and Meyer, 1991; Wiegner et al., 2009) and suggest this may be due to changes to bacterial communities during storms and a reduction in contribution of autochthonous DOC, considered to be more bioavailable than allochthonous carbon.

To our knowledge studies examining how DOC bioavailability changes with freshwater inflows in estuaries has not been examined. There is a need to characterise the bioavailability of DOC entering estuaries separately from upstream freshwater reaches as during transit time through the catchment significant biotic and abiotic alteration to the DOC pool may occur (Battin et al., 2008). Once DOC enters estuaries a range of factors may influence its biological degradation such as: the composition of the bacterial community, photobleaching, nutrient availability, retention time, mixing and dilution, flocculation, adsorbtion and aggregation (Day et al., 2012). Understanding how the bioavailability of DOC entering rivers and estuaries changes during inflow events is of particular importance, as it is during these events that the majority of annual carbon loads are delivered (Buffam et al., 2001; Hinton et al., 1997) and thus has significant implications in defining coastal carbon cycling. Understanding how bioavailability and microbial responses change during these periods is a key research frontier in carbon biogeochemistry (Marín-Spiotta et al., 2014).

In the Bega and Clyde Rivers, south-east Australia, we collected water from the tidal limit of the freshwater tidal zone of the two estuaries at multiple time points following inflow events and performed standardised *in-vitro* bioassay incubations. In this paper we hypothesise that: DOC bioavailability and bacterial growth rates are highest at the

peak of inflow events and decline towards base flow conditions. Globally these results will be an interesting comparison to temperate catchments in the northern hemisphere as south-east Australian catchments are distinct in having more highly episodic flow regimes.

3.3 Methods

3.3.1 Study sites

The Bega River estuary, NSW, Australia, (-36° 42' 43.64", +149° 54' 8.76") is a shallow, wave-dominated estuary (Fig. 3.1). The estuarine volume, 6371 ML, is relatively small compared to its catchment of 1941km^2 (Roy et al., 2001). The catchment is split into two sub-catchments, the Brogo River, regulated by Brogo Dam and Bega River which has two smaller dams, Cochrane and Candello. The upper sections of the catchment consist of mainly forested areas, whilst the middle catchment is mostly cleared for agriculture, dominated by the dairy industry (Brierley et al., 1999; Tozer et al., 2010). The freshwater tidal section has been mostly cleared for agriculture and irrigated farmland. Broad scale land clearing since colonisation in the late 1800's has drastically altered the river and estuary via the transportation of sandy top soils into the river channel (Brooks and Brierley, 1997). The Clyde River estuary, NSW, Australia, (-35° 33' 11.44", +150° 11' 12.11") is a tidally dominated estuary (Fig. 3.1). Its estuary volume, 50737 ML, is relatively large compared to its catchment size of 1791 km^2 (Roy et al., 2001). The river is unregulated, with no significant structures or water extraction. The catchment is almost entirely forested, with some forestry activities in the upper catchment. Discharge information was obtained from one gauging station on the Clyde River and two gauging stations on the Bega and Brogo Rivers, which were combined to give a single value for the Bega River. On the Bega River these gauging stations were located approximately 5 km upstream of the tidal zone, on the Clyde River the gauging station is located approximately 11 km up stream of the tidal zone. There are no tributaries located between the gauging stations and tidal zone.

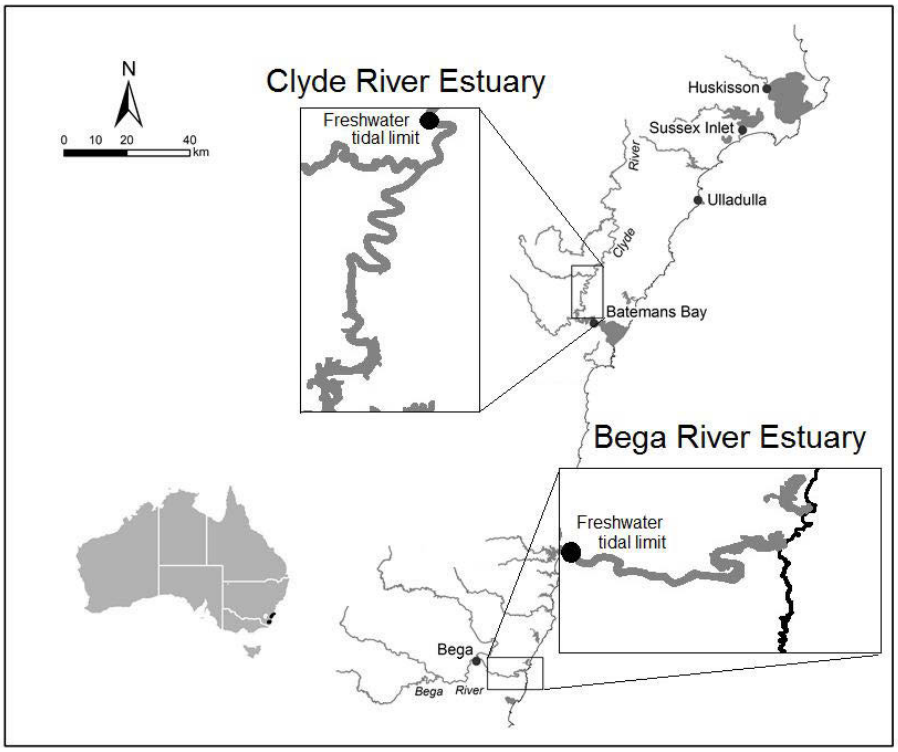

Figure 3.1. Map of the Bega and Clyde River catchments, NSW, Australia. Samples were taken at the tidal limit of each estuary.

3.3.2 Water Collection

Water was collected on eight occasions for each river at the tidal limit of the estuary (salinity was always 0). This location was chosen in order to capture the DOC pool that was flowing into the estuary before it mixed with receiving waters. Water collection occurred during a period encompassing two freshwater inflow events in each system (Fig. 3.2). Water collection was designed to cover the time period from the peak of inflow to the return to base flow conditions. Following the first inflow event on 4 March 2012 on each river, a second peak of inflow occurred on the falling limb of the hydrograph. We sampled on the first peak in discharge and then subsequently on the falling limb after the second peak, and here after refer to this period as a single event. Samples were stored on ice and then filtered within 1-2 hours using 0.2 µm polycarbonate filters and stored frozen in 1 L polyethylene bottles. Polycarbonate filters were pre-rinsed with Milli-Q water. All polyethylene bottles used in this study were cleaned with HCL 1%, rinsed with Milli-Q water and sample rinsed before use. Freezing DOC samples can potentially lead to precipitation of some materials; comparisons of DOC concentrations post freezing to those of non-frozen

DOC samples collected during the same time for a separate study (Chapter 2) showed freezing did not alter DOC concentrations.

3.3.3 Experimental design

Frozen water samples were defrosted at 4 ^{o} C and decanted into four 500 mL polyethylene bottles. Polyethylene bottles were chosen in order to minimise contamination and adsorption (Yoro et al., 1999). For each sample two bottles received additions of nitrogen (12 mg KNO_3 L^{-1}) and phosphorus (8 mg KH_2PO_4 L^{-1}) on days 0, 5 and 15 in order to ensure nutrient availability was not limiting bacterial metabolism. The remaining two bottles had no nutrient additions. A bacterial inoculum sourced from the freshwater tidal limit of each respective estuary was then added to each bottle, which were then placed in dark incubation chambers at 20ºC for 28 days.

Our bacterial inoculums were created by collecting additional water on the final day of sampling, 16 July 2012. Water was filtered in order to remove bacterial predators. Preliminary filtration using 0.7 µm glass fibre filters removed ~70% of bacterial abundance so we twice filtered our inoculums using a larger 2.7µm pre-combusted (500ºC, 4 hours) glass fibre filter which resulted in an inoculum ~90% of initial abundance. No visible flagellates or ciliates were found under initial examination. The inoculums were placed in dark growth chambers at 20ºC to adjust to experimental conditions for 72 hours. The inoculum was gently filtered onto pre-rinsed 0.2µm polycarbonate filters and then gently rinsed into Erlenmeyer flasks using Milli-Q water. The bacterial abundance was then quantified and added to each incubation bottle at approximately 10% abundance of ambient concentrations in each estuary on collection date.

BDOC was measured as a change in DOC concentration through time during the incubations. We used the basic conceptual framework proposed by Guillemette and del Giorgio (2011) where the amount of DOC consumed with 48 hours is considered short-term labile and the DOC consumed within 28 days long-term labile. We measured short-term BDOC (STC), long-term BDOC (LTC) and the percentage of total DOC that was bioavailable both for the short term (STB%) and long-term (LTB%) experiments. DOC not utilised within 28 days was classed as residual.

Bacterial growth rates (BGR) and bacterial growth efficiency (BGE) were determined during the initial stage of the experiment. Bacterial growth rate was calculated during the exponential growth phase which occurred between 0 and 36 hours. BGE is a measure of the carbon biomass production (bacterial production) relative to total carbon consumption (bacterial production + bacterial respiration) (del Giorgio and Cole, 1998). BGE was determined for the period between 12 and 24 hours. During this period lids were placed on all incubations and bottle manipulated to remove all air once the lid was applied. Respiration was calculated as a change in dissolved oxygen between 12 and 24 hours with a respiratory quotient of 1 assumed. Bacterial production was calculated as a change in bacterial biomass during the same period.

3.3.4 Sample processing and analysis

Bacteria samples were taken every 12 hours between 0 and 36 hours. Bacterial abundance and biomass samples (5 mL) were collected in sterile centrifuge tubes and fixed with 0.2 mL of concentrated 0.2 μm filtered formalin (37% Formaldehyde) and stored at 4ºC. In the laboratory, subsamples (2 mL) were stained with DAPI (4'6-diamindion-2-phenylindole) at a final concentration of 1 mg mL^{-1} for 15 minutes, and filtered through a polycarbonate black 0.2 μm pore-sized filter (Porter and Feig, 1980). Polycarbonate filters were mounted onto microscope slides and non-fluorescence immersion oil used. Slides were examined at a magnification of ×1000 using a fluorescence-equipped Olympus BX41 compound microscope. For each slide ≥500 total cells were captured using an Olympus DP72 camera and cellSens Standard software (version 1.3). Images were analysed for cell abundance and volume using CellC software (Selinummi et al., 2005). Bacterial biomass was calculated using the formula given by (Romanova and Sazhin, 2010). Dissolved oxygen measurements used to calculate bacterial respiration were taken using a calibrated HACH HQ20 LDO probe.

Sampling for DOC occurred on Days 0, 2 and 28. Samples were collected in pre-combusted (500ºC, 4 hours) 100 mL glass bottles, 0.45 μm filtered, acidified with 2N hydrochloric acid and refrigerated at 4°C. Samples were analysed in the laboratory by the high temperature combustion method (APHA, 2005). Nutrient samples were taken on day 0, 0.45 μm filtered and collected in pre-washed, sample rinsed 50 mL PET bottles. Samples were analysed for dissolved reactive phosphorus (DIP), dissolved

NO_2^- and NO_3^- (DIN), ammonium (NH_4), dissolved organic nitrogen (DON) and dissolved non-reactive organic phosphorus (DNP). All samples were analysed using a segmented flow analyser (OI Analytical Model FS3100) according to standard methods (APHA, 2005).

Specific ultraviolet light absorption (SUVA) was calculated for water collected on all sampling dates. SUVA provides a measure of the aromaticity of DOC. An increasing value of SUVA indicates increasing aromaticity (Traina et al., 1990). Filtered (0.2 μm) water samples were placed in quartz window cuvette and absorbance at 254 nm measured using a Varian Cary 50 Bio UV spectrophotometer. SUVA was calculated by dividing the UV absorbance at 254 nm (m^{-1}) by the concentration of DOC (mg C L^{-1}).

3.3.5 Data analysis

Multiple-regression analysis was performed using Sigmaplot ver.12. Multiple-regression analysis was carried out to create models for STC, LTC, STB%, LTB%, BGR and BGE. For each dependent variable we tested the incubations receiving nutrients separately from those without nutrients. Independent variables used included mean daily discharge (Q), mean 10 day antecedent discharge ($Q10$), DOC, DIN, NH_4, DON, DIP, DNP, SUVA and chlorophyll a. When performing analysis of BGR and BGE we also included STB% and STC. All data was first tested for normality using the Shapiro-Wilk test and non-normally distributed data was transformed (Ln [x+1]). Stepwise backward elimination was used to find the model which explained the most variance for each dependent variable.

STC, LTC, STB%, LTB%, BGR and BGE were analysed for differences between time (sampling date) and treatments (with and without nutrient addition) using permutational multivariate analysis of variance (PERMANOVA) with PRIMER + PERMANOVA software ver. 6 (Anderson, 2001). Euclidian distance was used as input for PERMANOVA analyses. Homogeneity of variance was tested using PERMDISP (Anderson et al., 2008). Data that was skewed was log transformed. Pair-wise t-tests were employed with PERMANOVA to test which treatments and time periods were significantly different. Monte Carlo asymptotic P-values were generated to increase the number of permutations used during t-tests.

3.4 Results

3.4.1 Initial Conditions

DOC concentrations varied between 2.95-12.98 mg L^{-1} across all experiments (Table 3.1). Concentrations were highest during the peak of flow for the larger inflow events occurring on 4 and 5 March 2012 on each river (Fig. 3.2). DOC concentrations became lower as conditions returned to base flow. On both rivers DIN and DON were always highest at the peak of flow, whilst NH_4 concentrations showed more variation (Table 3.1). On the Bega River DON dominated total nitrogen, whilst on the Clyde River, DIN contributed the most to total nitrogen concentrations. DIP was highest during the peak of flow on both Rivers, except during the first inflow event on the Clyde River where DIP remained low throughout March 2012. DNP values did not exhibit a clear pattern on either river. Chlorophyll *a* remained relatively low at all times on both rivers. Specific ultra-violet absorption (SUVA) varied between 3.10 and 4.81. On the Bega River the highest SUVA was recorded during both the highest discharge on 5 March and the lowest discharge on the 16 July 2012. On the Clyde River the highest SUVA values were recorded during base flow conditions on the 3 April 2012, and on the falling limb of the second inflow event on 14 June 2012.

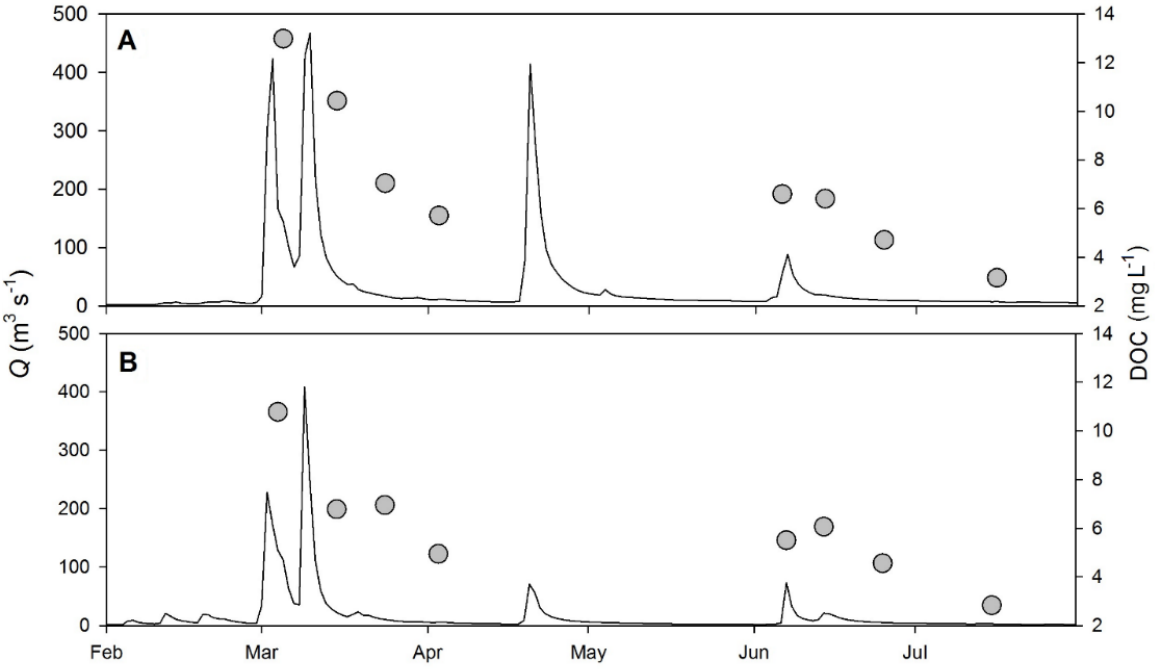

Figure 3.2. Time series of discharge and initial dissolved organic carbon values, March to July 2012 on the A) Bega River and B) Clyde River.

Table 3.1.Initial water quality and environmental variables on the Bega and Clyde Rivers, March to July 2012. Carbon and nutrient concentrations were taken at Day 0 of the experiment. Chlorophyll a, SUVA and discharge values reflect conditions on the initial day of sampling. Error is standard error, *n*=3.

	DOC (mg L^{-1})	DIN (µg L^{-1})	NH_4 (µg L^{-1})	DON (µg L^{-1})	DIP (µg L^{-1})	DOP (µg L^{-1})	C:N:P	Chlorophyll *a* (µg L^{-1})	SUVA (L mg^{-1} m^{-1})	*Q* (m^3 S^{-1})	Q_{10} (m^3 S^{-1})
Bega River											
5 Mar 2012	12.98	290	80	630	37	12	683:45:1	<1			
	±0.54	±20	±10	±50	±6	±4			4.81	143.60	107.80
15 Mar 2012	10.32	220	70	650	31	15	579:45:1	1.5			
	±1.08	±10	±10	±20	±5	±5		±0.28	4.17	50.42	168.05
24 Mar 2012	7.4	90	40	240	11	23	561:24:1	2.13			
	±0.54	±10	±0	±20	±2	±4		±0.35	4.23	14.63	26.01
3 Apr 2012	5.71	100	40	300	10	28	387:26:1	1.23			
	±0.27	±0	±0	±40	±0	±		±0.25	4.01	10.21	11.89
6 Jun 2012	6.59	180	130	280	29	51	212:16:1	3.3			
	±0.27	±20	±20	±20	±2	±8		±0.25	3.41	55.53	13.94
14 Jun 2012	6.79	190	40	280	16	37	330:36:1	1.2			
	±0.54	±10	±10	±20	±4	±6		±0.1	3.10	16.75	35.72
25 Jun 2012	4.71	150	220	140	14	26	304:28:1	1			
	±0.27	±10	±30	±10	±1	±2		±0	4.19	9.61	12.03
16 Jul 2012	3.15	120	30	200	6	19	325:55:1	2.2			
	±0.27	±0	±0	±10	±1	±2		±0.2	4.70	6.95	7.38
Clyde River											
4 Mar 2012	10.78	100	30	35	5	8	2138:28:1	<1			
	±1.08	±10	±0	±5	±0	±0			3.84	128.69	59.98
15 Mar 2012	6.78	160	30	23	5	6	1589:28:1	<1			
	±0.54	±20	±1	±2	±0	±0			3.70	18.15	100.81
24 Mar 2012	6.95	90	30	26	5	5	1792:32:1	<1			
	±1.08	±0	±1	±2	±0	±0			4.02	10.42	17.02
3 Apr 2012	4.95	60	20	7	6	10	798:32:1	<1			
	±0.27	±0	±0	±1	±0	±0			4.29	5.97	6.38
7 Jun 2012	5.51	50	20	27	30	29	241:4:1	1			
	±0.27	±10	±0	±10	±5	±5		±0	4.01	73.78	2.73
14 Jun 2012	6.06	40	20	33	15	29	355:5:1	<1			
	±0.54	±0	±0	±0	±4	±8			4.29	21.37	19.38
25 Jun 2012	4.56	40	10	15	7	19	452:3:1	1			
	±0.27	±0	±0	±0	±1	±2		±0	4.35	5.69	10.36
16 Jul 2012	2.95	50	20	12	7	10	447:11:1	1.33			
	±0.27	±10	±1	±4	±1	±1		±0.15	3.91	3.25	3.20

3.4.2 DOC bioavailability

BDOC significantly varied through time for all measures on both rivers (Table 3.1). Short-term BDOC (STC) varied between 0.13-2.22 mg C L^{-1} equivalent to STB% 2.5-22.8% (Fig 3.3). On the Bega River STC was highest on the 15 March and 14 June, one week after the peak of each main flow event, where most carbon consumption occurred during the first 48 hours. On the Clyde River STC was highest during the peak of each flow event. Long-term BDOC (LTC) varied between 0.40-3.62 mg L^{-1}, equivalent to LTB% between 9.1-31.0% (Fig 3.3). On both rivers LTC was greatest at the beginning of each flow event and decreased towards base flow conditions. The addition of nutrients (N and P) always led significantly increased carbon consumption on both rivers, with the exception of STC on the Bega River (Table 3.2). This was most pronounced on the Clyde River where the addition of nutrients led to BDOC a number of orders of magnitude higher than incubations without nutrients. Multiple-regression showed that mean 10 day antecedent discharge ($Q10$) and SUVA were the best factors for predicting STB%, STC and LTC on the Bega River (Table 3.3, Fig. 3.5A). On the Clyde River daily discharge (Q) and $Q10$ were used to create explanatory models for LTC (Table 3.3, Fig. 3.5B). All other bioavailability measurements were not significantly related to environmental variables.

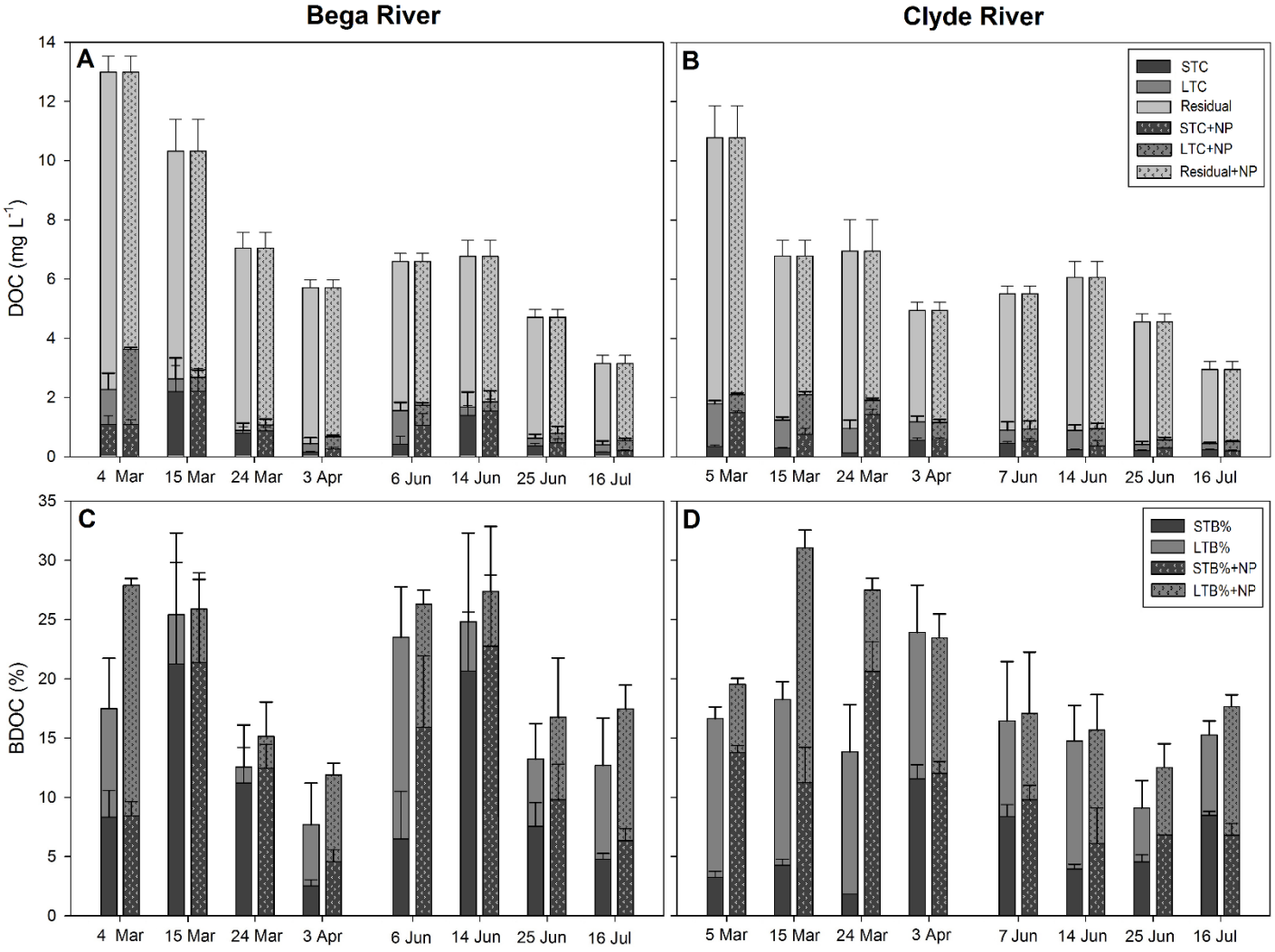

Figure 3.3. Mean Dissolved organic carbon bioavailability results on the Bega and Clyde Rivers, March to July 2012. A) short and long-term bioavailable DOC on the Bega River, B) short and long-term bioavailable DOC on the Clyde River, C) short and long-term percent bioavailability on the Bega River, D) short and long-term percent bioavailability on the Clyde River. Error bars are ± standard error, *n*=2.

3.4.3 Bacterial Response

Bacterial growth rates (BGR) significantly varied between sampling dates and ranged from 3.78-20.08 x10^7 cells h^{-1} L^{-1} on the Bega River (Table 3.2, Fig 3.4A). BGR was higher at the peak of discharge and reduced as conditions returned towards base flow. Multiple-regression analysis found *Q* to be the best factor for explaining variance in BGR on the Bega River (Table 3.3, Fig 3.5a). The addition of nutrients led to no significant differences in BGR on the Bega River. On the Clyde River BGR significantly varied through time and ranged between 1.59-17.44 x10^7 cells h^{-L} (Table 3.2, Fig 3.4B). In incubations receiving nutrient additions, BGR was higher towards the peak of flow and reduced towards base flow conditions. During the first three incubations on the Clyde River BGR was 4-fold higher when receiving nutrient additions compared to incubations without nutrients. In the incubations without the addition of nutrients, BGR were positively related to STC, and negatively related C:P (Table 3.3). When nutrient limitation was relieved *Q* was the best factor in creating explanatory models (Table 3.3, Fig 3.5B).

Bacterial growth efficiency (BGE) ranged between 3.46-68.18%, and significantly varied through time (Fig 3.4C,D). The highest BGE coincided with the lowest values for STB% on both rivers. On the Bega River, in incubations receiving nutrients, BGE increased from the peak of each inflow and was negatively related to initial DOC concentrations (Table 3.3). In the incubations not receiving nutrients and on the Clyde River there was no consistent pattern of variance in BGE through time. On the Clyde River, additions of nutrients led to significant increases in BGE on the 3 April and significant decreases on 7 June and 16 July (Table 3.2).

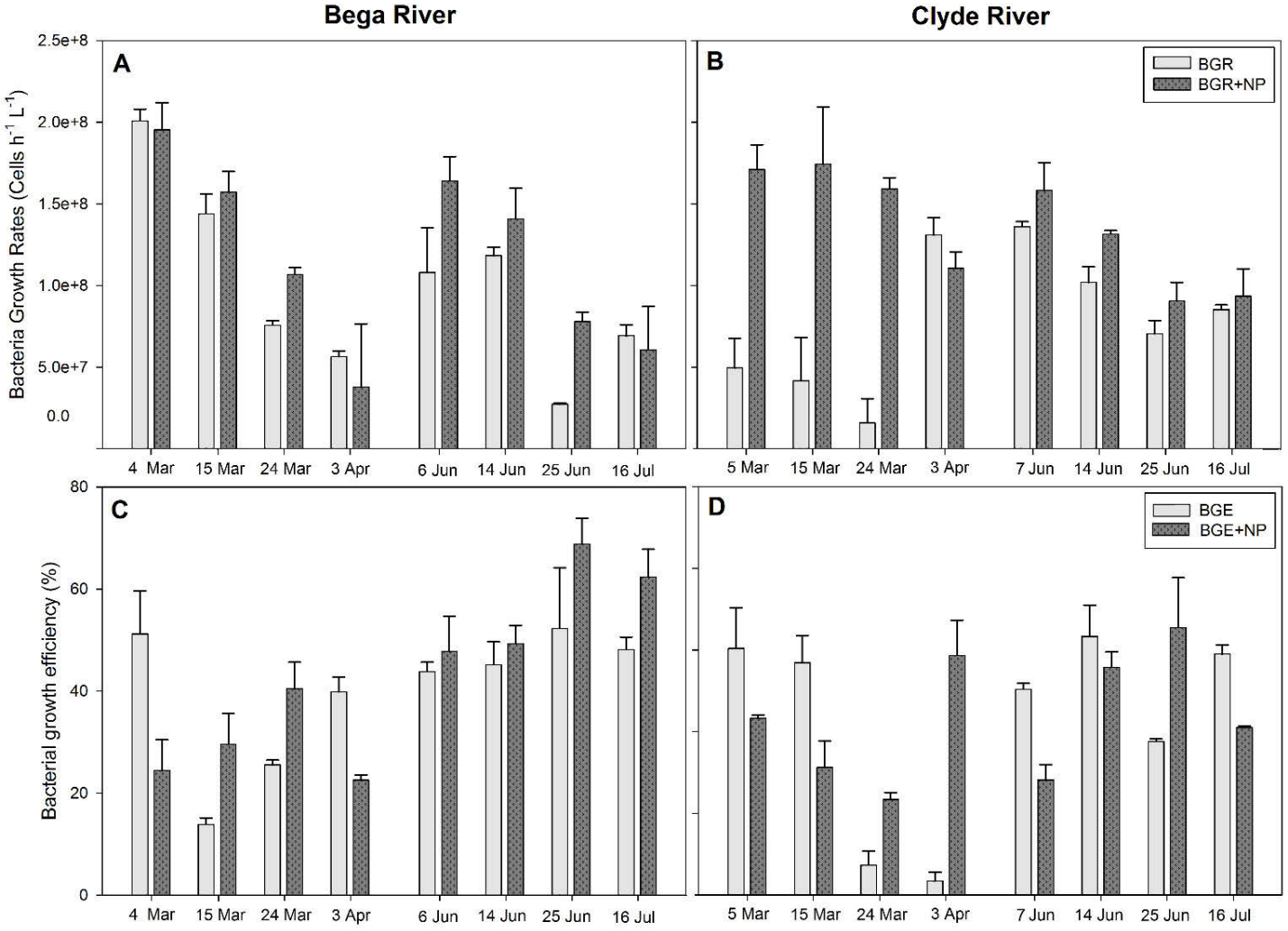

Figure 3.4. Mean Bacterial growth rates and efficiencies on the Bega and Clyde Rivers March to July 2012. A) bacterial growth rates on the Bega River, B) bacterial growth rates on the Clyde River, C) bacterial growth efficiency on the Bega River, D) bacterial growth efficieny on the Clyde River. Error bars are ± standard error, *n*=2.

Table 3.2. Results of PERMANOVA analysis. Pseudo *F*-values, degrees of freedom (df) and *P*-values for DOC bioavailability and bacterial growth rate and efficiency on Bega and Clyde Rivers.

	Between Time				Between Treatment				Time/Treatment interaction			
	Pseudo *f*	df	*P*	Perms	Pseudo *f*	df	*P*	Perms	Pseudo *f*	df	*P*	Perms
Bega												
ST%	8.58	**7**	**0.001**	999	3.87	1	**0.07**	999	0.54	7	0.792	998
LT%	5.04	**7**	**0.002**	997	5.53	1	**0.002**	999	0.26	7	0.96	999
STC	12.57	7	**0.001**	999	1.68	1	0.205	996	0.31	7	0.952	999
LTC	22.66	7	**0.001**	998	4.72	**1**	**0.049**	999	0.40	7	0.885	999
BGR	3.37	7	**0.028**	998	0.02	1	0.992	994	1.14	7	0.393	998
BGE	17.93	7	**0.001**	998	1.95	1	0.17	995	8.71	7	**0.001**	998
Clyde												
ST%	5.00	7	**0.005**	998	54.72	1	**0.001**	996	10.92	7	**0.001**	999
LT%	10.43	7	**0.011**	998	5.16	1	**0.003**	999	0.38	7	0.356	999
STC	67.46	7	**0.001**	997	12.21	1	**0.001**	999	13.55	7	**0.001**	997
LTC	12.51	7	**0.002**	998	21.39	1	**0.001**	999	1.89	7	0.161	999
BGR	26.69	7	**0.001**	997	3.37	1	**0.031**	997	5.47	7	**0.008**	998
BGE	9.46	7	**0.01**	997	19.81	1	**0.001**	998	18.40	7	**0.001**	999

Table 3.3. Multiple-regression results for the Bega and Clyde Rivers. Stepwise backward elimination was used to find the model which explained the most variance for each dependent variable. Only models that that were significant, $P<0.05$, are listed.

	Addition	Model	R^2	Adjusted R^2	V_{max}	N	F	p
Bega River								
STB	-	STB = 13.059 + 4.696LnQ_{10} - 5.601 SUVA	0.712	0.597	1.011	8	6.188	0.044
		S.E 12.744 2.842 1.512						
	NP	STB = 30.611 – 8.220 SUVA – 3.640 LnQ_{10}	0.796	0.714	1.011	8	9.729	0.019
		S. E 10.349 2.308 1.227						
LTB	-	-	-	-	-	-	-	-
	NP	-	-	-	-	-	-	-
STC	-	STC = -1.665 + 0.580 LnQ_{10}	0.829	0.801	1.000	8	29.154	0.002
		S.E 0.473 0.107						
	NP	STCN = 0.612 + 0.543 LnQ_{10} - 0.485 SUVA	0.902	0.862	1.011	8	22.909	0.003
		S.E 0.722 0.085 0.161						
LTC	-	LTC = -1.684 + 0.699 LnQ_{10}	0.845	0.819	1.000	8	32.694	0.001
		S.E 0.539 0.122						
	NP	LTCN = -2.052 + 0.858 LnQ_{10}	0.788	0.752	1.000	8	22.264	0.003
		S.E 0.801 0.182						
BGR	-	BGR = 16.333 + 0.470LnQ	0.649	0.590	1.000	8	11.082	0.016
		S.E 0.598 0.141						
	NP	BGR = 16.638 + 0.442 LnQ	0.681	0.628	1.000	8	12.807	0.012
		S.E 0.523 0.124						
BGE	-	-	-	-	-	-	-	-
	NP	BGE = 70.938 – 3.886 DOC	0.505	0.422	1.000	8	6.117	0.048
		S.E 12.151 1.571						
Clyde River								
STB	-	-	-	-	-	-	-	-
	NP	-	-	-	-	-	-	-
LTB	-	-	-	-	-	-	-	-
	NP	-	-	-	-	-	-	-
STC	-	-	-	-	-	-	-	-
	NP	-	-	-	-	-	-	-
LTC	-	LTC = 0.0596 + 0.245 LnQ	0.500	0.417	1.000	8	6.006	0.050
		S.E 0.396 0.100						
	NP	LTC = - 02.207 + 0.413 LnQ_{10}	0.647	0.589	1.000	8	11.021	0.016
		S.E 0.473 0.124						
BGR	-	BGR = 17.764 + 0.000633C:P + 2.756 STC	0.890	0.846	1.037	8	20.296	0.004
		S.E 0.310 0.000144 0.746						
	NP	BGR = 18.082 + 0.163 LnQ	0.610	0.545	1.000	8	9.372	0.022
		S.E 0.211 0.053						
BGE	-	-	-	-	-	-	-	-
	NP	-	-	-	-	-	-	-

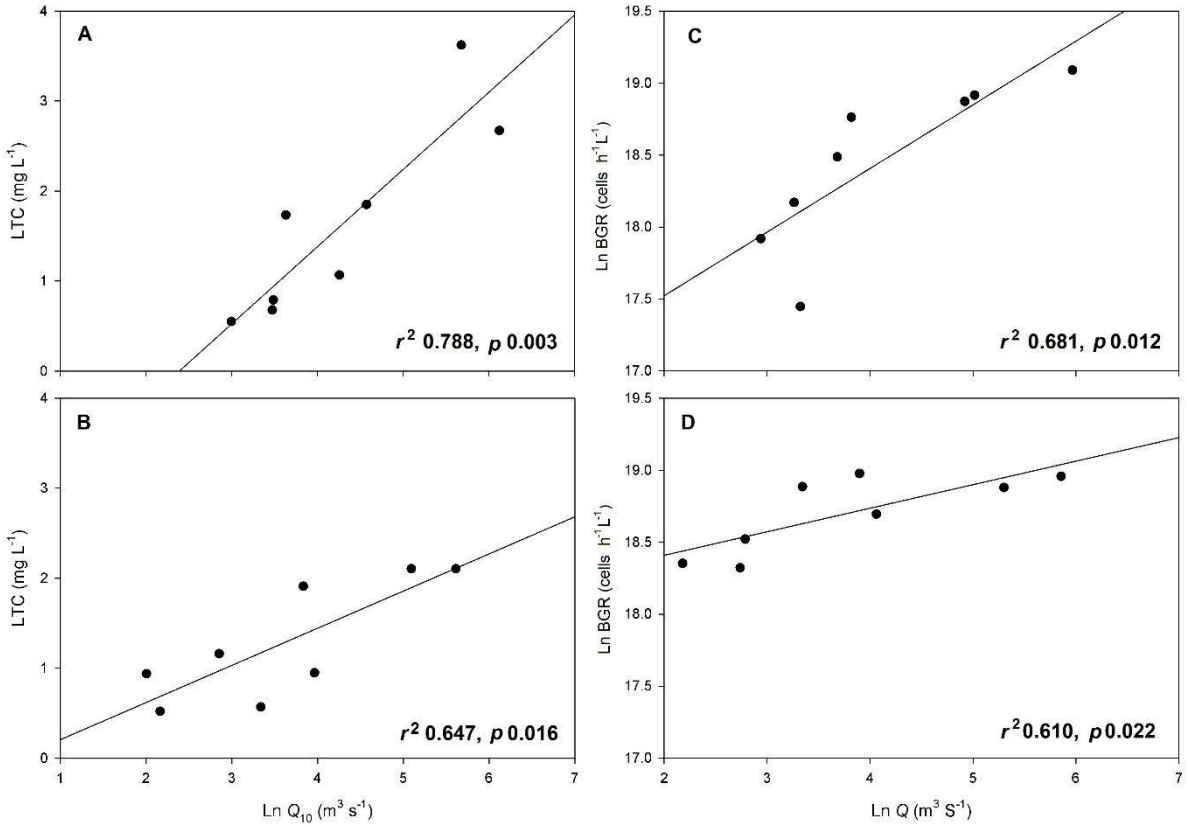

Figure 3.5. Multiple-regression analysis for long term bioavailable DOC and bacterial growth rates. A) LTC vs Ln *Q*10 on the Bega River, B) Ln BGR vs Ln *Q* on the Bega River, C) LTC vs Ln *Q*10 on the Clyde River, D) Ln BGR vs Ln *Q* on the Clyde River.

3.5 Discussion

Estuaries are important hotspots for the transformation and transportation of materials between the land and ocean. Much of the organic carbon transported from the catchments to the coast in temperate zones occurs during episodic inflow events (Raymond and Saiers, 2010; Webster et al., 1987). In the Bega and Clyde catchments we have previously found that discharge is the main factor correlated variance of DOC (Chapter 2). The role that these fluctuations have in the broader carbon cycle and the estuarine food web is largely controlled by the bioavailability of organic matter.

The bioavailability of DOC on the Bega and Clyde Rivers was highly dynamic. BDOC varied by a factor of nine in this experiment (Fig 3.3). LT% varied between 9-31%, traversing the full range of bioavailability reported in other estuaries (Cole et al., 2011; Hanisch et al., 2010; Sanderson et al., 2009). Hydrology appeared to play an important role in determining the lability of DOC to which discharge was strongly correlated.

Consistently LTC was highest at the peak of flow and reduced as conditions returned to base flow (Fig 3.3A,B). Whilst we are unaware of other studies in estuaries comparing bioavailability under different hydrologic conditions, some work has been conducted in upland stream systems. 30-day bioassay incubations on streams in Massachusetts (Wilson et al., 2013) and Alaska, USA (Fellman et al., 2009), both found DOC bioavailability increased in most circumstances during storms and was tightly coupled to discharge. Others have used different methods, such as plug flow bioreactors to find similar results from streams in Pennsylvania, USA (Gremm and Kaplan, 1998; McLaughlin and Kaplan, 2013). Changes in hydrologic flow paths during storm and inflows are cited by all authors as a key reason for changing DOC bioavailability. The results from the Bega and Clyde River estuaries presented here also indicate hydrologic flow paths are a likely controlling factor in estuarine DOC dynamics. During storm flows, precipitation percolates and travels horizontally through upper soil horizons and across terrestrial surfaces bringing relatively young DOC in rivers and streams which is transferred rapidly downstream to the estuary (Aitkenhead-Peterson et al., 2003; Sanderman et al., 2008). During base flow conditions water enters rivers mostly through lower mineral dominated layers bringing lower yields of DOC which are delivered downstream over longer times, allowing for microbial degradation in stream before export to estuaries (Battin et al., 2008).

Where the results from this study differ to those from upstream systems appears to be during the conditions following the peak of flow. The studies that have looked at finer scale temporal changes during storm events (Fellman et al., 2009; McLaughlin and Kaplan, 2013) have shown a rapid reduction in bioavailability during the falling limb of the hydrograph back to base flow conditions. Whilst BDOC (mg L^{-1}) reduced on the falling limb of the inflow events in our study, the bioavailability as a percentage (STB% and LTB%) remained high or increased (Fig 3.3). This is symptomatic of the hydrology in estuaries where the falling limb of inflow events is much more prolonged than in the upper catchment. Whilst longer transit times may allow for increased biological transformation of the DOC pool, the longer transit times required to reach the estuary, may also result in photodegradation which has been shown to increase the bioavailability of terrestrial DOC (Wetzel et al., 1995). This is supported by the fact that mean 10 day antecedent discharge provided a better factor than daily discharge for explaining variation in BDOC concentrations (Table 3.3, Fig 3.5).

Not all studies have shown a positive relationship between bioavailability and discharge. Wiegner et al. (2009) conducted 4 day bioassay incubations on the Wailuka River, Hawaii, USA, finding that base flow DOC was around 2.5 times more bioavailable than DOC during storms. The authors suggest this may be due to a switch from the DOC pool consisting of primarily autochthonous DOC during base flow to mainly allochthonous DOC during high flow conditions. Similarly Farjalla et al. (2006) tested the response of bacteria to water from the high and low point of the flow pulse in a flood plain lake on the Amazon River, Brazil. They found bacterial response and DOC bioavailability was much higher at the low water point due to higher primary production and autochthonous DOC input. Chlorophyll *a* concentrations were generally low all times during this study (<1-3.3 μg L^{-1}), making it unlikely that autochthonous DOC contributed to the total DOC pool to the same magnitude as allochthonous riverine DOC. The assumption that autochthonous DOC is always more bioavailable than allochthonous DOC may not always be true depending on the source of autochthonous carbon (Catalán et al., 2013).

The effect of changing hydrology on bioavailability is mediated through changes to DOC chemical composition. SUVA is one of the most commonly used measures to describe quality, and indicates humicity. SUVA has been shown to increase during storms reflecting an increased contribution to the DOC pool from sources high in humic substances (Vidon et al., 2008). There was no clear relationship between SUVA and discharge or DOC concentrations on the Bega and Clyde rivers. For example, the highest SUVA measurements recorded on the Bega River occurred on the two days with both the highest and lowest discharge (Table 3.1). This may indicate that changed flow paths during storms may not be the only source of humic substances to these estuaries. Other potential sources in estuaries can be related to outwelling (Cunha et al., 2003) or the resuspension of detrital material (Wainright and Hopkinson Jr, 1997). SUVA has also regularly been shown to have a negative relationship with DOC bioavailability (Fellman et al., 2008; Inamdar et al., 2011). SUVA was negatively related to STC and STB% on the Bega River though exhibited no clear relationship on the Clyde River or for long term measurements (Table 3.3).

The availability of nutrients such as nitrogen and phosphorus also affects the bioavailability of DOC (Thingstad et al., 1997) by limiting bacterial metabolism (Elser

et al., 1995). These nutrients may occur freely or may be bound in other organic compounds. The addition of nutrients in this experiment regularly led to increases in measurements of BDOC and bacterial growth. This can be seen most clearly on the Clyde River during the first inflow event between 4-24 March 2012 (Fig 3.3, Fig 3.4). These dates also corresponded with the highest C:N:P ratios (Table 1). These results highlight important differences between the catchments and potential influence of catchment land use. The Clyde River is a mostly undisturbed forested catchment, whist large parts of the Bega have largely been cleared for agriculture; agricultural land use and discharge from sewage treatment plants are the likely causes of higher nutrient concentration in the Bega River. These results suggest that land use changes that lead to increased nutrient inputs have the potential to greatly influence microbial processing of DOC.

These results support previous work on both rivers that suggested bacteria growth switched from carbon limitation to nutrient limitation after inflows delivered DOC to the estuary (Hitchcock and Mitrovic, 2013; Chapter 4). The results also support previous findings by Zweifel et al. (1993a) who showed in seawater culture experiments that the addition of nitrogen and phosphorus when excess available DOC was present, led to increased BGR and DOC bioavailability. There are only a few examples that perform tests of bioavailability both with and without nutrient additions (eg. Farjalla et al., 2006). Removing inorganic nutrient limitation clearly gives a better measure of potential lability of the carbon in the dissolved organic matter pool. Which technique provides a better reflection of *in-situ* conditions however is difficult to determine as both the DOC and nutrient pool *in-situ* is in constant flux, and often bound together in same compounds.

DOC bioavailability can also be indicated using bacterial growth rates, or bacterial production and respiration (Buffam et al., 2001; Farjalla et al., 2006). On the Bega and Clyde Rivers BGR were highest at the peak of discharge and reduced towards base flow conditions (Fig 3.4A,B). BGR was closely coupled with discharge (Table 3.3). The results for BGR showed a similar relationship to discharge as for BDOC, suggesting BGR could act as a good indicator of BDOC. Similar results were found by Buffam et al. (2001) who examined the response of bacteria in a bioassay of water collected during base and storm flow conditions. The authors found that BGR were

higher in the water collected during storm flow compared to base flow conditions. They suggest this was due to the larger volumes of BDOC present during storm flows.

Bacterial growth efficiency is often measured in bioavailability experiments and is used to indicate the proportion of carbon that is utilised for catabolic versus anabolic activities. BGE varied on the Bega and Clyde Rivers between 3.46-68.84%, similar to both the lower and upper limits of the range reported elsewhere for estuaries (del Giorgio and Cole, 1998, and references therein). Though the highest BGE coincided with lowest values for STB (Fig 3, Fig 3.4) the only time we were able to create any explanatory models via multiple-regression was on the Bega River where BGE was negatively related to initial DOC concentrations in incubations receiving nutrient additions. Farajalla et al. (2009) found BGE to be higher in Brazil during the low water period following a hydrological pulse coinciding with when DOC bioavailability was higher. The authors suggest that along with the presence of BDOC from autochthonous sources, nutrient supply has a strong influence on whether bacteria utilised DOC for growth, thus increasing BGE or whether carbon was remineralised via respiration, decreasing BGE.

The addition of nutrients in our experiments led to both decreases and increases in BGE. One explanation for this may be that when nutrient limitation is relieved during a period when DOC concentrations are low, those resources are partitioned primarily into growth. However, when nutrient limitation is relieved when DOC concentrations are high then both growth and respiration may increase. BGE also appeared not to be greatly influenced by BGR, which is consistent with previous findings of a review on BGE in aquatic systems (del Giorgio and Cole, 1998). The fact that BGE was highest when STB% was lowest does support to some degree Del Giorgio and Coles (1998) contention that bacteria maximise carbon consumption rather than growth efficiency. It appears that BGE is controlled by a complex set of factors including bioavailability and nutrient concentrations.

Another key factor that has been cited as influencing DOC bioavailability is the bacterial inoculum used in experiments. Leff and Meyer (1991) tested the response of bacteria to water from low and high discharge from a river in Georgia, USA. They used both a natural bacterial inoculum and another defined inoculum created with a range of bacteria from numerous sites. They found that discharge had little effect on

growth and production of the defined inoculum, however on a number of occasions the native bacterial response was significantly lower during high discharge. The authors suggest that the bacteria in these cases were likely inactive and unable to utilise BDOC, rather than the quality of the DOC itself causing the differences in measured bioavailability. Different bacterial communities can utilise different components of the DOC pool due to the varying range of enzymes they secrete for DOC degradation (Judd et al., 2006). In these experiments we used a native bacterial population for each river that was collected on the final sampling date during base flow conditions. It's possible that we may have received different measurements had we utilised bacteria from a different time or place. However, given the consistency of the relationship between bioavailability and discharge, it would be unlikely to change the major findings of this study.

The results presented here have important implications for our understanding of carbon cycling. In the Bega and Clyde estuaries, as in many coastal catchments, the majority of allochthonous DOC is delivered during episodic inflow (Chapter 2). This study demonstrates that the DOC during these periods is more bioavailable than during base flow conditions. As many studies of DOC bioavailability in estuaries have been conducted during base flow or unspecified flow conditions, it is possible that estimates of the flux of carbon between estuaries and the atmosphere has been significantly underestimated. There are also implications for estuarine food webs. The historical viewpoint that allochthonous DOC is largely unavailable to bacteria has influenced the idea that it is of little importance to higher trophic consumers. The presence of BDOC may potentially support important food web intermediaries such as zooplankton in some systems (Faithfull et al., 2012; Mitrovic et al., 2014). If the high growth rates recorded in this study are representative of those within the estuary following freshwater inflow events, then increased bacterial production may constitute a substantial resource subsidy to the heterotrophic food web.

Chapter 4 Different resource limitation by carbon, nitrogen and phosphorus between base flow and high flow conditions for estuarine bacteria and phytoplankton

4.1 Abstract

Freshwater inflows can deliver substantial inputs of allochthonous organic carbon to estuaries. The role that allochthonous dissolved organic carbon (DOC) has on structuring bacterial and phytoplankton communities is still not well understood. We performed a series of 1.25 L bioassay limitation experiments on the Bega and Clyde River estuaries in NSW, Australia, examining what resources limit bacteria and phytoplankton growth. We hypothesised that during base flow conditions bacteria would be carbon limited, and after high flow conditions they would be nutrient limited. A full factorial design was used with additions of carbon (glucose), nitrogen (KNO_3) and phosphorus (KH_2PO_4). During the experiments that took place during base flow conditions bacteria were always primarily C-limited. After high flow conditions, bacteria were P-limited on the Clyde River, and remained C-limited on the Bega River. Phytoplankton growth was limited at all times in each estuary, tending towards N-limitation on the Bega River and P-limitation on the Clyde River. During high flow conditions on the Clyde River, when bacteria and phytoplankton were both primarily P-limited, it appeared that bacteria were able to outcompete phytoplankton for nutrients. These results suggest that freshwater inflows and allochthonous DOC maybe important in structuring estuarine microbial ecosystems and individual estuaries may behave differently in terms of their limiting resources.

4.2 Introduction

Understanding the ways in which essential substrates such DOC and nutrients regulate bacterial production is crucial for understanding how estuarine systems function. In marine systems, bacterial productivity is closely coupled with primary productivity as bacterial populations are predominantly reliant on autochthonous carbon (Cole et al., 1988; Tranvik, 1992). In estuarine and coastal environments this relationship has been

shown to be less consistent due to the subsidy of allochthonous carbon (Almeida et al., 2005; Ameryk et al., 2005) .

The delivery of allochthonous DOC to estuaries can be conceptualised as two compartments, base flow and high flow (Buffam et al., 2001; Hinton et al., 1997). During base flow conditions water generally travels through lower mineral dominated soils collecting more refractory carbon (Frank et al., 2000; Ivarsson and Jansson, 1994). Hydrologic flow paths may differ between high and low flows (Aitkenhead-Peterson et al., 2003) with some evidence that allochthonous carbon is more bioavailable than base flow organic carbon (Kaplan and Newbold, 1995; Wikner et al., 1999; Chapter 3). Further, high flows have been found to deliver greater total annual loads of DOC than that of base flows to estuaries (Raymond and Saiers, 2010; Wiegner et al., 2009).

Resource bioavailability and stoichiometry are important for understanding resource limitation (Jansson et al., 2006; Kankaala et al., 2010; Thingstad and Lignell, 1997). Estuarine bacterial growth has been shown to be limited by carbon (Hitchcock et al., 2010; Hoikkala et al., 2009), nutrients (Cotner et al., 2000), co-limited by a combination of carbon and nutrients (Pinhassi et al., 2006), and limitation may vary both spatially and temporally (Pinhassi et al., 2006; Sala et al., 2002). The 'bacteria-phytoplankton paradox' suggests that because bacteria rely on phytoplankton for DOC they are in-essence controlled by primary production (Bratbak and Thingstad, 1985; Mindl et al., 2005). However, in the presence of excess allochthonous DOC, this reliance may be removed or diminished and in doing so change the relationship between phytoplankton and bacteria from one of commensalism to competition. In situations where bacteria and phytoplankton are competing for the same limiting nutrient, bacteria have been shown to be able to outcompete phytoplankton due to the greater surface area to volume ratio and the high affinity of bacteria for phosphorus, potentially suppressing primary production(Currie and Kalff, 1984; Jansson, 1993; Thingstad et al., 1993).

Enrichment bioassays are commonly used to investigate factors limiting phytoplankton (Hecky and Kilham, 1988) and bacterial growth (Kuparinen and Heinänen, 1993; Pomeroy et al., 1995; Zweifel et al., 1993b). Whilst a number of studies examining the differences between base and high flow have taken place in

freshwater systems (Buffam et al., 2001; Leff and Meyer, 1991; Wiegner et al., 2009), few have been conducted in estuaries (Wikner et al., 1999).

In this study, we performed bioassay limitation experiments in two south-eastern Australian estuaries with the aim of determining which key resources limit bacterial and phytoplankton production and how limitation may change following periods of high flow. To our knowledge this is the first bioassay limitation study that explicitly investigates changes in estuarine resource limitation between base and high flow conditions. With freshwater inflows to estuaries greatly reduced around the world and climate change causing changes to precipitation patterns, understanding this key question is of particular importance.

4.3 Methods

4.3.1 Study Sites

The Bega River estuary, NSW, Australia, (-36° 42' 43.64", +149° 54' 8.76") is a wave dominated estuary (Fig. 4.1) and drains a catchment 1941km^2 (Roy et al., 2001). The catchment is split into two sub-catchments, the Brogo River, regulated by Brogo Dam, and Bega River which has two smaller dams, Cochrane and Candello. The upper sections of the catchment consist of mainly forested areas, whilst the middle catchment is mostly cleared for agriculture, dominated by the dairy industry. The freshwater tidal section has been mostly cleared for agriculture and irrigated farmland, whilst the catchment surrounding the estuary itself is largely unmodified. Broad scale land clearing since colonisation has drastically altered the river and estuary via the transportation of sandy top soils into the river channel (Brooks and Brierley, 1997). The Clyde River estuary, NSW, Australia, (-35° 33' 11.44", +150° 11' 12.11") is a tidally dominated estuary and drains a catchment 1791km^2 (Fig. 4.1). The Clyde River is unregulated with no significant structures or water extraction. The catchment is almost entirely forested, with some forestry activities in the upper catchment. The lower sections of the estuary have a small urban settlement as well as oyster and other fisheries activities.

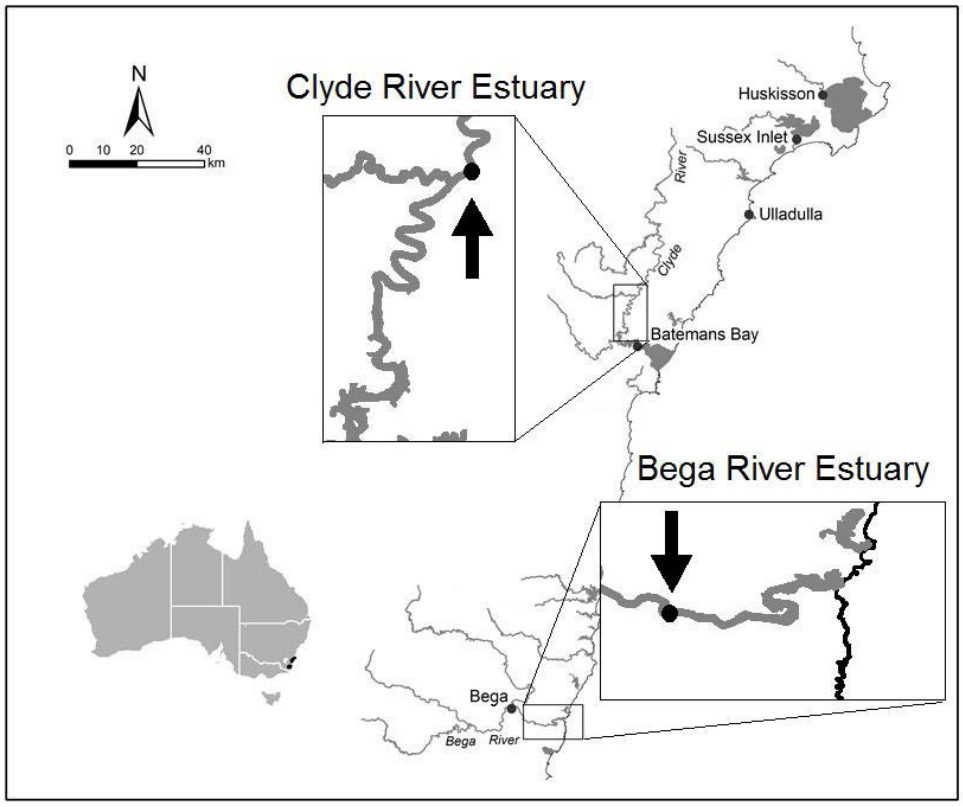

Figure 4.1 Map of the Bega and Clyde River catchments, NSW, Australia. Arrows indicate location of experimental sites which were in the freshwater tidal zone.

4.3.2 Experimental Design

In each estuary a single site was chosen at the lowest end of the freshwater tidal region where all experiments were conducted. Sites were chosen in the freshwater tidal zone as this is the first region that processes allochthonous material in the estuary (Muylaert et al., 2005). Four bioassay enrichment experiments were conducted in each estuary between November and January in both 2010 and 2011 under various discharge conditions (Fig. 4.2). This period was chosen as it is the time of maximum extraction within the Bega River estuary, and thus the period when the results are most pertinent to water management in the catchment. Experiments took place using 1.25 L clear PET bottles, containing estuary water collected from a depth of approximately 0.2-0.5 m which was filtered at 63µm using a plankton net to remove large zooplankton. Phytoplankton samples were taken before and after filtration to test the effect of pre-filtration with no observable effects on the phytoplankton community. The bottles were placed randomly in aluminium racks suspended from an anchored float in the

water column at a depth of 0.2 m at each site. They were positioned approximately 3 to 4 m from the shoreline with a northerly aspect to minimise shading.

A full factorial design was used with treatments including control with no additions, carbon (C) (10 mg glucose L^{-1}), nitrogen (N) (1 mg KNO_3 L^{-1}) and phosphorus (P) (0.6 mg KH_2PO_4 L^{-1}), in all possible combinations. These concentrations were chosen in order to sufficiently release bacteria or phytoplankton from any substrate limitation, and closely resemble concentrations within the Bega estuary after a period of high flow in June 2010 (Chapter 2). The experiments were conducted over a 72 hour period. Bacterial abundance and dissolved oxygen were measured at 24 hour intervals. Sampling for carbon, nutrients and chlorophyll *a* was conducted at 72 hours. All initial samples were taken from surrogate bottles, including surrogates to test C, N and P amendments.

In this paper we refer to limitation as a restriction on growth, based on an increase in biomass, measured as bacterial biomass and chlorophyll *a* over time (Cullen, 1991). We conceptualized 'primary limitation' as the first resource addition needed to significantly increase bacterial biomass or chlorophyll *a* above that of the control, and 'secondary limitation' as the next resource needed to create another significant increase once the primary limitation has been relieved.

4.3.3 Sample collection and analysis

Dissolved oxygen and temperature was measured using a HACH HQ20 LDO probe at 24 hour intervals. Conductivity and pH were measured with a Hydrolab Surveyor and MS5 Sonde probe at 0 and 72 hours. DOC (filtered at 0.45µm) and particulate organic carbon (POC) samples were collected in pre-combusted 100 mL glass bottles, acidified with 2N hydrochloric acid and refrigerated at 4°C. Samples were analysed in the laboratory by the High Temperature Combustion Method (APHA, 2005). Nutrient samples were collected in pre-washed and sample rinsed 50 mL PET bottles. Samples were immediately filtered (0.45µm) for dissolved reactive phosphorus, here in referred to as dissolved inorganic phosphorus (DIP), dissolved NO_2^- and NO_3^- here in referred to as dissolved inorganic nitrogen (DIN) and ammonium (NH_4). All samples were analysed using a segmented flow analyser (OI Analytical Model FS3100) according to standard methods (APHA, 2005). Carbon, nitrogen and

phosphorus ratios (C:N:P) are calculated in moles where C is DOC, N is DIN+NH_4 and P is DIP.

Bacterioplankton biomass samples (10 mL) were collected in sterile centrifuge tubes, fixed with 0.4 mL of concentrated 0.2 µm filtered formalin (37% formaldehyde) and stored at 4°C. In the laboratory, subsamples (2 mL) were stained with DAPI (4'6-diamindion-2-phenylindole) at a final concentration of 1 mg mL^{-1} for 15 minutes and filtered through polycarbonate black 0.2 µm pore-sized filters (Porter and Feig, 1980). Polycarbonate filters were mounted on to microscope slides and non-fluorescence immersion oil used. Slides were examined at ×100 using a fluorescence-equipped Olympus BX41 compound microscope. For each slide at least ten pictures of random views (≥500 total cells) were captured using an Olympus DP72 camera and CellSens Standard software (version 1.3). Images were analysed for cell abundance and volume using CellC software (Selinummi et al., 2005). Bacterial biomass was calculated using a conversion factor of 0.28 pg C μm^{-3} (Simon and Azam, 1989). Samples for chlorophyll *a* were determined by filtering 400 mL of water onto GF/C filters and were frozen until subsequent determination by Standard Methods (APHA, 2005) using the grinding technique and acetone as a solute with correction for phaeophytin. A detection limit of 1 µg L^{-1} was used for chlorophyll *a* analysis.

Discharge information was obtained from one gauging station on the Clyde River and two gauging stations on the Bega and Brogo Rivers, which were combined to give a single value for the Bega River. Flow duration curves were created for each river using daily flow values (ML d^{-1}) for the period 1960-2012 on the Clyde River and 1976-2012 on the Bega River. We conceptualised 'high flow' conditions as the period in the four weeks prior to the experiment where daily flow averages had exceeded the 75% percentile, and 'base flow' conditions were they remained under the 75% percentile.

4.3.4 Statistical analysis

Bacterial biomass and chlorophyll *a* were analysed for differences between treatments and time during each experiment using permutational multivariate analysis of variance (PERMANOVA) with PRIMER + PERMANOVA software ver. 6 (Anderson, 2001). Bray-Curtis distance was used as input for PERMANOVA analyses. Homogeneity of variance was tested using PERMDISP (Anderson et al., 2008). Data that was skewed

was log transformed. Where unequal dispersion was still present after transformation the chances of a type-I error were reduced by rejecting the null hypotheses at a probability of 0.01. Pair-wise t-tests were employed with PERMANOVA to test which treatments and time periods were significantly different. Monte Carlo asymptotic *P*-values were generated to increase the number of permutations tested during t-tests.

4.4 Results

4.4.1 Initial conditions

On the Bega River two experiments were conducted after a period of base flow in November 2010 and 2011 (Fig. 4.2). During these experiments DOC concentrations were at their lowest at 3.37 and 4.07 mg L^{-1}, respectively (Table 4.1). The January and December 2011 experiment took place after a period of high flow, where DOC concentrations were higher at 4.37 and 11.67 mg L^{-1} respectively. Dissolved inorganic nitrogen (DIN) concentrations varied between 40-70 µg L^{-1} and were highest in December 2011. Ammonium (NH_4) varied between 10-40 µg L^{-1}. Dissolved inorganic phosphorus (DIP) concentrations ranged from 11-15.4 µg L^{-1} with elevated values after high flows.

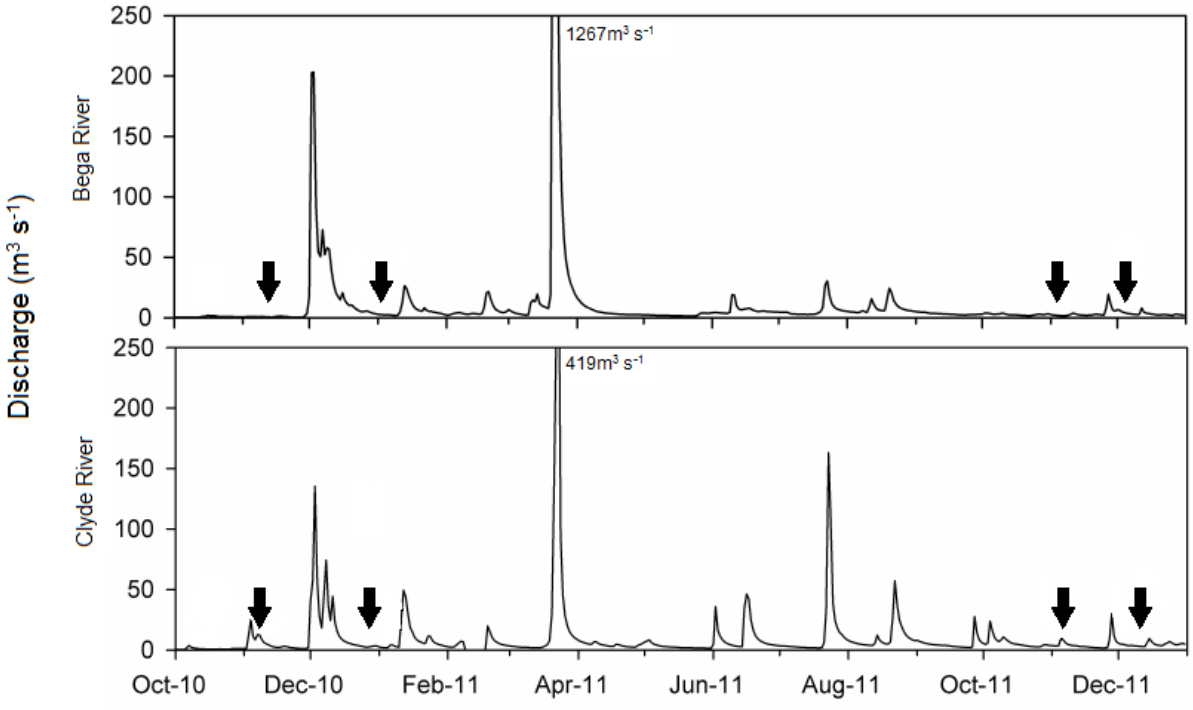

Figure 4.2 Discharge for the Bega and Clyde Rivers, October 2010 to December 2011. Arrows indicate the dates experiments commenced.

Table 4.1. Initial conditions and ambient carbon and nutrient mean concentrations at the start of the bioassay experiments, Bega and Clyde Rivers. Error is standard error (*n*=3). **n*=1, other replicates <1 µg L^{-1}.

	Flow Condition	DOC(mg L^{-1})	POC (mg L^{-1})	DIN (µg L^{-1})	DIP (µg L^{-1})	NH_4 (µg L^{-1})	Chlorophyll *a* (µg L^{-1})	C:N:P (mol)	Water Temp. (°C)
Bega									
09/11/2010	Base	4.07±0.12	0.26±0.22	50.0±0.0	11.3±0.3	40.0±0.0	4.93±0.30	929:18:1	21.5
04/01/2011	High	11.67±0.67	1.33±0.33	63.4±3.3	12.0±0.6	36.7±6.7	2.67±0.24	2587:18:1	24.1
05/11/2011	Base	3.37±0.12	0.63±0.12	66.7±3.3	11.0±1.0	10.0±0.0	1.37±0.20	790:15:1	21.4
06/12/2011	High	4.37±0.03	0.63±0.07	70.0±0.0	15.4±0.3	20.0±0.0	2.12±0.17	732:13:1	21.5
Clyde									
08/11/2010	High	9.00±0.06	2.10±0.09	20.0±0.0	5.0±0.0	80.0±0.0	<1	4642:44:1	22.6
27/12/2010	High	9.80±1.64	1.53±0.44	33.3±3.3	5.0±0.0	13.4±3.3	<1	5054:21:1	23.4
01/11/2011	Base	3.76±0.03	1.23±0.03	26.7±3.3	5.3±0.3	20.0±5.8	1.6*	1829:19:1	21.0
11/12/2011	High	5.30±0.00	1.90±0.00	40.0±0.0	5.3±0.3	20.0±0.0	1.8±0.18	2579:25:1	22.7

On the Clyde River the November 2011 experiment took place after a period of base flow (Fig. 4.2) which recorded the lowest DOC concentration at 3.76 mg L^{-1} (Table 4.1). All other experiments occurred after periods of high flow where DOC concentrations varied between 5.30 and 9.8 mg L^{-1}. DIN concentrations varied between 20-40 μg L^{-1} and DIP remained low ranging between 5-5.3 μg L^{-1}. Nutrient concentrations were always lower on the Clyde River than the Bega River, except in November 2010 when NH_4 concentrations on the Clyde were 80 μg L^{-1}.

4.4.2 Bacterial response

The addition of carbon or nutrients led to significantly different responses between treatments for all experiments (Table 4.2). On the Bega River, during both base and high flow experiments, bacteria were limited primarily by carbon. The treatments receiving carbon had approximately a 3-fold increase in bacterial biomass above the control in each experiment (Fig. 4.3A,C,E,G). Bacteria were secondarily limited by nitrogen in November 2010 and December 2011 with the addition of carbon and nitrogen being significantly higher than the control (Fig. 4.3B,D). Bacteria were equally limited by nitrogen and phosphorus in November and December 2011 where the treatments receiving either nitrogen or phosphorus in combination with carbon were both significantly higher than the control (Fig. 4.3F,H). At all times the treatments receiving the addition of carbon, nitrogen and phosphorus in combination stimulated the largest increases in bacterial biomass varying from 3-10 fold higher than the control (Fig. 4.3A,C,E,G).

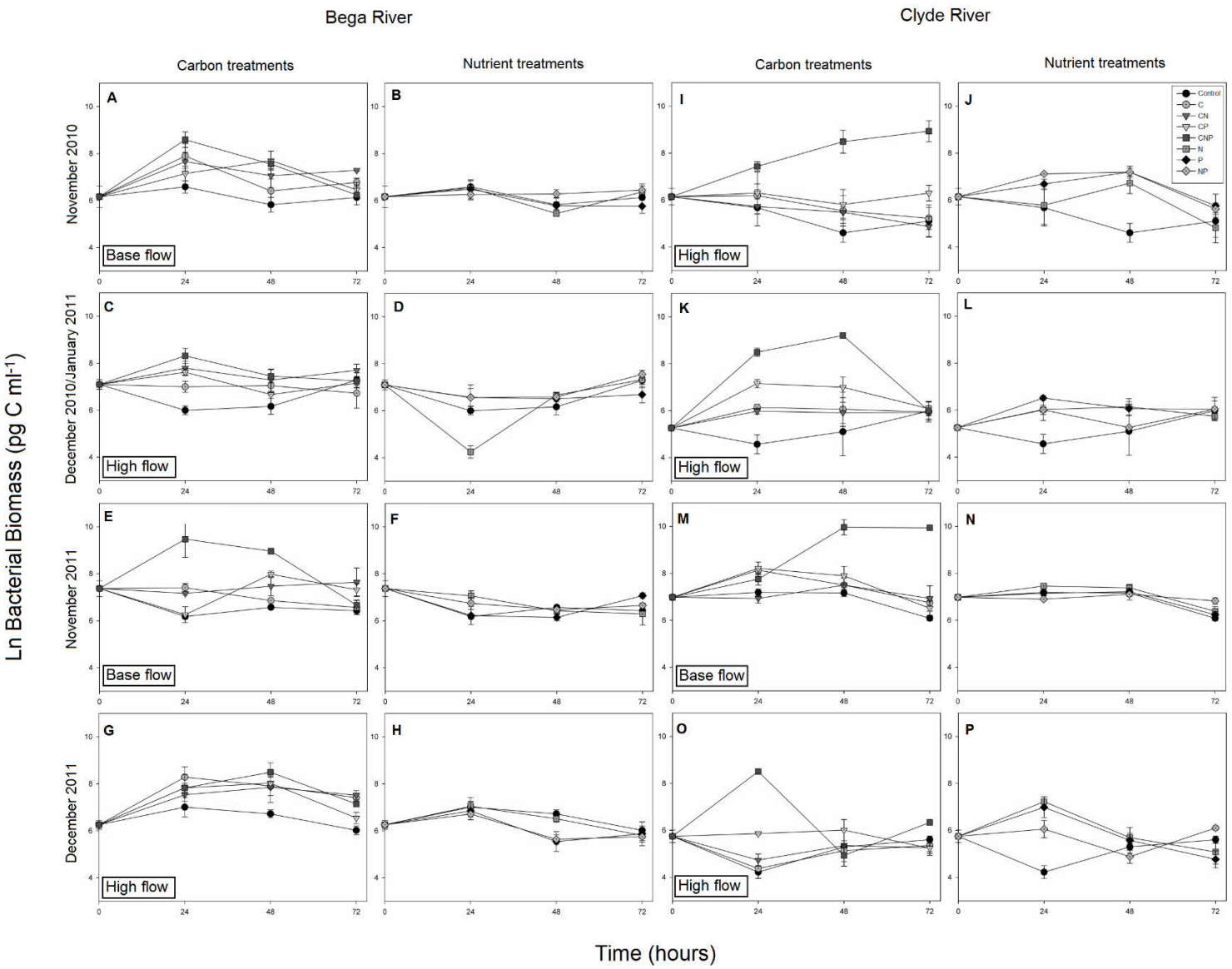

Figure 4.3 Bacterial biomass for each bioassay experiment. For each experiment treatments receving glucose (C) alone or in combination with nitrate (N) and phosphate (P) are seperated from treatments which received N and P alone or in combination for ease of viewing. (A-B) Bega River November 2010, (C-D) Bega River Januaray 2011, (E-F) Bega River November 2011, (G-H) Bega River December 2011, (I-J) Clyde River November 2010, (K-L) Clyde River December 2010, (M-N) Clyde River Novemeber 2011, (O-P) Clyde River December 2011. Controls are displayed on each graph. Error bars are Error bars are ± standard error (*n*=3).

On the Clyde River bacteria were primarily limited by phosphorus for two experiments taking place after high flow periods (November 2010 and December 2011) with additions of phosphorus leading to increases of 12 and 4 fold above the control, respectively (Fig. 4.3M,P). During these experiments, nitrogen was the secondary limiting factory in November 2010 and carbon in December 2011. During the other high flow period in December 2010, bacteria were equally limited by phosphorus alone and carbon alone where the addition of phosphorus led to a 6 fold increase of bacterial biomass above the control (Fig. 4.3L), and the addition of carbon to a 4 fold increase (Fig. 4.3K). During the base flow experiment, carbon was the primary limiting factor, with the addition of glucose leading to a 3 fold increase in bacterial biomass above the control (Fig. 4.3M). Phosphorus was the secondary limiting factor during this experiment. The highest bacterial biomass yield was always recorded for the treatment receiving combined enrichment of carbon, nitrogen and phosphorus which resulted in a 15-50 fold increase in bacterial biomass above the control (Fig. 4.3I,J,K,L). These results are summarised in Table 3.

4.4.3 Phytoplankton response

On the Bega River during the base flow experiments, phytoplankton were primarily limited by nitrogen in November 2010 (Fig. 4.4A) and expressed co-limitation between nitrogen and phosphorus in November 2011 (Fig. 4.4C) where chlorophyll *a* concentrations were significantly higher than the control. No secondary limitation was shown during base flow conditions. During high flow conditions, phytoplankton were primarily nitrogen limited and secondarily phosphorus limited in January 2011 (Fig. 4.4B), and equally limited by both in December 2011 (Fig. 4.4D). When phytoplankton were relieved from limitation, chlorophyll *a* concentrations were around 2-fold higher than the control, except during January 2011 when the treatments receiving nitrogen and phosphorus together were 12-fold higher than the control (Fig. 4.4B). The dominant phytoplankton genera remained similar for all experiments which included: *Ankistrodesmus, Cryptomonas, Cyclotella Dictyosphaerium, Monoraphidium, Nitzschia, Oocystis, Peridinium* and *Scenedesmus*

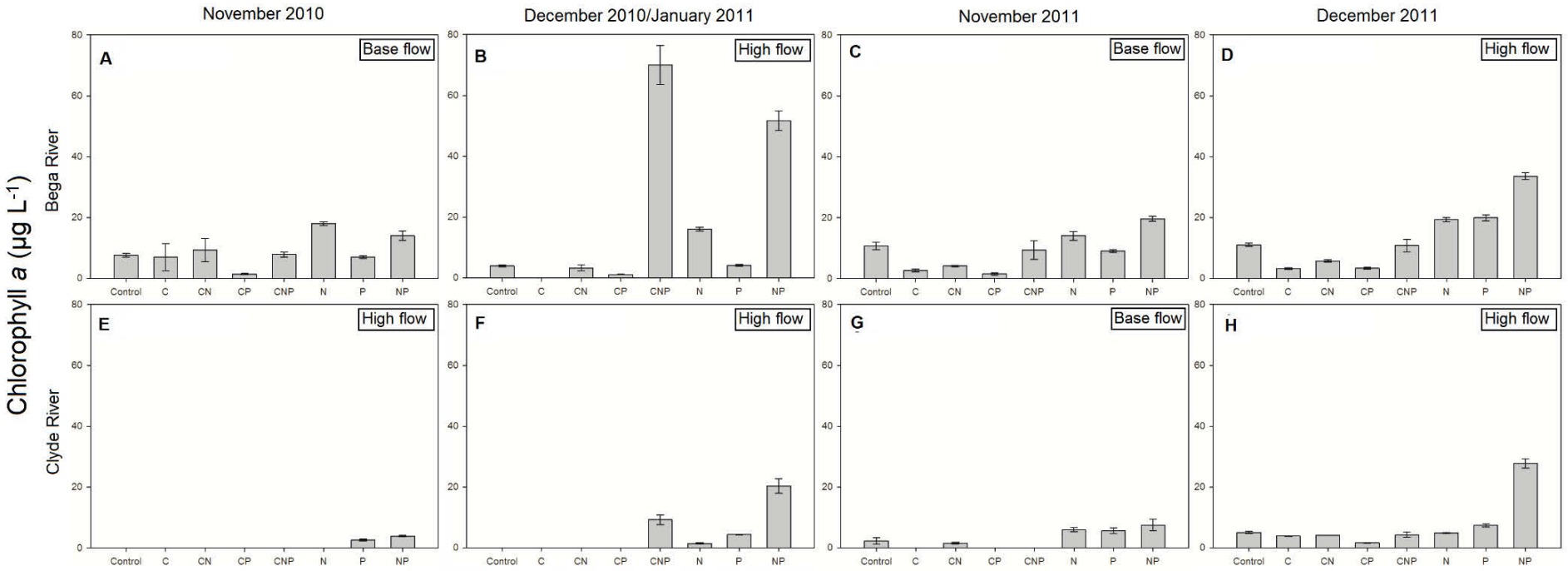

Figure 4.4 Chlorophyll *a* concentrations at 72 hours for each experiment. Where no bar/data is displayed chlrophyll *a* was below methodolgical detections limits. A) Bega River November 2010, B) Bega River Januaray 2011, C) Bega River November 2011, D) Bega River December 2011, E) Clyde River November 2010, F) Clyde River December 2010, G) Clyde River Novemeber 2011, H) Clyde River December 2011. Error bars are ± standard error (*n*=3)

On the Clyde River during base flow conditions, phytoplankton were equally limited by both nitrogen and phosphorus where the treatments receiving N, P and N+P had chlorophyll *a* concentration equally significantly higher than the control (Fig. 4.4G). During the high flow experiments phytoplankton were primarily limited by phosphorus during the two 2010 experiments where the treatment receiving P was significantly higher than the control (Fig. 4.4E,F). Co-limitation was expressed during the December 2011 experiment where only the treatment receiving N+P had chlorophyll *a* concentrations significantly higher than the control. During all these experiments chlorophyll *a* concentrations were consistently lower than those recorded for the Bega River (Fig. 4.4). In some cases chlorophyll *a* concentrations were below the detection limit, particularly during the November 2010 experiment. Results for phytoplankton limitation are summarised in Table 4.3. The dominant phytoplankton genera remained similar for all experiments which included: *Chroomonas, Cryptomonas, Cyclotella, Nitzschia, Oocystis* and *Peridinium*.

Table 4.2. Pseudo *F*-values, degrees of freedom (df) and *P*-values of bacterial biomass and chlorophyll *a* of main factors and interactions from PERMANOVA analyses for the bioassay experiments. nt= not tested

	Between treatments				Between hours				Treatment/hour interaction			
	Pseudo *f*	df	*P*	Perms	Pseudo *f*	df	*P*	Perms	Pseudo *f*	df	*P*	Perms
Bacteria biomass												
Bega												
Nov'10	5.5239	7	**0.001**	997	9.5079	3	**0.001**	998	1.8633	21	0.21	999
Jan'11	8.7848	7	**0.001**	999	6.6327	3	**0.002**	999	6.1246	21	**0.001**	998
Nov'11	9.5132	7	**0.001**	997	5.0375	3	**0.001**	998	4.5282	21	**0.001**	998
Dec'11	14.336	7	**0.001**	999	21.564	3	**0.001**	999	2.8654	21	**0.001**	998
Clyde												
Nov'10	8.1308	7	**0.001**	999	2.9527	3	**0.041**	999	1.9535	21	**0.015**	999
Dec'10	8.8343	7	**0.001**	999	14.249	3	**0.001**	998	3.0814	21	**0.001**	998
Nov'11	25.665	7	**0.001**	998	27.225	3	**0.001**	999	8.1219	21	**0.001**	999
Dec'11	5.0596	7	**0.001**	999	4.5567	3	**0.005**	999	5.3419	21	**0.001**	999
Chlorophyll *a*												
Bega												
Nov'10	8.4424	7	**0.001**	999	12.516	1	**0.001**	999	8.4424	7	**0.001**	999
Jan'11	72.36	7	**0.001**	997	188.89	1	**0.001**	998	72.36	7	**0.001**	998
Nov'11	7.7854	7	**0.001**	997	215.03	1	**0.001**	998	7.7854	7	**0.001**	999
Dec'11	25.88	7	**0.001**	998	812.42	1	**0.001**	999	25.88	7	**0.001**	999
Clyde												
Nov'10	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
Dec'10	40.006	3	**0.002**	966	nt	nt	nt	nt	nt	nt	nt	nt
Nov'11	5.98	4	**0.011**	990	nt	nt	nt	nt	nt	nt	nt	nt
Dec'11	17.716	7	**0.001**	998	329.14	1	**0.001**	998	17.716	7	**0.001**	998

Table 4.3. Summary of primary and secondary factors limiting bacterial and phytoplankton growth on the Bega and Clyde Rivers. *Co-limitation.

	Bega River				Clyde River			
	Nov-10	Jan-11	Nov-11	Dec-11	Nov-10	Dec-10	Nov-11	Dec-11
Bacterial Production								
Primary limitation	C	C	C	C	P	C,P	C	P
Secondary Limitation	N	N,P	N,P	N	N	N	P	C
Primary Production								
Primary limitation	N	N	NP*	N,P	P	P	N,P	NP*
Secondary Limitation	none	P	none	none	none	N	none	none

4.5 Discussion

4.5.1 Bacterial limitation, inflows and allochthonous DOC

On both the Bega and Clyde Rivers, ambient DOC concentrations were lower during the experiments at base flow, than they were in those that took place after high flows (Table 4.1). This is consistent with what was expected after high flows which have been shown to deliver significantly higher concentrations of DOC to estuaries than is transported during base flow conditions (Jassby et al., 1993), and is consistent with the relationship between DOC and discharge in both these estuaries (Chapter 2).

We hypothesised that during base flow conditions, when DOC concentrations were low, bacteria would be primarily C-limited. This was confirmed as on both rivers bacterial biomass was significantly higher in treatments receiving the addition of glucose compared to the control, indicating carbon was the primary limiting factor. This supports other studies that have shown bacteria are primarily limited by carbon when ambient bioavailable DOC concentrations are low (Hoikkala et al., 2009; Sala et al., 2002; Westhorpe et al., 2010), including across seasons in a similar temperate Australian estuary (Hitchcock et al., 2010). During these times bacterial carbon demands are likely to be primarily supported from autochthonous sources as allochthonous inputs are generally lower during base flow conditions.

Conversely, we hypothesised that after periods of high flow the associated high inputs of allochthonous DOC would relieve bacterial carbon limitation. The experiments that took place on the Clyde River generally support this hypothesis. Phosphorus was a limiting factor during high flow periods, as additions of phosphate led to significant increases of bacterial biomass above the control (Fig. 4.3J,L,P). P-limitation has been found in a number of other estuarine and coastal studies (Cotner et al., 2000; Pinhassi et al., 2006; Pomeroy et al., 1995; Tanaka et al., 2009). As ambient DOC concentrations were much higher during the high flow experiments it appears there was ample supply of bioavailable DOC to meet bacterial carbon demands. During these periods C:P ratios increased 2-3 fold as organic carbon increased and phosphorus concentrations remained at levels similar to base flow conditions (Table 4.1). Whilst DOC loads to estuaries have been correlated to discharge and precipitation, increases in phosphorus during discharge are more varied with undisturbed catchments, such as

the Clyde River, receiving lower inputs of phosphorus, than more disturbed and regulated catchments (Harris, 2001 and references therein). It therefore appears that in the Clyde River estuary bacterial growth is limited by available carbon during base flow periods, which is relieved during periods of high flow due to inputs of dissolved organic material, high in bioavailable carbon and low in phosphorus, resulting in bacteria being P-limited after high flow periods.

Complicating the results during high flow periods on the Clyde River is that on one of these occasions, bacteria appeared to also be equally limited by carbon as phosphorus, as the addition of glucose alone and phosphorus alone both led to significant increases in bacterial biomass compared to the control (Fig. 4.3K,L). We suggest this may be due to synergistic degradation of glucose and ambient DOM. Farjalla et al. (2009) conducted bacterial regrowth experiments on fresh macrophyte leachate and humic DOM. They found that whilst the bacterial production was higher in treatments with fresh leachate than humic DOM, the highest bacterial response occurred when both substrates were combined. One of the possible hypotheses they offer to explain those results is that extracellular enzymes such as β-glucosidase synthesised to degrade low-molecular weight DOC, may have assisted with the degradation of more complex compounds. If this is the case here, a proportion of high-molecular weight DOM such as humic and fulvic acids which contain phosphorus, may have been degraded in the glucose treatment which were not degraded in the control, temporally relieving the bacterial community of P-limitation. Another factor may be that whilst we only measured changes to the bacterial community as a whole, individual species may react differently. Bacterial communities are diverse and each species may have specialised requirements and DOC preferences (Judd et al., 2006). One bacterial group or species with lower phosphorus requirements may have responded to the addition of glucose as it is a readily degradable carbon source, but not to the likely less labile ambient DOC. Whilst testing the changes in community composition were beyond the scope of this experiment our observations during microscopy showed a shift towards larger ‘sausage-shaped’ bacteria on a number occasions in the presence of excess glucose as has been previously found by Havskum et al. (2003), indicating potential differential responses by some bacteria types.

Differing from the Clyde River, the high flow experiments on the Bega River do not support our hypotheses that high flows may relieve carbon limitation. During both experiments that took place after high flow periods bacteria were C-limited (Fig. 4.3C,G). We suggest these results are most likely due to a large proportion of the ambient DOC being unavailable to bacteria. We hypothesised that high flow DOC would be distinctly different to that which enters estuaries during base flow. Whilst this may be true, the peak of this high flow period occurred around 3 weeks prior to the beginning of the January 2011 experiment, and ambient DOC concentrations had reduced from 18.3 mg L^{-1} in the days after the peak of the flow (Chapter 2) to 11.67 mg L^{-1} three weeks later, suggesting the bulk of labile DOC may have already been utilised leaving mostly refractory DOC, possibly explaining these results. Additionally, we conceptualised flow conditions as either base or high flow, however, natural conditions may not fit so well into these dichotomous categories. For example whilst the two high flow experiments on the Bega River took place after inflow events, the sizes of these events differed greatly (Fig. 4.2) and the initial DOC concentration after the smaller inflow in December 2011 was only marginally higher than base flow conditions (Table 4.1). Understanding how the magnitude of an inflow event and the time since peak flow influence the biochemical nature of DOC is an important area for future study.

4.5.2 Phytoplankton, inflows and nutrients

Concentrations of chlorophyll *a* were fairly low at the start of all experiments, particularly in the Clyde River, where concentrations were below the detection limit on several occasions (Fig. 4.4E,F,G).The likely reason for these low initial chlorophyll *a* concentrations is due to the influence of riverine water during high flow periods. Monitoring work on the Clyde River suggests chlorophyll *a* concentrations are higher during base flow conditions (Chapter 2). Despite these low initial concentrations, the addition of nutrients stimulated phytoplankton growth in all experiments suggesting some limitation was always present (Fig. 4.4). The specific limiting nutrient varied through time on both rivers with phytoplankton being N-limited on the Bega River and P-limited on the Clyde River in 2010, and then equally limited or co-limited by both nitrogen and phosphorus in 2011 (Fig. 4.4). No clear pattern of a particular nutrient being limiting was seen for either base or high flows.

The role of nutrients and nitrogen to phosphorus ratios (N:P) in structuring estuarine phytoplankton communities has been thoroughly discussed in the literature (Elser et al., 2007; Howarth, 1988; Howarth and Marino, 2006; Justić et al., 1995; Nixon, 1995). In short, nitrogen limitation is the most commonly reported factor limiting primary production in estuaries, though phosphorus limitation has also been reported (Howarth, 1988; and references therein). This is due to the general pattern of low N:P ratios being common in estuaries. In this study N:P ratios mostly varied between 13 and 25 (Table 4.1), within a fairly close range of the theoretical optimal ratio of 16:1 (Redfield, 1958), and thus likely not pronounced enough to lead to a predictable pattern of limitation. The exception was for the first high flow experiment on the Clyde River where high concentrations of dissolved NH_4 led to N:P of 44:1 where phytoplankton where P-limited (Figure 4.4). Overall the results indicate that the N-limitation that is wide spread in northern hemisphere estuaries may potentially be less common for the phytoplankton communities of the Bega and Clyde Rivers.

4.5.3 Changing relationship between bacteria and phytoplankton

During the base flow periods, and at all times in the Bega estuary, bacterial and phytoplankton communities had different primary limiting factors, therefore no competition between the two was likely to be present. During these experiments, when bacterial C-limitation was released by the addition of glucose, the secondary limiting factor for bacteria and primary limiting factor for phytoplankton became aligned, likely leading to direct competition for available nutrients. Under these conditions it appears that bacteria were able to out-compete phytoplankton for nutrients on most occasions. For example the November 2010 experiment on the Bega River, phytoplankton were primarily and bacteria secondarily N-limited. In the treatment receiving nitrate alone, chlorophyll *a* was significantly higher than the control, however in the treatment receiving both glucose and nitrate combined, chlorophyll *a* was not significantly different from the control (Fig. 4.4). Previous studies have also found that when C-limitation has been relieved by the addition of glucose, primary production have been suppressed as bacteria have outcompeted phytoplankton for nutrients (Caron et al., 2000; Joint et al., 2002), most likely due to bacteria having a greater surface area to volume ratio compared to phytoplankton and a greater affinity for phosphorus (Currie and Kalff, 1984; Thingstad et al., 1993). Phytoplankton may

even release DOC when under stress from competing with bacteria for nutrients, in turn potentially supporting further increased bacterial production and exacerbating competition for nutrients (Mindl et al., 2005).

The potential exception to this appears to be when carbon, nitrogen and phosphorus are in-excess of bacterial and phytoplankton demands, as was the case during the high flow experiment on the Bega River in January 2011 (Fig. 4.3C and Fig. 4.4B). During this experiment, both the highest bacterial biomass yield and chlorophyll *a* results were achieved for the treatment receiving carbon, nitrogen and phosphorus combined. This was the only time chlorophyll *a* was higher with the addition of carbon combined with nutrients, than just the combination of nitrogen and phosphorus. There are a number of possible explanations for this result. First, nitrogen and phosphorus were in such surplus supply they exceeded the demand for both communities allowing high growth for both. Secondly, the phytoplankton community may not have been outcompeted for nutrients. Whilst many studies have shown bacteria can outcompete phytoplankton for available nutrients it seems some species, in particular small diatoms, that have a high surface area to volume ratio may in fact be able to outcompete bacteria under certain conditions (Havskum et al., 2003). Thirdly, the results may also be partially explained by a mutualism between phytoplankton and bacteria. The presence of glucose stimulating bacterial activity may have led to the remineralisation of organic nitrogen to inorganic forms thus supporting increased primary production (Kirchmann, 2000; Wheeler and Kirchman, 1986), in turn producing autochthonous DOC which may support increased bacterial production (Baines and Pace, 1991).

Competition between bacteria and phytoplankton appeared to be present during the high flow experiments on the Clyde River. During this time both bacteria and phytoplankton were primarily phosphorus limited and likely in direct competition (Fig. 4.3J,L,P, Fig. 4.4E,F,H). If allochthonous inputs of DOC associated with high flow periods lead to P-limitation of bacteria, and they are able to outcompete phytoplankton, it is possible that for the period that allochthonous labile DOC is present, primary production may be suppressed and the system tilted towards net heterotrophy. This suppression of primary production may be further exacerbated during periods of high flow due to increases in turbidity reducing light penetration

(Cole and Cloern, 1984; Jassby et al., 1993). Various studies have linked high flow periods and allochthonous inputs to shifts towards heterotrophy in coastal and estuarine systems (Almeida et al., 2005; Howarth et al., 1996; Russell et al., 2006). Wikner and Andersson (2012) recently found in a long term study of the Baltic Sea that increasing precipitation resulted in a consistent pattern of bacterial biomass production in excess of primary production. They suggest that increases in precipitation associated with climate change resulting in higher discharge and DOC loading to estuaries and coastal areas may have negative effects on primary production. A monitoring study of the Bega and Clyde River estuaries however indicates that discharge and allochthonous inputs do not appear to have negative impacts on primary production (Chapter 2).

4.5.4 Implications for flow management

Discharge and periods of high flow are regularly correlated with DOC loading to estuaries (eg. Jassby et al., 1993). Despite the common finding that bacterial growth is C-limited in estuaries (Hitchcock et al., 2010; Hoikkala et al., 2009), few studies have taken place examining how substrate limitation may vary between base and high flow conditions. In this study we found that during base flow periods, when bacteria were C-limited, the addition of DOC stimulated increased bacterial growth. We also found that bacterial resource limitation may shift between base and high flow conditions. These results suggest that discharge and allochthonous DOC may play an important role in the functioning of estuarine bacterial dynamics and this may have implications for microbial food webs.

Globally, regulation and extraction in coastal catchments has drastically changed the patterns of freshwater inflows to estuaries (Gillanders and Kingsford, 2002, and references therein). Whilst a range of impacts associated with reduced flows have received a great deal of attention, the impact of a potential reduction in allochthonous DOC delivery to estuaries remains an area where more research is needed (Scheltinga et al., 2006). The Bega River catchment is regulated by a number of dams, and water is extracted for agricultural and urban purposes, which has had significant impacts of ecosystem health (HRCN, 1998). As a result, environmental flow rules have been developed which include provisions to protect minimum low flows (NOW, 2011). Based on the results of these experiments, the protection of high inflows may support

increased bacterial production in the estuary. If nutrient loading is high during these flows primary production may also be stimulated, though any potential stimulation may be limited by competition for nutrients with bacteria. Such changes in productivity may also have positive implications for higher trophic levels if bacterial production is efficiently consumed by zooplankton such as cladocerans and copepods (eg. Bartels et al., 2012; Hoffman et al., 2008).

4.6 Conclusion

The results of these bioassay experiments supported our hypothesis that bacterial growth would be C-limited during base flow conditions. During high flow conditions bacterial growth was P-limited on the Clyde River, however remained C-limited on the Bega River. Phytoplankton growth was limited at all times in each estuary, tending towards N-limitation on the Bega River and P-limitation on the Clyde River. During low flow conditions bacteria and phytoplankton had different limiting factors. However, during the experiments after high flow conditions on the Clyde River, bacteria and phytoplankton were both P-limited. It appeared that bacteria were able to out-compete phytoplankton for nutrients when in competition. These results suggest that freshwater inflows may be important to the functioning of estuarine microbial ecosystems.

Chapter 5 Zooplankton responses to freshwater inflows and organic matter pulses in a wave-dominated estuary

5.1 Abstract

Freshwater inflow events play a major role in structuring estuarine zooplankton communities. Inflows affect zooplankton directly through advective forcing and changes in salinity, and indirectly through changes to resources via the delivery of organic carbon and nutrients which can stimulate microbial and primary production. Understanding how these multiple driving forces influence zooplankton community structure, density and diets during freshwater inflow events is an area that requires further study. Here we investigate changes to estuarine zooplankton assemblage structure, density and $\delta^{13}C$ stable isotopes during a period of highly variable freshwater inflow in the Bega River estuary, Australia. High inflows resulted in a reduction of salinity and a shift in the zooplankton assemblage structure from purely estuarine taxa towards freshwater taxa. The density of rotifers, cladocera, and in the upper estuary copepods, increased following inflows, concurrent with increases in dissolved organic carbon concentration and bacterial biomass. Redundancy analysis found environmental variables including discharge, salinity and bacterial biomass explained 66-73% of zooplankton variation. Stable isotope results indicated all copepod and cladocera species tested were predominantly supported by allochthonous carbon from terrestrial sources. These results provide important evidence that freshwater inflows play a critical role in structuring zooplankton assemblages and supporting increased production through the delivery of allochthonous organic carbon.

5.2 Introduction

Freshwater inflows (FWI) are a major structuring force in estuaries and have a strong influence over many biogeochemical processes. FWI increase hydrological connectivity between aquatic and terrestrial environments by wetting benches and floodplains and supplying resources to rivers (sensu Junk et al. 1989). FWI events, such as high inflows following storms, can lead to large, short-lived pulses in organic matter entering estuaries (Ford et al., 2005). The fate of this large flux of energy,

predominantly in the form of dissolved organic carbon (DOC), and its effects on food webs is a key question in estuarine science. It has important implications for our understanding of estuarine productivity, basal resources for food webs and coastal carbon cycling (Bianchi, 2013).

Zooplankton are key components of estuarine food webs and act as important intermediaries between fish and lower trophic levels (Murphy et al., 2012). There is a tight coupling between zooplankton and biogeochemical changes (Sugimoto and Tadokoro, 1997). FWI events can rapidly alter the composition of planktonic communities (Hoover et al., 2006). Direct changes induced by FWI events can be a result of physical forcing such as advection, which may deliver freshwater species from upstream to the estuary and flush zooplankton within the estuary to the coastal zone (Kobayashi et al., 1998). Changing salinity related to inflows can also lead to successional changes between freshwater, estuarine and marine zooplankton assemblages (Primo et al., 2009).

FWI can also influence zooplankton indirectly through changes in availability of key food resources such as phytoplankton and heterotrophic protists. Inflows may deliver increased nutrients promoting phytoplankton growth or conversely they may deliver suspended sediment, which reduces light availability and primary production (Saeck et al., 2013). DOC is also delivered during FWI, which drives microbial food web production (Findlay et al., 1992). Responses of zooplankton to these flow-influenced changes in basal food resources can occur over a period of days to weeks (Hitchcock et al., 2010; Karlsson et al., 2007; Mitrovic et al., 2014) and may vary depending on a range of complex factors such as temperature, season, geomorphology and community composition (Lawrence et al., 2004; Lloyd et al., 2004).

The role and fate of DOC delivered to estuaries during FWI events is currently a hot topic in the literature. Until recently the general view in estuarine and coastal systems has been that allochthonous organic carbon loading does not support zooplankton production due to it being a poorer food source than phytoplankton, often requiring more trophic steps in the food web before reaching zooplankton consumers (Fenchel, 2008). There have however been recent studies in estuaries that have shown that the carbon needs of zooplankton can be met by a mixture of autochthonous and allochthonous carbon sources (Abrantes et al., 2013). Studies using stable isotope

signatures to trace the sources of organic carbon supporting zooplankton have shown changes in the contribution of allochthonous DOC to zooplankton diets following FWI events. For example Hoffman et al. (2008) showed that zooplankton were predominately supported by autochthonous DOC during dry years, with a switch towards greater use of allochthonous DOC during wet years.

Despite their fast response times and links to higher trophic levels, there have been relatively few studies investigating zooplankton responses to freshwater inflow events in estuaries. Kimmel and Roman (2004) reported on a 16 year data set of approximately monthly sampling that found that freshwater inflows mainly effected the habitat parameters and predators of copepods in Chesapeake Bay, USA. Similarly a long-term study in San Francisco estuary found discharge induced no bottom-up resource influence over zooplankton (Kimmerer, 2002). Other shorter term studies that have focused on higher sampling frequency have provided insights into zooplankton responses to specific inflow events . Indeed, Hoover et al. (2006) showed that nutrient loading following storms in Kanohe Bay, Hawaii, led to rapid successional changes which altered the bottom-up and top-down factors controlling zooplankton. Appropriate temporal resolution, monitoring changes in zooplankton across days to weeks, is required to better understand how resource availability influence zooplankton assemblages and alter the contribution of autochthonous verse allochthonous carbon after inflow events.

The Bega River estuary, NSW, Australia, is a wave-dominated estuary with a small estuarine volume to catchment ratio, suggesting it is highly susceptible to environmental changes associated with FWI. Previous work we have conducted in this estuary has found discharge to be a main factor influencing organic carbon dynamics (Chapter 2) and during these periods organic carbon is more bioavailable (Hitchcock and Mtrovic 2015; Chapter 3). The estuary bacterioplankton have been shown to be limited by carbon resulting in increased bacterial production following increases in DOC (Hitchcock and Mitrovic, 2013; Chapter 4). The main objective of this study was to investigate how the estuarine zooplankton community would respond to a period of highly episodic FWI on the Bega River. We hypothesised that during high FWI: 1) zooplankton assemblage structure would be dominated more by freshwater species; 2) the zooplankton density would increase with organic carbon loading; and 3) the

zooplankton would be more supported by terrestrial carbon compared to low flow periods. To test these hypotheses we monitored discharge, physical and chemical changes in the estuary, zooplankton density, assemblage structure and $\delta^{13}C$ stable isotope signatures during a period of highly episodic freshwater inflow.

5.3 Methods

5.3.1 Study site

The Bega River estuary, NSW, Australia, (-36° 42' 43.64", +149° 54' 8.76") is a wave dominated estuary (Fig. 5.1). The estuarine volume, 6371 ML, is relatively small compared to its catchment of 1941km^2. The catchment is split into two sub-catchments, the Brogo River, regulated by Brogo Dam and Bega River which has two smaller dams, Cochrane and Candello. The upper sections of the catchment consist mainly of forested areas, whilst the middle catchment is mostly cleared for agriculture, dominated by the dairy industry (Brierley et al., 1999; Tozer et al., 2010). The freshwater tidal section has been mostly cleared for agriculture and irrigated farmland however, the rest of the estuary is largely unmodified. Broad scale land clearing since colonisation has drastically altered the river and estuary via the transportation of sandy top soils into the river channel (Brooks and Brierley, 1997). Mean annual rainfall is 597 mm and was above average in 2012 at 844 mm. We selected two sampling stations for this study, station 1 which is oligohaline under base flow conditions and station 2 which is mesohaline (Fig. 5.1).

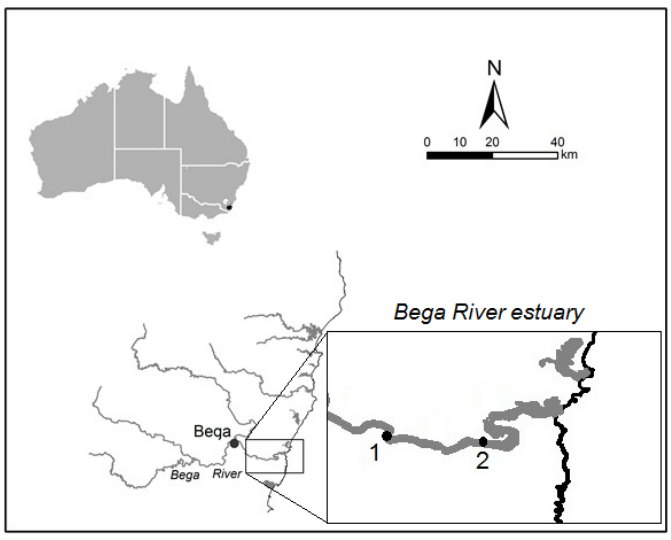

Figure 5.1. Bega River estuary, south-east Australia. Inset: the tidal section with sampling stations 1 and 2.

5.3.2 Sampling procedures and analysis

Between January 2012 and October 2012, sampling was performed on 10 occasions during a period of highly episodic inflow. Sampling was performed moving upstream on the ebb tide within 0-2 hours of high tide. Discharge information was obtained from two gauging stations on the Bega and Brogo Rivers, approximately 5km upstream of the tidal limit, which were combined to give a single value. Daily water temperatures were also recorded by the Bega River gauging station. Conductivity, dissolved oxygen, pH and site specific temperature were measured with a Hydrolab Surveyor 4 and MS5 Sonde probe. Turbidity samples were measured using a Hach 2100 turbidimeter.

Samples for nutrients, chlorophyll a, bacterial biomass and phytoplankton were taken in triplicate by hand at 20 cm below the water surface. Depth integrated samples were deemed not necessary due to the shallow nature of the sampling sites (0.5 to 1.0 m depth). Dissolved organic carbon (DOC) samples were collected in pre-combusted and sample rinsed 100 mL glass bottles, 0.45 µm filtered, acidified with 2N hydrochloric acid and refrigerated at 4°C. Samples were analysed in the laboratory by the High Temperature Combustion Method (APHA, 2005). Nutrient samples for dissolved reactive phosphorus (DIP), dissolved NO_2^- and NO_3^- (DIN), ammonium (NH_4), total nitrogen (TN), total phosphorus (TP) were collected in pre-washed and sample rinsed 50 mL PET bottles, dissolved samples filtered to 0.45 µm frozen and later analysed using a segmented flow analyser (OI Analytical Model FS3100) according to standard methods (APHA, 2005).

Chlorophyll *a* was determined by filtering 500 mL of water onto GF/C filters. Filters were frozen until subsequent determination by standard methods (APHA, 2005) using the grinding technique and acetone as a solute with correction for phaeophytin. Chlorophyll *a* had a detection limit of 1 µg L^{-1}. Phytoplankton samples were preserved with Lugols iodine and subsequently counted using a calibrated Lund cell and compound microscope after concentration by sedimentation in a measuring cylinder (APHA 1998). Counting precision was ± 20% (Hötzel and Croome, 1999). Phytoplankton were identified to genus level (Prescott, 1984).

Bacterial biomass samples were collected in sterile 10 mL centrifuge tubes and fixed with 0.4 mL of concentrated 0.2 μm filtered formalin (37% Formaldehyde) and stored at 4°C. In the laboratory, subsamples (2 mL) were stained with DAPI (4'6-diamindion-2-phenylindole) at a final concentration of 1 mg mL^{-1} for 15 minutes, and filtered through a polycarbonate black 0.2 μm pore-sized filter (Porter and Feig, 1980). Polycarbonate filters were mounted onto microscope slides with non-fluorescence immersion oil. Slides were examined at ×100 using a fluorescence-equipped Olympus BX41 compound microscope. For each slide images were captured using an Olympus DP72 camera (>500 total cells per sample) and cellSens Standard software (version 1.3). Images were analysed for cell abundance and volume using CellC software (Selinummi et al., 2005). Bacterial biomass was calculated using the formula of Romanova and Sazhin (2010).

Samples for zooplankton enumeration were taken in duplicate at each station by 1 minute horizontal surface tows at two knots using a 40 cm diameter 45 μm plankton net and preserved with 50% ethanol. Surface tows were used due to the shallow water depth (<1 m) at both stations. Zooplankton density (individuals m^3) was estimated by counting consecutive aliquots until 100 specimens of each taxon were counted or until 50% of the sample was counted. Organisms were identified to the highest taxonomic resolution feasible. Density was not determined for samples taken on 5th March 2012 due to difficulty in sorting because of high levels of suspended sediment and particulate material.

Zooplankton samples for stable isotope analysis of $\delta^{13}C$ (^{13}C:^{12}C) were taken on 8 occasions using single 10 minute horizontal tows at 2 knots with a 30 cm diameter 125 μm plankton net. A larger mesh net was used as we were only interested in collecting mesozooplankton that could be sorted easily by hand. Samples were placed in 0.45 μm filtered water for 4 hours for gut clearance before being preserved with 85% ethanol (Feuchtmayr and Grey, 2003). When sorting samples for stable isotope analysis, specimens were first thoroughly rinsed with reagent grade water to remove ethanol. Dominant copepods and cladocera were sorted into species by hand using a stereo microscope and forceps before being placed in 1 mol L^{-1} HCL bath for 2 hours to demineralise dissolved inorganic carbon. Samples were then washed with reagent grade water and placed directly into tin cups and dried at 60°C for 24 hours. Samples

were only analysed if at least 1 mg of dry weight was generated. Samples for potential source materials were collected in December 2011. Samples of terrestrial material were collected of dominant tree and grass species (5 species) and soil (2 samples) surrounding the estuary. Autochthonous carbon samples were sourced from benthic algae collected at three separate, shallow, near shore areas. Each sample was dominated by the same assemblage of *Geitlerinema* and *Leptolyngbya* with smaller numbers *Dictyosphaerium*, *Scenedesmus* and *Nitzschia* present. Phytoplankton were not analysed due to the inability to separate taxa from other components of particulate organic carbon; a common issue for phytoplankton in isotope studies (Karlsson et al., 2014). However, the species present in the benthic algal samples were also present in the phytoplankton samples during the study. Samples were dried for 24 hours at 60°C, ground with a mortar and pestle, weighed and placed in tin capsules. All processed samples were shipped to Griffith University where they were analysed using a continuous flow-isotope mass spectrometer (GV Isoprime Eurovector EA 3000, Manchester, UK). All results were determined against laboratory standard reference material IAEA-CH-6.

5.3.3 Data analysis

Redundancy analysis was performed using CANOCO ver 4.5 to analyse the environmental factors that affected zooplankton density and assemblage structure (Braak and Šmilauer, 2002). Separate analysis was performed for copepods and nauplii, cladocera and rotifers. All samples and variables were subjected to Shaprio-Wilk's normality test and all samples with a non-linear distribution were log transformed (Ln x+1). The explanatory environmental variables were selected using automatic forward selection. The variables used in all analysis included mean 10 day antecedent discharge (*Q*10), temperature, DOC, bacterial biomass, chlorophyll *a* and salinity. *Q*10 was chosen as we have previously found it to be the best measure to integrate the effects of discharge in the days prior to sampling (Chapter 2). We eliminated TN, TP and turbidity from all analyses due to strong co-linearity between them and discharge. All remaining factors had variable inflation factors of <20. Additional variables were added for each analysis where appropriate. For rotifer analysis, total copepods (excluding harpacticoida as they are not known to prey of rotifers) and total cladocera were added as they likely exert a top down influence. For

cladocera analysis, total rotifers were added as they are a potential prey species and copepods (excluding harpacticoida) were added as they may feed on cladocera. For copepods and nauplii analysis, total rotifers and total cladocera were added as explanatory factors. Monte-Carlo permutation (999 permutations without restriction) was used to test the significance of canonical axis and environmental variables on zooplankton communities.

5.4 Results

5.4.1 Water Quality, Bacteria and Phytoplankton

Discharge was highly variable during the study period and was defined by a series of large FWI events which occurred between March and June, the largest of which peaked at a daily average of 463 m^3 s^{-1} (Fig. 5.2A). Discharge had a strong influence in structuring the biogeochemical conditions within the estuary. Salinity at station 1, the most upstream station, shifted from 2 to 0 on the practical salinity scale between January and February as discharge increased moderately, and remained dominated by freshwater throughout the rest of the study (Fig. 5.2B). At the mid-estuary site, station 2, salinity was more variable, peaking at 13 in February before declining to 0 during the first inflow event in March and remained between 0 to 5 for the rest of the study. Water temperatures fluctuated from a high of 25.5°C in January to a low of 7.5°C in July.

Nutrient concentrations were closely coupled with discharge. The highest concentrations were recorded at the beginning of March with total nitrogen (TN) 113.3 µg L^{-1} at station 2 and total phosphorus (TP) 10.3 µg L^{-1} at station 1 (Fig. 5.2C). Similarly, dissolved organic carbon (DOC) concentrations also peaked during this period with 12.2 mg L^{-1} at station 1 coinciding with the peak of the first inflow event in March (Fig. 5.2D). The smaller inflow in June also resulted in an increase in DOC concentration, though was much lower than during the larger floods in March at 5.8 mg L^{-1}. Bacterial biomass concentrations followed a very similar pattern to DOC (Fig. 5.2E). Bacterial biomass was highest following the peak of the first inflow event in March at 310.4 µg C L^{-1}, before declining to pre-inflow concentrations by April. Bacterial biomass increased again during the June flood and smaller inflow in October.

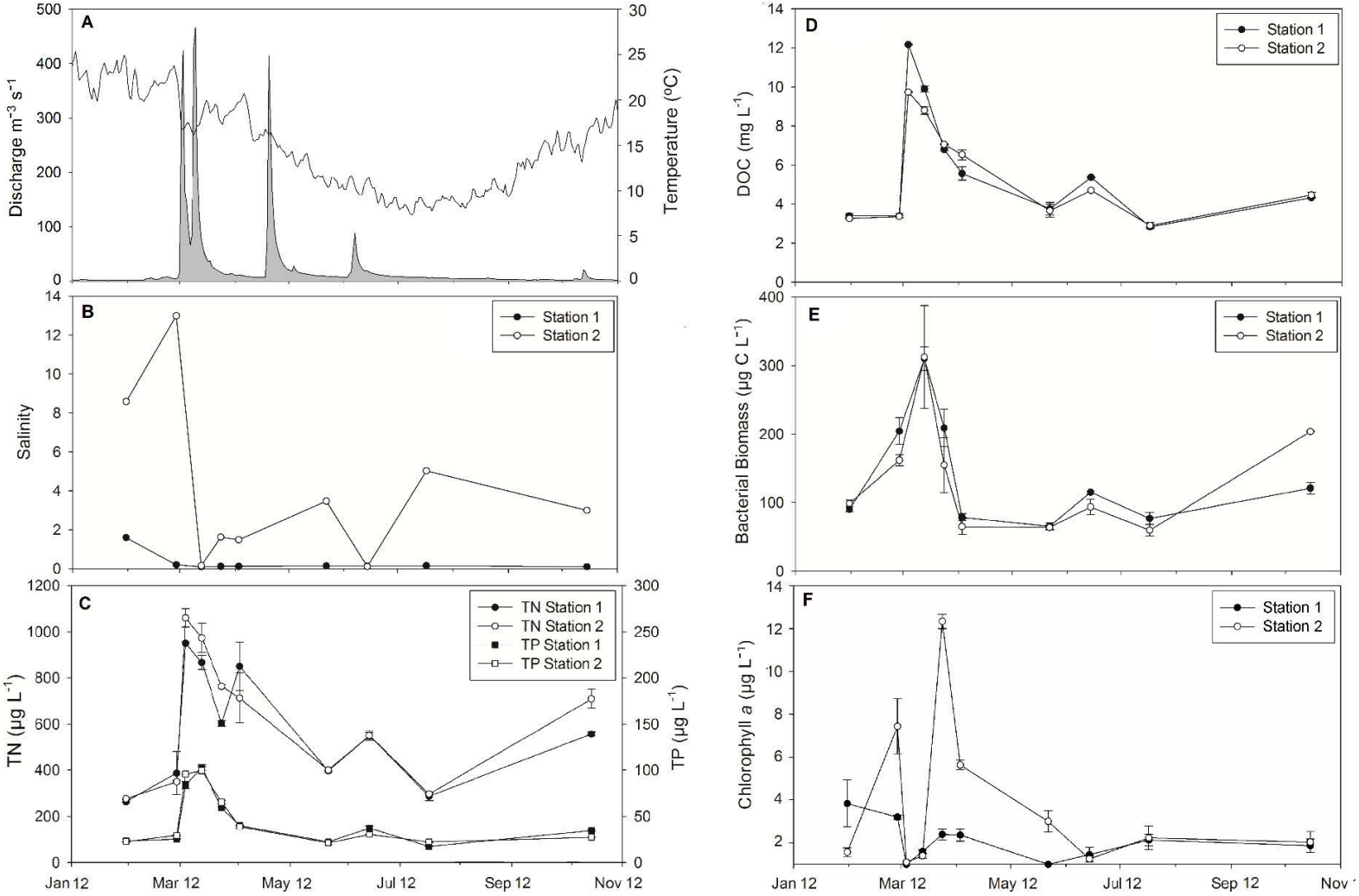

Figure 5.2. Water quality and biological parameters on the Bega River, January to October 2012. A) discharge and temperature; B) salinity; C) total nitrogen and total phosphorus; D) dissolved organic carbon; E) bacterial biomass; F) chlorophyll a. Error bars are ± standard error, *n*=3

Chlorophyll *a* concentrations at station 1 and station 2 showed differing patterns. At station 1, chlorophyll *a* was highest in January at 3.8 μg L^{-1}, when discharge was lowest and temperatures warmest and then remained under 3 μg L^{-1} for the remainder of the study (Fig. 5.2F). At station 2, chlorophyll *a* had an initial peak in February at 7.43 μg L^{-1} before declining to 1 μg L^{-1} during the peak of inflow in March. Chlorophyll *a* then peaked again on the falling limb at the end of March rising to 12.3 μg L^{-1}. For the second half of the study chlorophyll *a* at station 2 remained below 3 μg L^{-1}.

There were also shifts in the dominance of phytoplankton taxa throughout the study period (Fig. 5.4A,B). In January and February, during the initial low flow period, phytoplankton was dominated by Chlorophyta. During the peak of the two inflow events in March, and during the peak of later inflow event in June, the community became dominated by cyanobacteria, in particular *Geitlerinema* (Table 5.1). In the period following floods in March, Cryptophyceae, in particular *Cryptomonas*, as well as taxa belonging to Chlorophyta dominated.

5.4.2 Zooplankton density

Rotifer densities varied 10 fold during the study period. At both stations the lowest densities were recorded during the low flow periods at the beginning of the study and then in May between flood events (Fig. 5.3A). The highest densities occurred during March and April, peaking at 1.1 $x10^7$ m^3 at station 2. At all times the dominant rotifer genera was *Asplanchna*. Redundancy analysis explained 65.9% of variation in rotifer density (Table 5.1). The first canonical axis explained 37.2% of the variation in rotifer density and was dominated by the negative influence of salinity on all taxa and negative influence of copepods on *Asplanchna*, *Colurella*, *Epiphanes* and *Philidinae* (Fig. 5.5a). The second axis explained 10.6% of variance and shows *Brachinous*, *Keratella* and *Synchaeta* closely positively related to bacterial biomass, DOC and 10 day antecedent discharge.

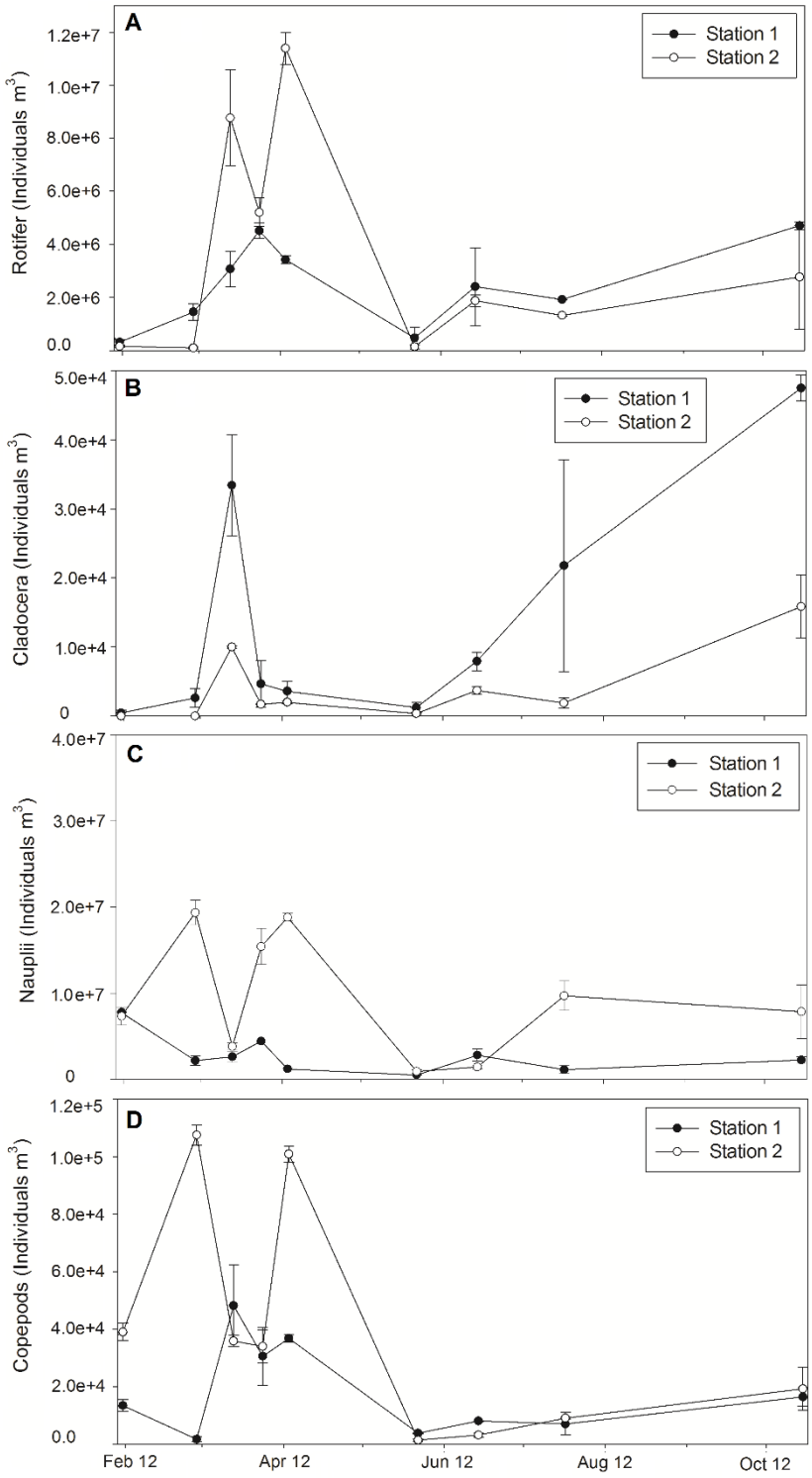

Figure 5.3. Zooplankton density on the Bega River, 31 January to 17 October 2012. A) rotifer density; B) cladocera density; C) nauplii density; D) copepod density. Error bars are ± standard error, *n*=3

Cladocera density varied greatly during the study period ranging from 0 to $4.8x10^4$ m^3. During the low flow period in January and February cladocera density was low at both stations (Fig. 5.3B) and the assemblage was dominated by *Moina tenucornis* and *Chydorus sp*. at station 1, and *M. tenucornis* and *Bosmina meridionalis* at station 2 (Fig. 5.4B). Cladocera density rose sharply at both stations at the start of March during the peak of the first inflow event and was dominated by *Chydorus* sp. at station 1, and a mixture of *Daphnia carinata*, *B. meridionalis* and *Ilyocryptus* sp. at station 2. Cladocera density remained low from the end of March through to May before rising to its highest point in October when the community was dominated by *B. meridionalis* (Fig. 5.4B). Redundancy analysis explained a total 67.9% of variation in cladocera density (Table 5.1). The first canonical axis explained 33.4% of variation showing *D. carinata*, *Chydorus* sp. and *M. tenucornis* negatively correlated to salinity, and *D. carinata* positively related to rotifer density and *Q*10 (Fig. 5.5B). The second axis explained 19.2% of variance and showed *Chydorus* sp. and *M. tenucornis* positively related to DOC and bacterial biomass, and *B. meridionalis* and *Ilyocryptus* sp. negatively to temperature.

Nauplii numerically dominated the zooplankton community with densities at times over 1000 fold higher than adults and copepodites. At station 1, nauplii density peaked at the beginning of the study period in January when temperatures were highest (Fig. 5.3C). Nauplii density at station 2 peaked in February during the low flow period and then in March and April in the weeks following inflow events. The density of copepods at station 1 was lowest during the low flow period in February and highest following the inflow events in March and April peaking at $4.8 x10^4$ m^3 (Fig. 5.3D). At station 2, copepod density showed a slightly different pattern with peaks in density in both February and April before declining during the second half of the study.

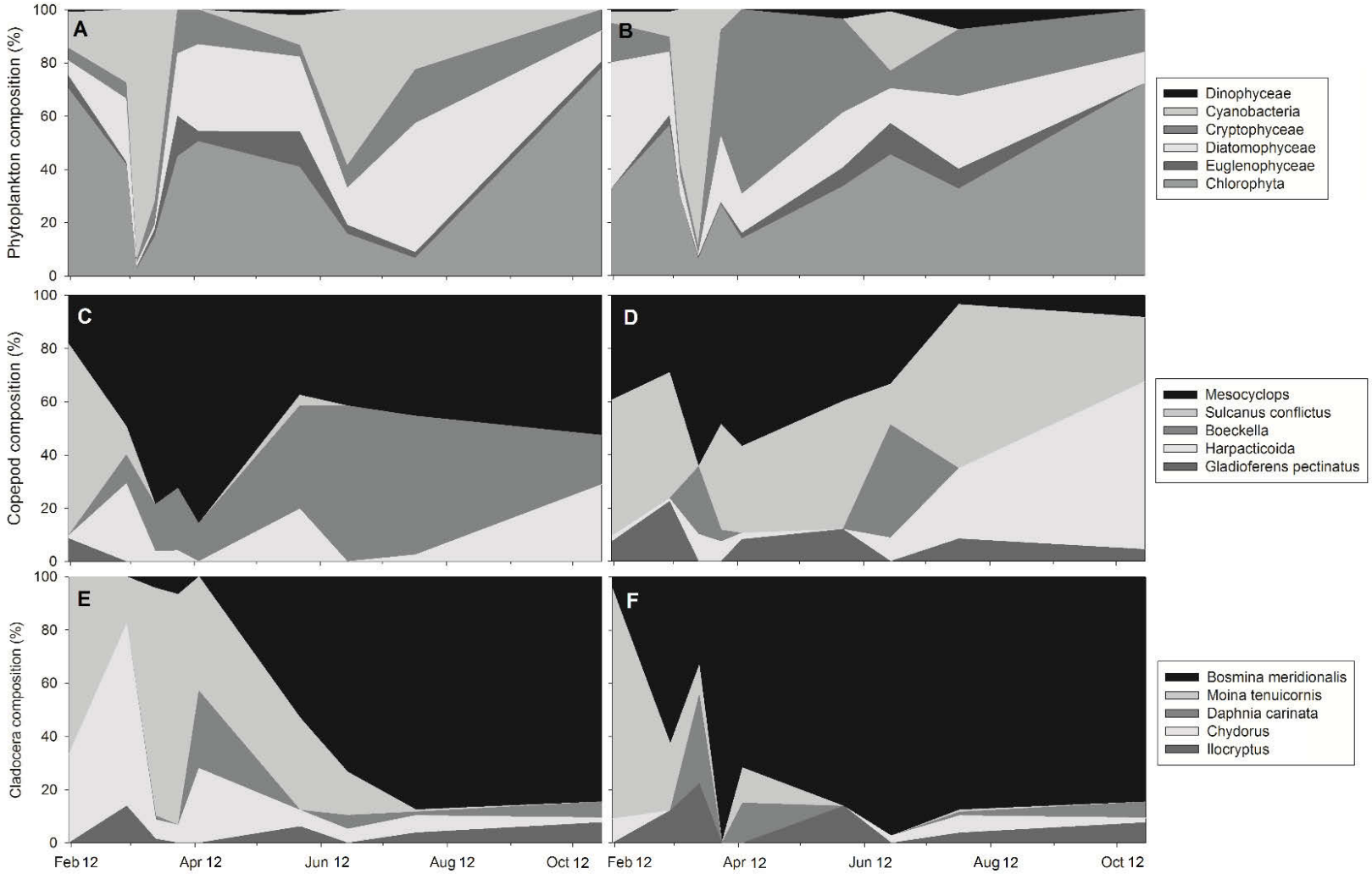

Figure 5.4. Relative assemblage structure on the Bega River, 31 January to 17 October 2012. A) Phytoplankton genera at station 1 and B) station 2; C) copepods at station 1 and D) station 2; E) cladocera at station 1 and, F) station 2

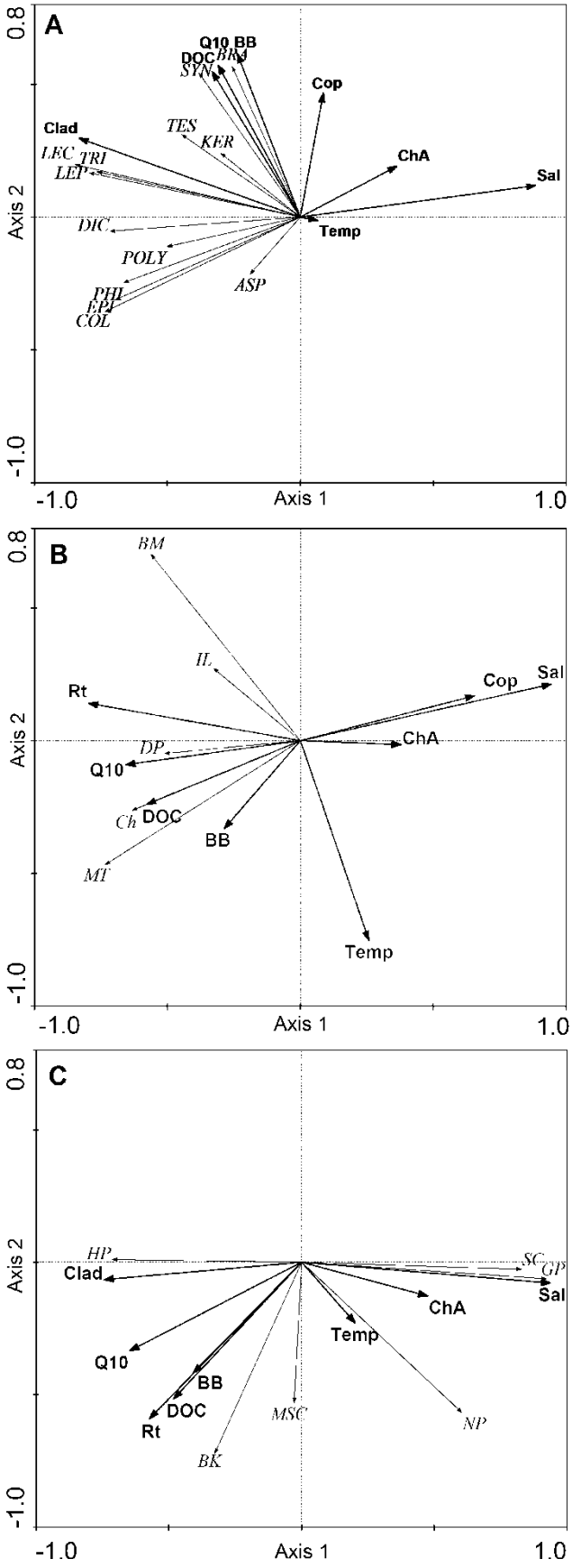

Figure 5.5.Redundancy analysis of zooplankton and environmental variables for samples collected from the Bega River between 31 January and 17 October 2012. A) Rotifer analysis; B) cladocera analysis; C) copepod analysis. Abbreviations for environmental variables: Temp= temperature, Q10= mean 10 day antecedent discharge, ChA= chlorophyll a, Sal= salinity, Cop= copepods copepod, Clad= cladocera, NP= nauplii, Rt= rotifers. Abbreviations for zooplankton tested: A) ASP= *Asplanchna*, BRA= *Brachionus*, COL= *Colurella*, DIC= *Dicranophorus*, EPI= *Epiphanes*, KER= *Keratella*, LEC= *Lecane*, LEP= *Lepandella*, PHI= *Philodinae*, POLY= *Polyarthra*, SYN= *Synchaeta*, TES= *Testudinella*, TRI= *Trichocera*; B) BM= *Bosmina meridionalis*, MT= *Moina Tenuicornis,* DP= *Daphnia carinata, IL= Ilocrptus* sp., CH= *Chydros* sp.; C) BK= *Boeckella sp*., MSC= *Mesocyclops sp.*, GF= *Gladioferens pectinatus*; SC= *Sulcanus conflictus*, HP= harpacticoida.

There were large shifts in which species of copepod were present during the study. In January and February during the low flow period, the estuarine species *Sulcanus conflictus*, *Gladioferens pectinatus* and *Mesocyclops* sp. dominated the copepod assemblage. At station 1, the copepod community shifted during the inflow events in March to being dominated by *Mesocyclops* sp. and later on *Boeckella* sp. (Fig.4C). At station 2 during the peak of inflow in March and June, *G. pectinatus* disappeared and was replaced by *Boeckella* sp.. Through April and May the copepod community was a mixture of *S. conflictus* and *Mesocyclops* sp. and towards the end of the study a mixture of *S. conflictus* and harpacticoida species (Fig.4D). Redundancy analysis explained a total of 73.3% of variation in nauplii and copepod density (Table 5.1). The first canonical axis explained 57.6% of variation, with *G. pectinatus* and *S. conflictus* density positively related to increasing salinity and chlorophyll *a* and harpacticoida negatively related to salinity (Fig.5C). The second axis explained 8.6% of variance and shows nauplii density positively related to temperature, and *Boeckella* sp. and *Mesocyclops* sp. positively related to rotifer density and bacterial biomass. Other zooplankton taxa were present during this study, but because their presence was rare they were excluded from the main analysis (Table 5.2).

Table 5.1. Results of redundancy analysis for rotifers, cladocera and copepods on the Bega River, January to October 2012.

	Eigen Value Axis 1	Eigen Value Axis 2	Eigen values Total canonical axis	Axis 1 F	Axis 1 *P* value	Total canonical axis F	Total canonical axis *P*
Rotifer	0.372	0.106	0.659	5.326	**0.002**	2.176	**0.004**
Cladocera	0.334	0.192	0.679	4.513	**0.010**	2.376	**0.002**
Copepod	0.576	0.086	0.733	12.243	**0.004**	3.094	**0.007**

5.4.3 Stable isotope data

The stable isotopes results showed clear separation in the δ^{13} C signatures of the allochthonous and autochthonous source material. The terrestrial plants ranged between -27.8‰ to -31.8‰, soils between -22.4‰ to -24.9‰ and all benthic algal samples returned values of -18.2‰ (Fig. 5.6). Throughout the study period allochthonous C from terrestrial plants was the dominant source of carbon utilised for the zooplankton measured. At station 1 both *Boeckella* sp. and *D. carinata* δ^{13}C signatures fell within or above the range of terrestrial plant δ^{13}C, varying between -27.8‰ to -32.8‰ (Fig. 5.6A). The only other species that we were able to collect enough biomass for isotope analysis at station 1 was *Mesocyclops* sp. which showed a signature between terrestrial plants and soils of -25.7‰ in March. At station 2 these taxa had similar signatures with *Boeckella* sp. returning values ranging from -29.6 to -31.9‰, *D. carinata* -30.4‰, and *Mesocyclops* sp. -25.5‰ to -29.4‰ (Fig. 5.6B). The cladocera *M. tenucornis* appeared to be supported by terrestrial carbon, returning values ranging between -27.0‰ to -30.1‰ in March and April. *S. conflictus* showed the greatest variation in δ^{13}C during the study. Its δ^{13}C signature was -27.6‰ in February during the low flow period, which shifted at the end of March following the inflow events to -31.7‰, and then lowered to -29.3‰ in May (Fig. 5.6B). *S. conflictus* signature reached its lightest point in July at -25.7‰ and was similar to *G. pectinatus* which was -25.4‰. The δ^{13}C signature of *S. conflictus* rose again to -28.5‰ by the end of the experiment.

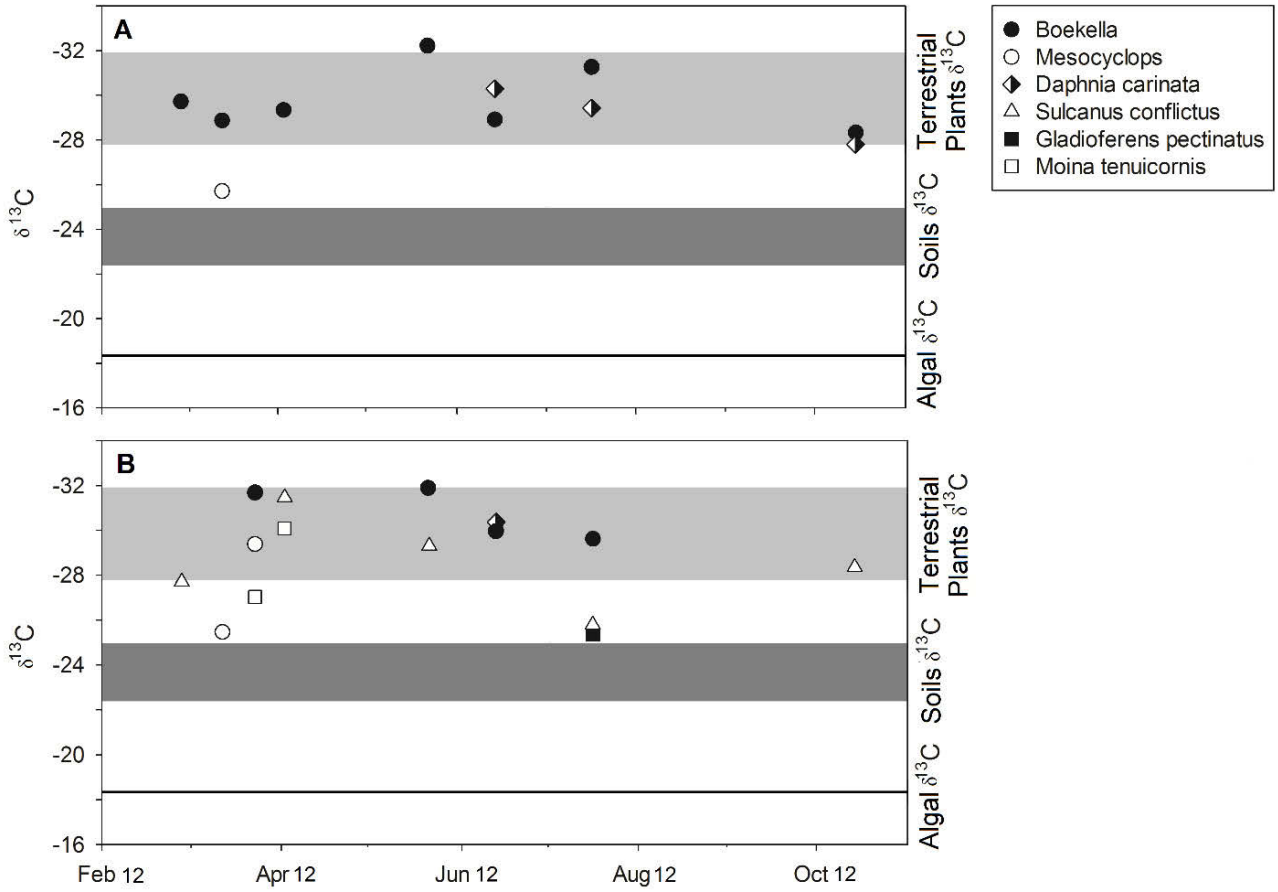

Figure 5.6. Stable isotope $\delta^{13}C$ results on the Bega River between 28 February and 17 October, at A) station 1 and; B) station 2. Shaded areas represent the range of $\delta^{13}C$ signatures from source materials, light grey= terrestrial plants; dark grey= soils; black= algal.

Table 5.2. List of phytoplankton and zooplankton taxa found on the Bega River Estuary, January to October 2012.

Phytoplankton	
Chlorophyta	**Diatomphyceae**
Actinastrum	*Acanthoceras*
Ankistrodesmus	*Achnanthes*
Botryococcus	*Amphora*
Chlamydomonas	*Aulacoseira*
Closterium	*Cocconeis*
Coelastrum	*Cyclotella*
Cosmarium	*Dinobryon*
Crucigenia	*Diploneis*
Dictyosphaerium	*Entomoneis*
Gloeoactinium	*Fragilaria*
Kirchneriella	*Gyrosigma*
Monoraphidium	*Mallomonas*
Oocystis	*Melosira*
Scenedesmus	*Navicula*
Tetraedron	*Nitzschia*
Cyanobacteria	*Skeletonema*
Aphanocapsa	*Synura*
Cyanodictyon	***Dinophyceae***
Geitlerinema	*Peridinium*
Gloeothece	***Euglenophyceae***
Leptolyngbya	*Euglena*
Merismopedia	*Lepocinclis*
Microcystis	*Trachelomonas*
Myxobaktron	**Cryptophyceae**
Planktolyngbya	*Chroomonas*
Pseudanabaena	*Cryptomonas*
Raphidiopsis	
Romeria	
Spirulina	
Synechococcus	
Zooplankton	
Rotifera	**Cladocera**
Asplancha	*Chydorus sp.*
Asplanchnopus	*Daphnia carinata*
Brachionus	*Bosmina meridionalis*
Cephelodella	*Ilocryptus sp.*
Colurella	*Moina tenucornis*
Dicranophorus	**Copepoda**
Epiphanes	*Boeckella sp.*
hexathra	*Gladioferens pectinatus*
Keratella	Harpacticoida
Lecane	*Mesocylops sp.*
Lepadella	*Sulcanus conflictus*
Mytillina	nauplii
Philodinae	**Other**
Polyarthra	Barnacle nauplii
Synchaeta	Bivalve
Testudinella	Chironomidae larvae
Trichocera	Crab zoea
	Hyperiid (juvenile)
	Isotomidae larvae
	Mysidacea
	Oligochaeta
	Ostracoda

5.5 Discussion

5.5.1 Changes to zooplankton assemblage structure

We hypothesised that following high FWI, the estuarine zooplankton assemblage would become more dominated by freshwater species. Our results support this hypothesis which showed immediate and broad shifts in zooplankton community structure. The best example of this can be seen between the pre-flood date of 28 February and just after the peak of the flood on 4 March (Fig. 5.2A). Both rotifer and cladocera density sharply increased between these dates (Fig. 5.3A,B). Reduction in salinity following the inflow is likely an important reason for this as all rotifer and cladocera genera were strongly negatively related to salinity (Fig. 5.5A,B). The majority of cladocera and rotifer genera generally prefer freshwater environments. For example, Silva et al. (2009) found that the density of both rotifers and cladocera were negatively correlated with salinity in the Mossoró River Estuary, Brazil, and Park and Marshall (2000) found rotifer density to be negatively correlated to salinity in Chesapeake Bay, USA. The preference of rotifers and cladocera for a more freshwater environment is also evidenced during the second half of our study between June and October where the total densities of both groups was higher at the predominantly freshwater station 1 compared to station 2 (Fig. 5.3A,B). A similar pattern was found by Holst et al. (1998) in the Elbe estuary, Germany, where rotifer density was greatest in the freshwater sections and reduced in density towards the mouth.

Changes in salinity driven by freshwater inflows appeared to cause shifting copepod species composition. During low flow periods when salinity was >2 at station 1, *S. conflictus* and *G. pectinatus* were the dominant calanoid copepod species (Fig. 5.4D) and were positively related to salinity (Fig. 5.5C). Both species, common to Australian estuaries, can tolerate a wide range of salinities (Brand and Bayly, 1971). During periods of intense freshwater inflow that occurred in March and June these species were mostly replaced with the freshwater *Boeckella sp.*, which was only encountered when salinities were low during the study. Similar results were found by Hall and Burns (2002) in the coastal Lake Waihola, New Zealand, where salinity was the major determinant of zooplankton distribution; as water became more saline the zooplankton

community switched from one dominated by freshwater *Boeckella hamata* and *D. carinata* to one dominated by *G. pectinatus*.

The changes witnessed on the Bega River are also likely influenced by advective forcing. During inflow events advective forcing may influence zooplankton communities in two ways; delivering zooplankton from upstream freshwater environments, and washing out zooplankton from the estuary to the coastal zone. In many systems advective forcing due to inflows has been associated with reductions in zooplankton numbers from estuaries (Kobayashi et al., 1998; Pace et al., 1992). The loss of zooplankton due to advection in the Bega River does not, however, seem as significant as in other systems. In fact, most groups appeared to increase in density following inflows. The only group at a coarse level that experienced losses during this period was nauplii at station 2, whose density sharply declined during the first weeks of March (Fig. 5.3C). The reduction in nauplii during inflows may be due to their slow swimming speeds, leaving them susceptible to washout (Richardson, 1992). In the Bega River, freshwater inflows both delivered rotifers and cladocerans downstream to the lower estuary, whilst concurrently the salinity was reduced creating a favourable environment for their survival.

The most persistent copepod present during this study was *Mesocyclops* sp., which was present at all times at both sampling stations on the Bega estuary (Fig. 5.4C,D). The persistence of *Mesocyclops sp*. even after high flows may in part be explained by its ability to withstand high water velocities and maintain position (Richardson, 1992). Other species of copepods have developed strategies for dealing with periods of intense inflow. For example, Ueda et al. (2004) found that during floods in Chikugo River estuary, Japan, the calanoid *Pseudodiaptomus inopinus* was able to avoid being washed out by aggregating on the submerged banks of the flooding estuary, allowing it to quickly repopulate once flood waters subsided.

5.5.2 Responses to changing resources

Our second and third hypotheses were that following inflows zooplankton density and support from terrestrial carbon sources would be higher compared to low flow periods. The results presented here broadly support these hypotheses with most zooplankton increasing in density and those tested for stable isotope signatures showing strong

support from terrestrial carbon sources during and after FWI. Rotifer density increased in the period following high inflows in March (Fig. 5.3A). Whilst rotifers were negatively related to salinity, at station 1 salinity remained low at all times (Fig. 5.2A), giving us an insight into other influencing factors independent of salinity. Here changes in rotifer densities closely follow the changes through time in carbon and bacterial concentrations (Fig. 5.2C,D,E). Holst et al. (1998) studied rotifer responses to changes in food resources in the Elbe estuary, Germany, finding that rotifer density was closely linked to food availability which included bacteria, detritus, flagellates and phytoplankton.

Rotifer diets are species dependent and vary from passive filter feeders to selective predators (Arndt, 1993). Key rotifer genera responsible for grazing on bacteria include *Brachionus* and *Keratella,* which feed on particles 0.5 – 20 μm and *Synchaeta* feeding on particles ranging from 1 – 40 μm (Arndt, 1993, and references therein). On the Bega River these genera were positively related to bacterial biomass suggesting that these microbes directly, or via other small bacteriovores such as nanoflagellates, represent an important part of their diet (Fig. 5.5A). As a result, pulses of DOC, leading to increases in bacterial production, may be an important food source for rotifers following FWI. This assertion is supported by the findings of Mitrovic et al. (2014), who recently demonstrated in a mesocosm study in a lowland river in Australia that DOC additions led to significant increases in the density of rotifer genera including *Asplanchna*, *Brachionus*, *Polyarthra* and *Keratella*, which were linked to successional increases in bacteria and ciliate density. Similarly Hofmann and Höfle (1993) showed that DOM additions to mesocoms in a eutrophic lake led to increases in the density of *Keratella* species.

High FWI initially led to a decrease in nauplii density, though populations recovered by the end of March and April (Fig. 5.3C). Nauplii are major consumers of phytoplankton (Calbet and Landry, 2004), but they also consume bacteria and small protists (Roff et al., 1995). Nauplii density however appeared less influenced by food resources and more strongly influenced by temperature, to which it was positively related (Fig. 5.5C). Nauplii hatching and development rates are positively related to temperature for species of *Mesocyclops* (Maier, 1989), *Boeckella* (Jamieson, 1986) and *Gladioferens* (Rippingale and Hodgkin, 1974). Furthermore, *S. conflictus* has

diapause eggs which only hatch in the presence of warm temperatures and low salinities (Newton and Mitchell, 1999). Trying to separate these multiple influences in estuaries is a difficult task. The use of techniques such as stable isotope in unison with abundance data can prove useful in determining which food resources support communities and when their contributions change in response to FWI. However, stable isotope analysis is technically hard for microzooplankton due to the need to physically isolate the organism of interest from similarly sized plankton or source material and the need to attain sufficient mass to run through the mass spectrometer. As a result, stable isotope techniques are much more useful when examining larger or mature organisms.

The most comprehensive stable isotope results for before, after and during inflows were for *Boeckella sp*. which increased in density following flows, supporting our second hypothesis. The stable isotope analysis indicated that *Boeckella sp*. was mainly supported by allochthonous carbon at all times during the study period (Fig. 5.6). Similarly *Mesocyclops sp*. were also most abundant following FWI, although their smaller size meant that we did not have enough biomass to perform stable isotope analysis on them as extensively. However, of the samples that were analysed, it was clear that although they were supported by a mixture of carbon sources, terrestrial carbon sources were the most dominant. On the Bega River *Boeckella sp.* and *Mesocyclops sp.* were positively related to rotifers (Fig. 5.5C), suggesting that they may have been an important food source. *Mesocyclops* and *Boeckella* have indeed been shown to feed on rotifers (Brandl, 2005 and references therein), which are considered a preferred food source for some copepods (Williamson and Butler, 1986). The other important heterotrophic food source for copepods are smaller heterotrophic protists such as ciliates and flagellates which we did not account for in this study owing to their small size and the challenges associated with attaining clean samples of sufficient mass to analyse. Nevertheless, protists have been shown to be a preferred food source for copepods (Gifford and Dagg, 1988) and of potentially high nutritional quality (Müller-Navarra, 2008). And there is some evidence of the importance of protists as a resource in response to FWI too, as Reaugh et al. (2007) showed that during wet years in Chesapeake Bay, USA, zooplankton fed much more heavily on microzooplankton.

S. conflictus peaked in density before and in the weeks following March inflows (Fig. 5.3C, Fig. 5.4D). The stable isotope results for *S. conflictus* support our third hypothesis, showing that following the inflows in March its $\delta^{13}C$ signature more closely resembled terrestrial plant carbon. Furthermore, the $\delta^{13}C$ signature later shifted during the low flow period in July to resemble a mixture of carbon sources (Fig. 5.6B). *S. conflictus* is a predator and feeding studies have indicated its preferred food is nauplii, particularly of *G. pectinatus*, as well as copepodites including its own species (Ough and Bayly, 1989). *G. pectinatus,* which occurred less frequently and for which it was only possible to collect stable isotope samples once, also appeared to be supported by a mixture of carbon sources during the low flow period in July (Fig. 5.6). These temporal fluctuations in the $\delta^{13}C$ signature of *S. conflictus* may reflect the changing diet of its prey species throughout this period.

Cladocera populations increased following inflows in March, with sharp rises in the density of *M. tenucornis* and *D. carinata* (Fig. 5.3B, Fig. 5.4E,F), supporting our second hypothesis that zooplankton density would increase following FWI events. Cladocera species are generally considered to have wide diets that may include detritus, bacteria, protists, phytoplankton and in some case small protozoans such as rotifers (Kobayashi, 1991). For the technical reasons discussed earlier, we were not able to collect enough biomass to analyse stable isotope results for all cladocera species at all times. For example, *D. carinata* $\delta^{13}C$ results were only available for the second half of the study period. During this period, the isotope data suggest that *D. Carinata* were always supported by terrestrial sources of carbon (Fig. 5.6). *D. carinata* was positively related to rotifer density (Fig. 5.5B), and peaks in relative density of *D. carinata* (Fig. 5.4E,F) closely succeeded peaks in rotifer density (Fig. 5.3A) further suggesting that rotifers form an important part of their diet, as has been evidenced in other systems (Diéguez and Gilbert, 2011). Bacteria may also form a large part of juvenile *Daphnia* diets (Kankaala, 1988). Barry (1997) showed food availability to be the main limiting factor for *D. carinata* during warmer months, though when temperatures dropped below 15°C populations became temperature limited; this is a similar temperature point to which populations decreased in the Bega River.

Some caution should be taken when interpreting stable isotope data. We sampled a random selection of terrestrial vegetation and all data returned values within the range consistent with C3 plants. Our selection of soils however indicated a likely mix of sources from both C3 and C4 plants (O'Leary, 1988). What the exact contribution of C4 plants to total DOC pool is however unclear; in terms of the climate it could be expected that C3 plants would dominate. Additionally, we did not measure phytoplankton stable isotopes directly. This is common due to the difficultly in separating phytoplankton from similar sized heterotrophic organisms and detritus (Karlsson, 2014). Whilst most studies measure a size fraction of POM and assume phytoplankton is similar, we instead measured dense patches of algae growing on the sandy banks. Whilst this method provides a stronger assurance of isolation from unwanted material benthic algae may have $\delta^{13}C$ signatures 10% more positive than planktonic algae (France, 1995).

5.5.3 Freshwater inflows, allochthonous carbon and zooplankton

Allochthonous carbon support for zooplankton taxa, like that evidenced in this study, has also been reported in other estuarine systems. In Hilo Bay, Hawaii, Atwood et al. (2011) used $\delta^{13}C$ signatures of zooplankton to show increasing terrestrial support following a freshwater inflow event. Hoffman et al. (2008) showed a longer-term pattern in the York River estuary, USA, with zooplankton utilising more allochthonous carbon sources during wet years. Significantly, they also found that zooplankton had a preference for autochthonous carbon, suggesting that zooplankton utilisation of allochthonous carbon may be in part driven by the availability of autochthonous sources (Hoffman et al. 2008). Grey et al. (2001) found in Loch Ness, Scotland, that zooplankton were supported by terrestrial carbon during winter months but this changed following the spring phytoplankton bloom when zooplankton became mostly supported by autochthonous carbon.

For most of this study, and in particular at the predominantly freshwater station 1, chlorophyll *a* concentrations were low, which suggests that autochthonous sources of carbon may have been in short supply. This, in turn, could therefore explain the dominant use of allochthonous sources of carbon by zooplankton. However, chlorophyll *a* was high at the end of March at station 2, yet during this period zooplankton were still clearly supported by terrestrial carbon sources rather than by

the primary producers within the estuary. One explanation for this may be the dominance of mixotrophic flagellates during this period, as *Cryptomonas*, belonging to the class Cryptophyceae, dominated phytoplankton during this period (Fig. 5.4B). *Cryptomonas* are considered a high quality food source for zooplankton (Burnsd and Hegarty, 1994) and our results suggest that it is likely that they were being consumed by zooplankton in the Bega River during the study period. *Cryptomonas* are also known to be phagotrophic (Urabe et al., 2000). If *Cryptomonas* were consuming large amounts of bacteria during the study period we would expect it to have a $\delta^{13}C$ signature resembling that of terrestrial carbon (the dominant contributor to DOC) and, therefore, zooplankton $\delta^{13}C$ signatures that also resemble terrestrial sources. The potential food web importance of mixotrophic organisms was highlighted recently by Lee et al. (2012) who suggested that mixotrophic dinoflagellates in Chesapeake Bay utilise both autotrophic and heterotrophic energy pathways, providing a food source to mesozooplankton in low nutrient and low light conditions.

It is commonly suggested that allochthonous carbon is unlikely to support to zooplankton production as it is usually channelled through the microbial loop, requiring multiple trophic stages before reaching zooplankton, resulting in large amounts of the initial resource being mineralised through respiration (Ducklow et al., 1987). This is likely to be true in systems where allochthonous inputs are low, as the loss of carbon from the microbial loop through respiration would leave little available for higher trophic organisms. In contrast, where allochthonous inputs are large, such as during the FWI events in March on the Bega River, even large respiratory losses would still leave a substantial quantity of organic matter available to zooplankton.

This study provides evidence for a significant role of allochthonous sources of organic matter in supporting estuarine food webs. Our focus on zooplankton is deliberate, as they can link microbial and metazoan food webs and they are a key food source for juvenile fish in many estuarine and coastal systems (Castonguay et al., 2008; Murphy et al., 2012; Newton, 1996; Williams et al., 2013). Furthermore, stable isotope studies of estuarine fish have found increasing allochthonous support during periods of high inflows (Abrantes and Sheaves, 2010b; Hoffman et al., 2007), suggesting that changes in resource utilisation at the base of the food chain, in response to FWI events, may have far reaching trophic implications, at least in the short term. This is particularly

important as flow in the Bega River, like many coastal catchments around the world, is regulated via dams and extraction and this has greatly altered the quantity and quality of freshwater flowing into the estuary. Environmental flow rules may be developed to account for these hydrological changes, but at present they focus on delivering only a minimum level of FWI, with no current rules that expressly protect medium and high inflow events. Our results demonstrate FWI events play important roles in structuring the zooplankton community and facilitating cross-system subsidies of terrestrial carbon supporting zooplankton production, and presumably higher trophic levels as well. Protection of these larger inflow events will, therefore, be important in protecting estuarine health, processes and productivity.

Chapter 6 Additions of dissolved organic carbon from terrestrial sources subsidise estuarine zooplankton: an *in-situ* mesocosm study

6.1 Abstract

Freshwater inflows play an important role in delivering dissolved organic carbon (DOC) to estuaries. Episodic inputs of DOC may support increased bacterial production. However, the role of DOC in supporting zooplankton production is widely debated. To evaluate this role, we performed an *in-situ* mesocosm experiment in the Bega River estuary, Australia. We added a DOC leachate derived from terrestrial vegetation to 400 L mesocosm bags as treatments of +1.5, +3 and +16 mg C L^{-1} and monitored changes in carbon, nutrients, bacteria, chlorophyll *a* and zooplankton over 22 days. Bacterial biomass peaked at day 2 and was highest in the +16 mg treatment followed by +3 mg treatment. Chlorophyll *a* was not significantly different between treatments. Mesozooplankton was dominated by copepodites of *Gladioferens pectinatus* and *Sulcanus conflictus* between days 5-9 and by adults between days 9-15. Significantly higher numbers of copepods were present in the +16 mg treatment followed by the + 3 mg treatment compared to the controls. Stable carbon isotope signatures of copepods in the +16 mg treatment were significantly different from the control, and they more closely resembled the $\delta^{13}C$ signature of the DOC leachate. These results suggest that the impact of allochthonous DOC loading events on estuarine zooplankton occurs over short periods, and that the magnitude of the response is, in part, controlled by the quantity of bioavailable DOC loaded to the system. Our findings also underscore the importance of microbial dynamics stimulated by DOC loading events from freshwater inflows as a trophic path in estuarine food webs.

6.2 Introduction

Episodic inflow events play an important role in facilitating cross-boundary subsides between terrestrial and aquatic environments. Allochthonous organic matter transported during these “hot moments” has the potential to significantly subsidise ecosystem metabolism (Tank et al., 2010). The importance of allochthonous

particulate organic matter in supporting higher trophic organisms is well recognised (Vannote et al., 1980). However, the relative contribution of dissolved organic matter (DOM), primarily as dissolved organic carbon (DOC), remains highly contentious for organisms in pelagic food webs (Brett et al., 2009; Ducklow et al., 1986; Karlsson et al., 2014; Sherr et al., 1987). Currently, the dominant paradigm is that allochthonous DOC provides little support to higher trophic levels, as the majority of the DOC pool is considered to be recalcitrant and not available to metazoan consumers (Fenchel, 2008). Support for this view comes from studies that have compared the use of allochthonous DOC to the more bioavailable autochthonous DOC (Findlay and Sinsabaugh, 2003). These studies have shown that while allochthonous DOC can be utilised by heterotrophic bacteria, its incorporation into higher trophic levels requires intermediary organisms such as nanoflagellates and ciliates. These multi-trophic level microbial food webs are believed to be inefficient at transferring carbon to higher levels because much of the carbon is lost to the system via respiration (Ducklow et al., 1986).

Counter to the prevailing view that allochthonous carbon has no major role to play in supporting metazoan food webs, a number of recent stable isotope studies suggest that allochthonous DOC can provide a significant subsidy to pelagic zooplankton and fish in estuaries and coastal systems (Abrantes and Sheaves, 2010b; Hitchcock et al., 2010; Hoffman et al., 2007; von Biela et al., 2013). Significantly, many of these studies have identified that the size of allochthonous carbon subsidy provided to higher trophic levels varies with environmental conditions, especially hydrological events (Abrantes et al., 2013). For example, Hoffman et al. (2008) showed in the York River estuary, USA, that the contribution of allochthonous carbon to cladocera and copepod diets increased during wet years, while Atwood et al. (2011) showed that terrestrial carbon made a more significant contribution to zooplankton biomass following storms in the tropical estuary of Hilo Bay, Hawaii. Since the majority of annual carbon load is delivered to estuaries during episodic inflows, it may be that even with a greater number of steps in microbial food webs (where large amounts of organic carbon are respired), the total organic carbon pool may be large enough to represent a significant resource subsidy.

Manipulative mesocom experiments, such as the 'Link or sink' experiment by Ducklow et al. (1986), have been a crucial tool in evaluating the role of different sources of carbon in supporting higher trophic levels in aquatic systems. Researchers have used these approaches to address carbon use by heterotrophic and metazoan components of food webs by adding small amounts of glucose to act as a food web tracer (Berglund et al., 2007). However, the natural DOM pool differs significantly from glucose, containing a range of complex carbon compounds, of varying labilities and concentrations. as well as nutrients such as nitrogen and phosphorous,. Recent work in freshwater rivers by Mitrovic et al. (2014) has shown that glucose should not be considered equivalent to the natural DOM pool, demonstrating that additions leaf leachate led to significantly increased copepod density, whilst the same concentrations of glucose addition did not.

In addition to using artificial DOM, relatively few mesocosm studies have been designed to examine the effects of different concentrations of resource subsidies. This is important to consider, as Faithfull et al. (2012) showed in their mesocosm experiment that zooplankton support from DOC and the microbial loop was higher in the treatments with high glucose concentrations (2.1 mg C L^{-1}) compared to lower concentrations (0.42 mg C L^{-1}). Therefore, testing a gradient of DOC subsidies is important in order to understand the response of the food web to naturally occurring DOC pulses which vary in concentration with the scale of freshwater inflows to estuaries (Hitchcock and Mitrovic, 2014).

Given the limitations of mesocosm studies which have evaluated responses to relatively artificial carbon sources (glucose) and usually at low concentrations, there is a clear need for 'link or sink' experiments that more closely replicate the natural quality and quantity of allochthonous DOM pulses that estuaries receive. Understanding if variable concentrations of naturally occurring allochthonous DOC can support zooplankton, and what concentrations are required to witness significant effects, is an important next step in developing our understanding of the role of resource pulses in estuarine ecosystems.

The Bega River estuary is a shallow wave-dominated estuary in NSW, Australia. Previous work in the system has revealed that the major inputs of DOC occur during episodic freshwater inflow events (Chapter 2; Hitchcock and Mitrovic, 2014). During

these periods the DOC pool is more bioavailable than during low flows and this has been shown to support increased bacterial biomass (Chapter 3). However, it is not clear to what extent the DOC fraction, and in turn the microbial loop, directly supports zooplankton production. We also do not know what quantity of allochthonous carbon may be needed to satisfy zooplankton carbon requirements. In this study we answered these questions by examining whether DOC leachate additions can support increased estuarine zooplankton density in experimental mesocosms. Briefly, we added 3 different concentrations of DOC leachate sourced from dominant riparian vegetation, and then followed the response of bacteria, chlorophyll *a*, carbon, nitrogen, phosphorous, zooplankton and stable isotopes of $\delta^{13}C$ and $\delta^{15}N$ over a 22 day period. We hypothesised that no significant differences in zooplankton density would occur in the treatment receiving the lowest quantity of DOC addition as most carbon would be lost to respiration. In the treatments receiving the largest additions we hypothesised that, despite organic carbon losses through the food chain, the subsidy would be large enough to lead to significant increases in zooplankton density and changes in zooplankton $\delta^{13}C$ signatures.

6.3 Methods

6.3.1 Study Site

The Bega River estuary, NSW, Australia, (-36° 42' 43.64", +149° 54' 8.76") is a wave-dominated estuary. The estuarine volume, 6371 ML, is relatively small compared to its catchment of 1941km^2. The catchment is split into two sub-catchments, the Brogo River, regulated by Brogo Dam and the Bega River which has two smaller dams, Cochrane and Candello. The upper sections of the catchment consist of mainly forested areas, whilst the middle catchment is mostly cleared for agriculture and is dominated by the dairy industry (Brierley et al., 1999; Tozer et al., 2010). The freshwater tidal section has been mostly cleared for agriculture and irrigated farmland, however the rest of the estuary is largely unmodified. Broad scale land clearing since colonisation has drastically altered the river and estuary via the transportation of sandy top soils into the river channel (Brooks and Brierley, 1997). The mesocosm experiment was conducted *in-situ* in the mesohaline section of the estuary; salinity was 8 on the practical salinity scale.

6.3.2 Experimental design

The DOC leachate was created using leaves of the dominant vegetation of the upper estuary which included native tree species of Eucalyptus, *Casurina glauca* and the introduced agricultural grass *Pennisetum clandestinum*. The leachate was prepared in a similar method to Mitrovic et al. (2014). In brief, the leaves were collected 1 month prior to the experiment and stored at 4°C in the dark. In batches, the leaves were then soaked in 40 litres of reverse osmosis water for 48 hours at 4°C in the dark. After 48 hours the leaves were removed and new leaves added, with this process repeated four times. The resultant leachate solution was then filtered using 0.2 μm (polycarbonate membrane) filters to remove bacteria and other particles (Hauer and Lamberti, 2011). The DOC, total nitrogen and total phosphorus concentrations were then measured for the solution. In order to mimic more closely the stoichiometry of water delivered during floods we added additional nitrogen as KNO_3 and phosphorus as KH_2PO_4 to approximate relative proportions of C:N:P (332:14:1) received during floods (Hitchcock and Mitrovic, 2014).

The mesocosm experiment was performed between 11 November and 3 December 2012 after an extended period of low flows (>2 months). The experiment was performed using fourteen 400 L plastic mesocosm bags with a closed bottom (diameter 64cm, 1.3 m deep). The mesocosms were attached inside a larger support structure which was 4 m long, 2 m wide and 2 m deep, made from a frame of reinforced PVC tubing covered by polyester sailcloth material (Fig. 6.1). This allowed the natural movement of water from wave and tide action but protected the plastic mesocosm bags from damage. The outer structure was first filled on 10 November 2012 by tipping it on its side and lowering it slowly into the water until full. It was then moved into position and secured using anchors and ropes to the bank. This was done one hour before high tide. The mesocosm bags were then rinsed with estuary water before being filled by gently lowering them into the water column and lifting back to the surface, and then suspended within the protective structure at random locations. Filling all 14 bags took approximately one hour. The mesocosms were then allowed to settle overnight. On 11 November 2012 (day 0) the DOC leachate was added and mixed before sampling occurred. The experiment took place over 22 days. This was deemed to be long enough for zooplankton to respond to the additions as at the *in-situ*

temperatures, which were between 20-23°C, the dominant copepod species have been experimentally shown to have a full life cycle within 18-22 days (Newton and Mitchell, 1999; Ough and Bayly, 1989). Our treatments were control and DOC leachate additions equivalent to additions of +1.5 mg C L^{-1}, +3 mg C L^{-1} and +16 mg C L^{-1}. Hereafter these are referred to as the +1.5, +3, and +16 treatments. The control had five replicates and other treatments three replicates.

Figure 6.1. Experimental design for mesocosm experiment on the Bega River. The smaller plastic bags are the 400L mesocosm, whilst the larger outside structure protects the bags from damage. Inset adding DOC leachate at day 0.

6.3.3 Sampling procedures and analysis

Samples were collected from each mesocosm bag using a 5.5 L integrated sampler at a depth of 1 m. The collected water was placed into a bucket and homogenised before being subsampled for each parameter. All equipment was thoroughly rinsed with estuary water and then the water to be sampled between mesocosms to avoid cross contamination. Temperature, conductivity, dissolved oxygen and pH were measured with a Hydrolab Surveyor and MS5 Sonde probe. Samples for carbon, nitrogen and phosphorus were taken on days 0 through 6, and on days 9, 15 and 22. All carbon and nutrient samples were filtered using 0.45 μm polycarbonate filters. Dissolved organic carbon (DOC) samples were collected in pre-combusted, sample rinsed 100 mL glass bottles, acidified with 2 N hydrochloric acid and refrigerated at 4°C. Samples were analysed in the laboratory by the High Temperature Combustion Method (APHA 2005). Dissolved inorganic nitrogen (DIN), dissolved inorganic phosphorus (DIP), ammonium (NH_4) and dissolved organic nitrogen (DON) were collected in pre-

washed and sample rinsed 50 mL PET bottles. Samples were frozen and later analysed using a segmented flow analyser (OI Analytical Model FS3100) according to standard methods (APHA 2005).

Bacterial biomass samples (10 mL) were collected in sterile centrifuge tubes on days 0 through 5, and then on 9, 15 and 22. Samples were fixed with 0.4 mL of concentrated 0.2 μm filtered formalin (37% formaldehyde) and stored at 4°C. In the laboratory, subsamples (2 mL) were stained with DAPI (4'6-diamindion-2-phenylindole) at a final concentration of 1 mg mL^{-1} for 15 minutes, and filtered through a polycarbonate black 0.2 μm pore-sized filter (Porter and Feig, 1980). Polycarbonate filters were mounted onto microscope slides with non-fluorescence immersion oil. Slides were examined at ×100 using a fluorescence-equipped Olympus BX41 compound microscope. For each slide ≥500 total cells were captured using an Olympus DP72 camera and cellSens Standard software (version 1.3). Images were analysed for cell abundance and volume using CellC software (Selinummi et al., 2005). Bacterial biomass was calculated using the formula of Romanova and Sazhin (2010).

Chlorophyll *a* samples were taken on days 0, 3, 5, 9, 15 and 22 by filtering 500 mL of water onto GF/C filters. Filters were frozen until subsequent determination by Standard Methods (APHA 2005) using the grinding technique and acetone as a solute with correction for phaeophytin. A detection limit of 1 μg L^{-1} was used for chlorophyll *a* analysis.

Zooplankton samples were taken on days 0, 5, 9, 15 and 22. Subsamples of 4 L were collected and passed through 35 μm plankton net and stored in 125 mL PET bottles and preserved with a 70% v/v ethanol solution. In the laboratory, samples were concentrated via sedimentation to 10 mL. A 1 mL pipette and a Sedgewick-Rafter counting chamber were used for subsampling and counting of zooplankton. The tip of the pipette was cut to make a 4 mm diameter opening so larger zooplankton would not be under sampled. Zooplankton were counted until 100 individuals of each group were identified or at least 50% of the sample was counted. Copepods were identified as either nauplii, copepodites (stages i-iv) or adults (stage v – adults). Calanoid copepods were identified to species. Harpacticoida copepods were grouped together and not distinguished between copepodites and adults. Rotifers were identified to genera.

Other zooplankton occurred in very small numbers, < 2 individuals per sample, and were grouped together as 'other zooplankton'.

Zooplankton stable isotope samples for $\delta^{13}C$ and $\delta^{15}N$ were taken on the initial day the mesocosm bags were filled (i.e. effectively day -1) and on day 22. Samples were taken with a 30 cm diameter 125 μm plankton net performing vertical tows. Initial samples were taken by performing 20 vertical tows to a depth between 0 and 2 m in the area immediately adjacent to the experiment following the filling of the mesocosm bags. Stable isotope samples on day 22 of the experiment were collected after all other sampling was complete. For each mesocosm bag the plankton net was gently lowered to the bottom and pulled to the surface 15 times. All zooplankton samples were placed in 0.45 μm filtered mesocosm water for 4 hours for gut clearance before being preserved with a 85% v/v ethanol (Feuchtmayr and Grey, 2003).

When sorting samples, specimens were first thoroughly rinsed with reagent grade water to remove ethanol. Dominant copepods species were sorted by hand and placed in 1 mol L^{-1} HCL bath for 2 hours to demineralise dissolved inorganic carbon. Samples were then washed with reagent grade water and placed directly into tin cups and dried at 60°C for 24 hours. Where insufficient material (>1 mg dry weight) could be recovered for each species, replicates of the same treatment were grouped together. Stable isotope samples for the DOC leachate material were prepared by evaporating 500 mL of leachate material in a 500 mL measuring cylinder with a pre-combusted (4 hours at 450°C) GF/C filter placed at the bottom for 24 hours at 60°C. This was repeated 4 times (total of 2 L) using the same glass fibre filter, which was dried for 48 hours during the final procedure. The GF/C was then ground using a mortar and pestle and placed in tin cups. All samples were then shipped to Griffith University where they were analysed using a continuous flow-isotope mass spectrometer (GV Isoprime Eurovector EA 3000, Manchester, UK). All results were determined against laboratory standard reference material IAEA-CH-6.

6.3.4 Statistical analyses

Differences between treatments and time were tested using permutational multivariate analysis of variance (PERMANOVA) with PRIMER + PERMANOVA software ver. 6 (Anderson, 2001). Euclidean distance was used for environmental data and Bray-

Curtis distance for zooplankton density data, as input for the PERMANOVA analyses. Homogeneity of variance was tested using PERMDISP (Anderson et al., 2008) and data that was skewed was log transformed. Where unequal dispersion was still present after transformation the chances of a type-I error were reduced by rejecting the null hypotheses at a probability of 0.01. Pair-wise t-tests were employed with PERMANOVA to test which treatments and time periods were significantly different. Monte Carlo asymptotic *P*-values were generated to increase the number of permutations tested during t-tests.

6.4 Results

6.4.1 Physico-chemical, bacteria and chlorophyll a

Water temperatures during the experiment varied between 20.0-23.3°C and were not significantly different ($P>0.05$) between treatments (Fig. 6.2A). Initial DOC concentrations were 2.88 mg L^{-1} and did not change significantly in the control throughout the experiment, remaining between 2.77-3.36 mg L^{-1} (Fig. 6.2B). The +1.5 and +3 treatments had significantly higher ($P<0.01$) DOC concentrations compared to the control until day 15, whilst the +16 treatment was significantly higher ($P<0.01$) at all times (Fig. 6.2B, Table 6.1). The addition of the leachate led to significantly higher ($P<0.01$) concentrations of DIN, DON, NH_4 and DIP in the addition treatments compared to the controls (Fig. 6.2C-F). By day 1 nitrogen and phosphorus concentrations had greatly reduced and all remained low for the remainder of the experiment.

The addition of the DOC leachate immediately resulted in significantly lower ($P<0.001$) dissolved oxygen (DO) in all treatments compared to the control before returning to concentrations close to those at the beginning of the experiment (Fig. 6.3A, Table 6.1). The most pronounced response was in the +16 treatment which recorded the lowest DO concentrations at 3.76 mg L^{-1} on day 4. By day 22 all treatments had similar DO concentrations compared to the control (Table 6.2).

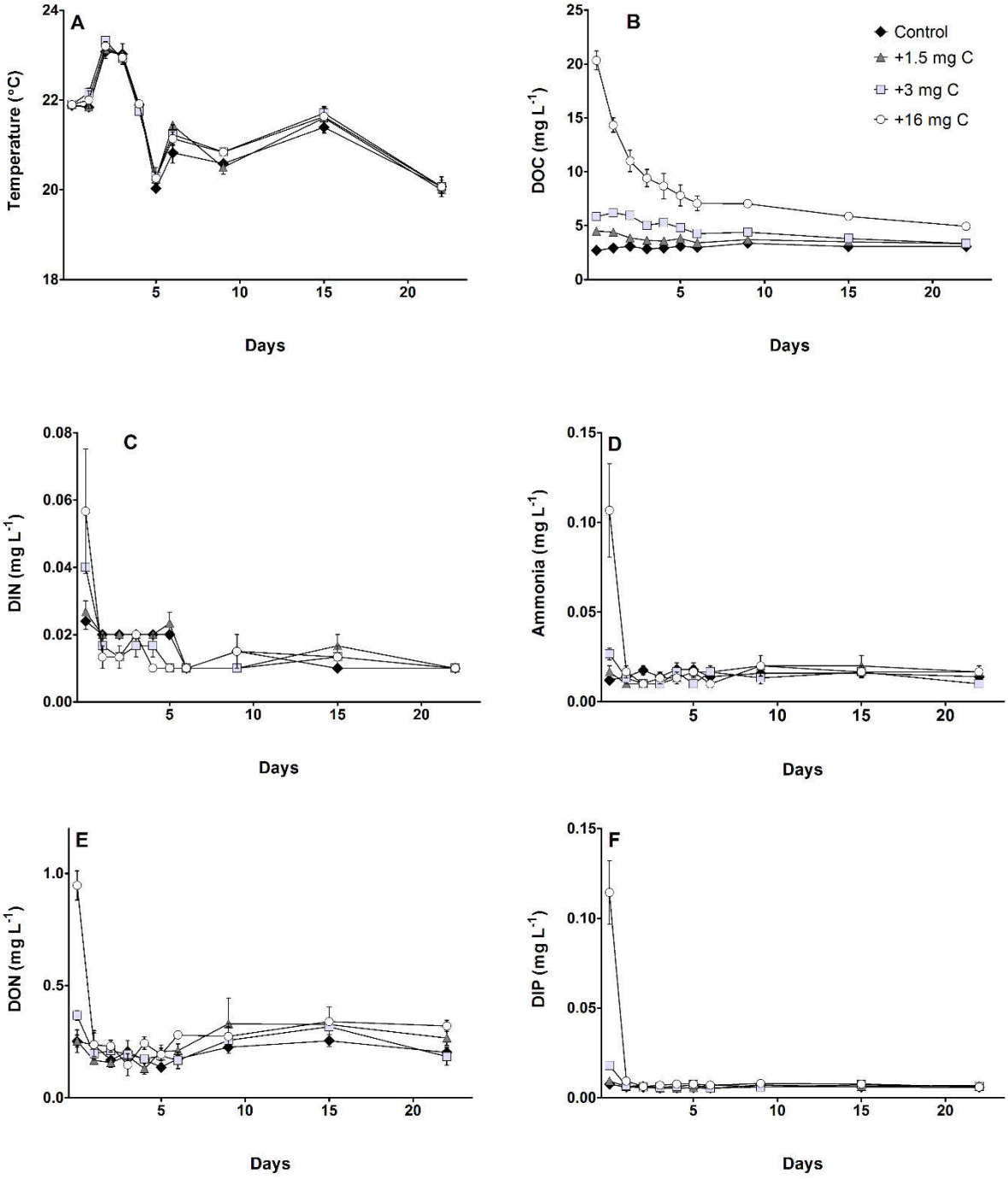

Figure 6.2. Water quality during the mesocosm experiment, Bega River. A) Temperature; B) DOC; C) DIN; D) NH_4; E) DON; F) DIP. Error bars are ± standard error, *n*=5 for control and *n*=3 for treatments.

Table 6.1 Pseudo F-values, degrees of freedom (df) and *P*-values for water quality and zooplankton from PERMANOVA analyses. GP = *Gladioferens pectinatus*, SC = *Sulcanus conflictus*. nt=not tested. *denotes failed Permdisp test and significance considered *P*<0.01

	Between treatments				Between Days				Treatment/Day interaction			
	Pseudo *f*	df	*P*	Perms	Pseudo *f*	df	*P*	Perms	Pseudo *f*	df	*P*	Perms
Temperature	2.00	3	0.112	998	202.42	9	**0.001**	999	0.56	27	0.970	999
DOC*	1261.4	3	**0.001**	999	72.54	9	**0.001**	998	25.50	27	0.001	999
DIN*	0.41	3	0.745	999	25.81	9	**0.001**	998	3.80	27	**0.002**	998
Ammonia*	8.91	3	**0.001**	998	13.15	9	**0.001**	998	9.13	27	**0.001**	999
DON	10.63	3	**0.001**	999	11.86	9	**0.001**	999	3.54	27	**0.002**	999
DIP*	16.10	3	**0.001**	998	19.43	9	**0.001**	999	12.40	27	**0.001**	998
DO*	157.22	3	**0.001**	998	60.43	9	**0.001**	996	16.44	27	**0.001**	997
Bacterial biomass	74.16	3	**0.001**	999	30.43	8	**0.001**	998	2.49	24	**0.001**	998
Chlorophyll *a*	2.20	3	0.131	998	17.67	5	**0.001**	999	1.37	15	0.191	997
Nauplii	2.75	3	**0.008**	998	8.76	4	**0.001**	998	1.17	12	0.256	998
Rotifers	2.54	3	**0.03**	999	13.301	4	**0.001**	997	1.49	12	0.069	998
GP copepodites	2.91	3	**0.011**	998	21.383	4	**0.001**	998	2.23	12	**0.002**	997
GP adults	3.92	3	**0.002**	998	32.62	4	**0.001**	999	1.64	12	0.041	999
SC copepodites	4.38	3	**0.01**	999	48.91	4	**0.001**	999	1.71	12	**0.027**	997
SC adults	2.23	3	**0.027**	998	31.15	4	**0.001**	998	2.20	12	**0.001**	998
GP $\delta^{13}C$	10.05	3	**0.003**	998	nt	-	-	-	nt	-	-	-
GP $\delta^{15}N$	1.64	3	0.26	998	nt	-	-	-	nt	-	-	-

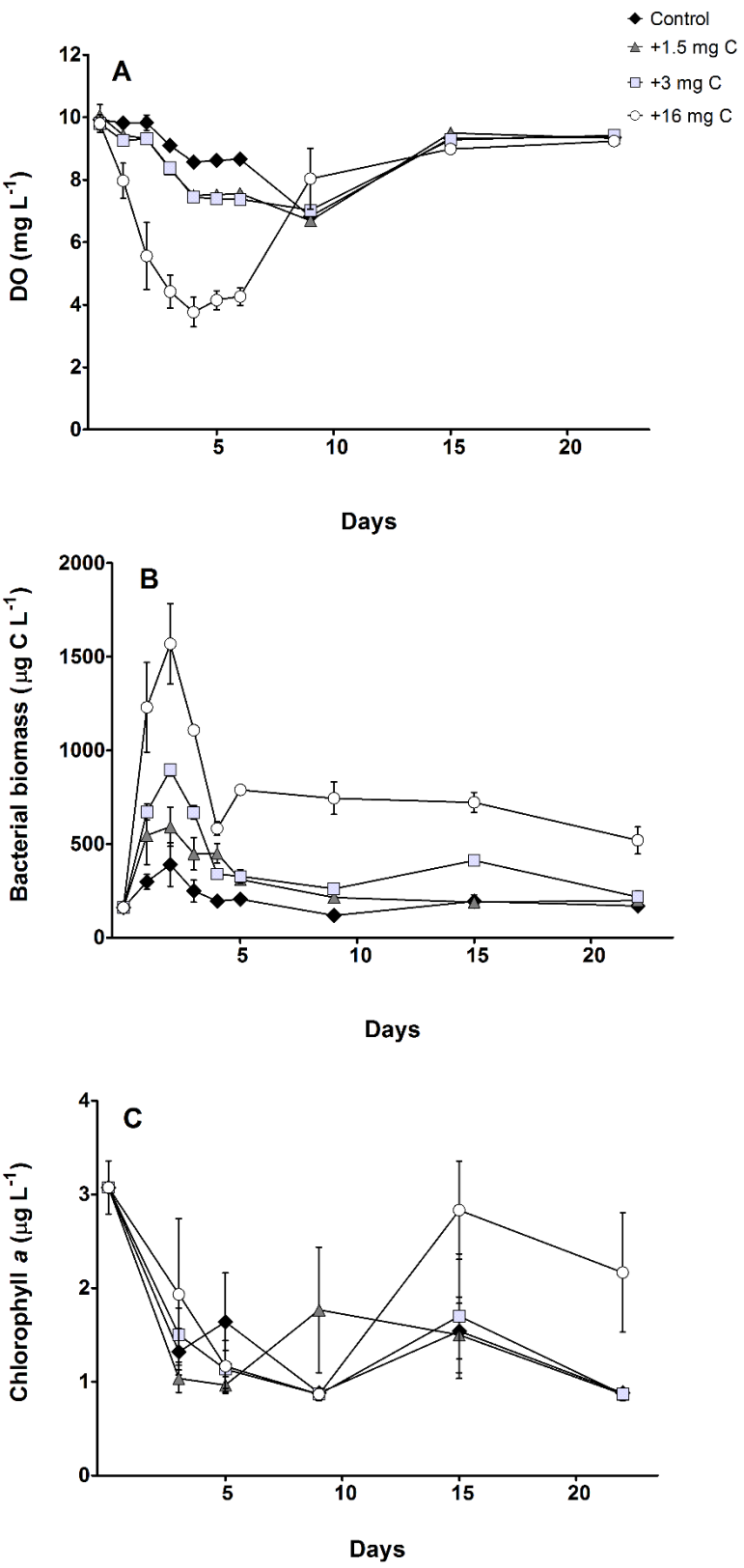

Figure 6.3. Biological parameters during the mesocosm experiment, Bega River. A) Dissolved oxygen; B) Bacterial biomass; C) Chlorophyll a. Error bars are ± standard error, n=5 for control and *n*=3 for treatments.

Bacterial biomass increased in all mesocosms, peaking at day 2, and differed significantly through time and between treatments (Fig. 6.3B, Table 6.1). The +16 treatment had significantly higher ($P<0.05$) bacterial biomass compared to the control at all times. The +3 treatment bacterial biomass was significantly higher ($P<0.05$) than the control on days 1, 3, 4, 9 and 15, whilst the +1.5 treatment was significantly higher on days 2, 4, 5 and 9 (Table 6.2).

Chlorophyll *a* concentrations varied significantly ($P<0.001$) through time though not between treatments (Fig. 6.3C, Table 6.1). Initial chlorophyll *a* concentrations of 3.1 $\mu g\ L^{-1}$ fell and remained low for the rest of the experiment. The exception was for the +16 treatment which had chlorophyll *a* concentrations moderately higher than the control and other treatments at the end of the experiment.

Table 6.2. Results of Permnova t-test pairwise comparisons, treatments vs days, for DOC, nutrients, dissolved oxygen and bacteria. Only significant (P<0.05) values are listed. *denotes failed Permdisp test and significance considered P<0.01.

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 9	Day 15	Day 22
DOC*										
+1.5	0.001	0.001	0.004	0.001	0.002	0.003	0.004	-	-	-
+3	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.01	-
+16	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.01	0.001
DIN*										
+1.5	-	-	-	-	-	-	-	-	-	-
+3	0.007	-	-	-	-	-	-	-	-	-
+16	-	-	-	-	-	-	-	-	-	-
Ammonia*										
+1.5	-	-	-	-	-	-	-	-	-	-
+3	0.004	-	-	-	-	-	-	-	-	-
+16	0.006	-	-	-	-	-	-	-	-	-
DON										
+1.5	-	-	-	-	-	0.048	-	-	-	-
+3	-	-	0.04	-	-	-	-	-	-	-
+16	0.003	-	0.036	-	-	-	0.004	-	-	0.043
DIP*										
+1.5	-	-	-	-	-	-	-	-	-	-
+3	0.001	-	-	-	-	-	-	-	-	-
+16	0.004	0.008	-	-	0.005	0.005	0.002	-	0.002	-
DO*										
+1.5	-	-	-	0.002	0.002	0.001	0.002	-	-	-
+3	-	-	-	0.009	0.002	0.001	0.001	-	-	-
+16	-	0.009	0.003	0.001	0.001	0.001	0.001	-	-	-
Bacteria										
+1.5	-	-	0.022	-	0.001	0.028	nt	0.042	-	-
+3	-	0.009	-	0.028	0.001	-	nt	0.015	0.028	-
+16	-	0.001	-	0.011	0.001	0.001	nt	0.001	0.006	0.002

6.4.2 Zooplankton density

Nauplii and rotifers dominated the microzooplankton. Densities of both were significantly different (P<0.05) between treatments and days (Table 6.1). Nauplii declined from initial densities during days 5 and 9 in the control, +1.5 and +3 treatments (Fig. 6.4A). Nauplii density remained relatively stable in the +16 treatment through days 0 to 9. At day 22, nauplii density had increased and was significantly higher (P<0.01) in the +3 and +16 treatments compared to the control (Table 6.3). Rotifer density showed a similar pattern to nauplii, with a decline from initial densities which remained low for most of the experiment (Fig. 6.4B). Rotifer density in the +3 and +16 treatments was significantly higher (P<0.05) than the control at days 15 and

22 (Table 6.3). Rotifer populations were dominated almost exclusively by *Asplananchna* sp. at days 0-9, whereas populations at days 15 and 22 were dominated almost exclusively by *Colurella* sp.

Two species of calanoid copepod dominated the mesozooplankton, the omnivorous *Gladioferens pectinatus* and the carnivorous *Sulcanus conflictus*. The density of both species varied significantly ($P<0.05$) between treatments and days (Table 6.1). Copepodites of *G. pectinatus* were initially low and increased in all treatments at days 5 and 9 before declining towards the end of the experiment (Fig. 6.4C). *G. pectinatus* copepodites were significantly higher ($P<0.01$) in the +16 treatment compared to the control at day 5 (Table 6.3). Density of adult *G. pectinatus* was highest at days 9 and 15 in all treatments (Fig. 6.4D). Numbers of adult *G. pectinatus* were significantly higher ($P<0.05$) in the +3 treatment at day 9 and in the +16 treatment at days 9 and 15 compared to the control.

Copepodites and adults of *S. conflictus* were not detected in any mesocosm at day 0 (Fig. 6.4E,F). *S. conflictus* copepodites were most numerous at days 5 and 9 before declining at days 15 and 22. *S. conflictus* copepodite densities were significantly higher ($P<0.01$) in the +16 treatment compared to the control at day 9 (Table 6.3). The density of *S. conflictus* adults peaked at day 9 and was significantly higher ($P<0.05$) in the +3 and +16 treatments compared to the controls.

Other mesozooplankton species occurred in small numbers (Fig. 6.4G). These included mostly harpacticoida copepods and never more than two specimens of *Bosmina meridionalis*, diptera larvae, polychaetes and juvenile hyperiid amphipods. Due to the low number of individuals recorded for these taxa, PERMANOVA analysis was not conducted on this group of 'other zooplankton'.

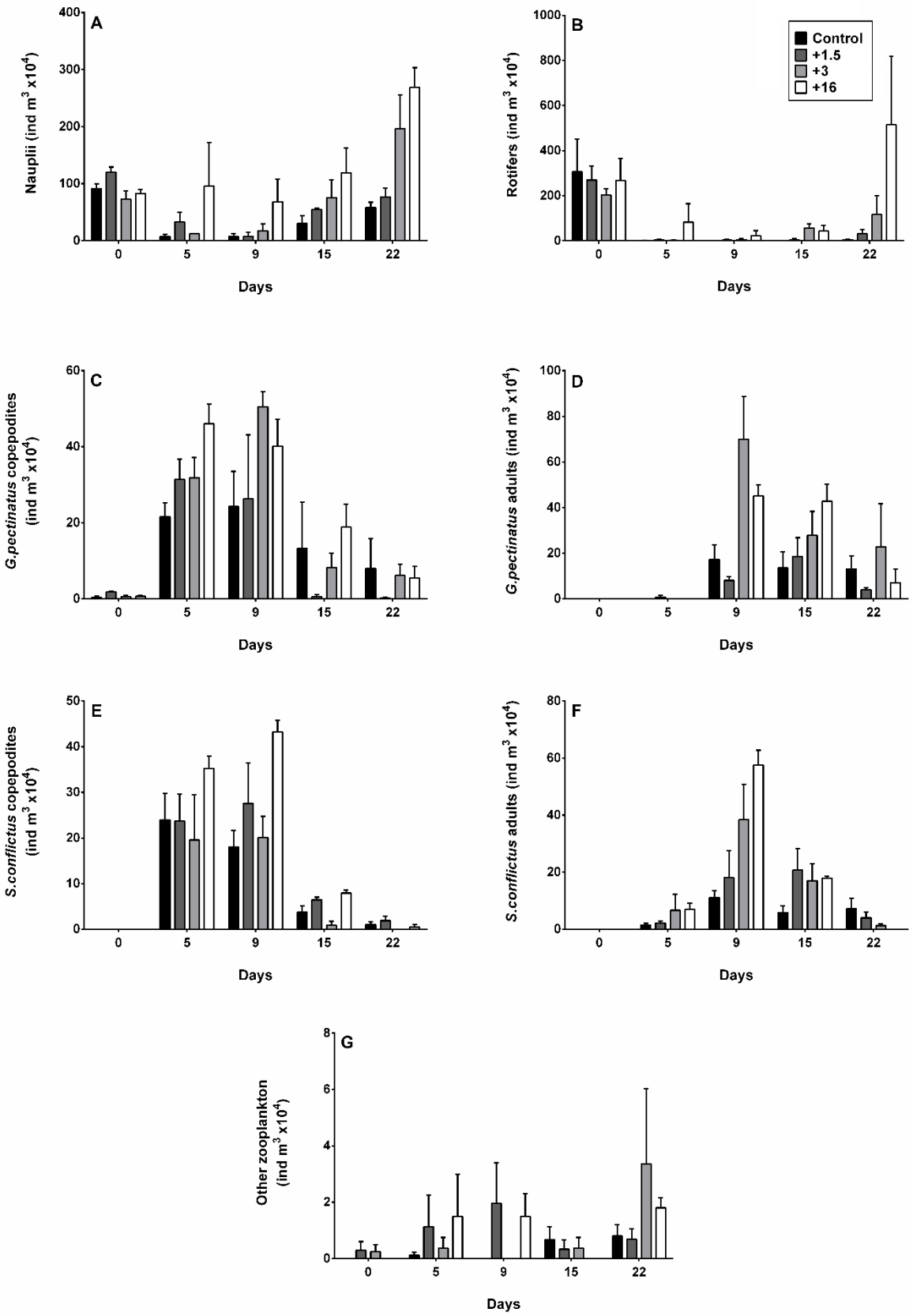

Figure 6.4. Zooplankton density during the mesocosm experiment, Bega River. A) Nauplii; B) Rotifers; C) *Gladioferens pectinatus* copepodites; D) *Gladioferens pectinatus* adults, E) *Sulcanus conflictus* copepodites, F) *Sulcanus conflictus* adults, G) Other zooplankton. Error bars are standard error, *n*=5 for control and *n*=3 for treatments.

Table 6.3. Results for Permanova monte-carlo pairwise comparisons treatments vs days. Only significant (*P*<0.05) values are listed. GP = *Gladioferens pectinatus*, SC = *Sulcanus conflictus*, Cop = copepodites.

	Day 0	Day 5	Day 9	Day 15	Day 22
Nauplii					
+1.5	-	-	-	-	-
+3	-	-	-	-	0.006
+16	-	-	-	-	0.001
Rotifers					
+1.5	-	-	-	-	-
+3	-	-	-	0.04	0.023
+16	-	-	-	0.032	0.006
GP Cop					
+1.5	-	-	-	-	-
+3	-	-	-	-	-
+16	-	0.009	-	-	-
GP Adults					
+1.5	-	-	-	-	-
+3	-	-	0.024	-	-
+16	-	-	0.034	0.043	-
SC Cop					
+1.5	-	-	-	-	-
+3	-	-	-	-	-
+16	-	-	0.007	-	-
SC Adults					
+1.5	-	-	-	-	-
+3	-	-	0.018	-	-
+16	-	-	0.001	-	-
GP $\delta^{13}C$					
+1.5	nt	nt	nt	nt	-
+3	nt	nt	nt	nt	-
+16	nt	nt	nt	nt	0.026

6.4.3 Zooplankton stable isotopes

Initial $\delta^{13}C$ signatures for *G. pectinatus* and *S. conflictus* were -25.3‰ and -25.8‰ respectively (Fig. 6.5). By day 22, the $\delta^{13}C$ signatures for both species in the control, +1.5 and +3 treatments were similar to their initial signatures. In contrast, the PERMANOVA results showed that the $\delta^{13}C$ signatures for *G. pectinatus* were significantly different ($P<0.05$) from the control in the +16 treatment, which had a value of -23.7‰ (Table 6.3). Stable isotopes for *S. conflictus* were pooled between replicates for each respective treatment due to a lack of material and as a result, PERMANOVA analysis was not conducted for this species. Nevertheless, the $\delta^{13}C$ signature of *S. conflictus* in the +16 treatment (22.8‰) was also substantially lighter than the values from the control treatment. The $\delta^{13}C$ signatures of both copepod species at day 22 in the +16 treatment showed a closer resemblance to the DOC leachate (which had a $\delta^{13}C$ signature of -21.5‰) than did the other treatments.

Initial $\delta^{15}N$ signatures for both species was 7.4‰ (Fig. 6.5). At day 22, the $\delta^{15}N$ values ranged from 7.9‰ to 8.8‰. The exception was for the +3 treatment, where *S. conflictus* signatures remained the same as those at the beginning of the experiment.

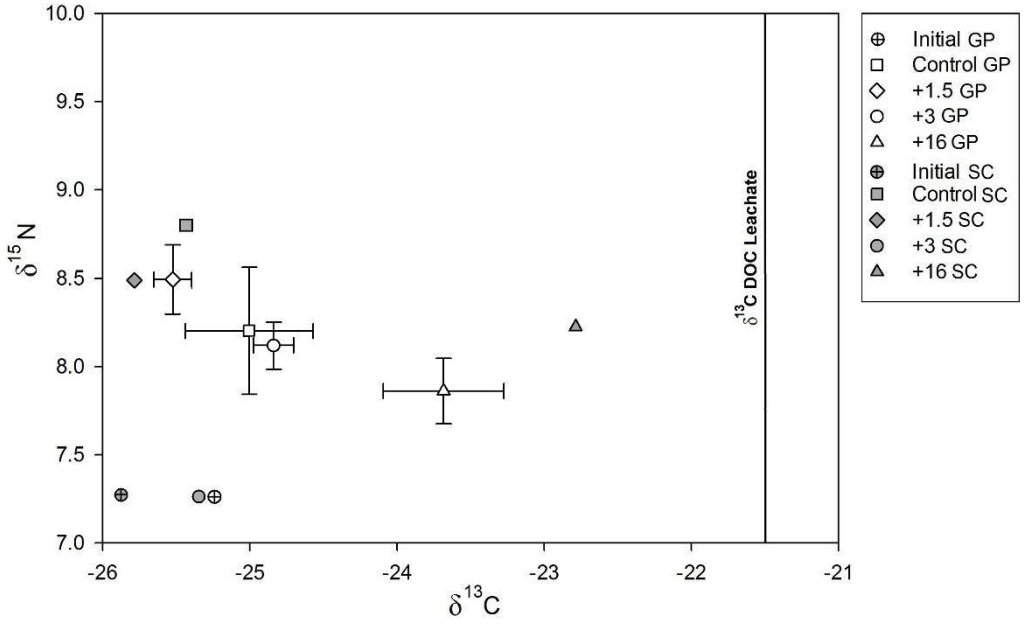

Figure 6.5. Initial and day 22 stable isotope $\delta^{13}C$ and $\delta^{15}N$ signatures during the mesocosm experiment, Bega River. Horizontal line indicates $\delta^{13}C$ signature of the DOC leachate. GP = *Gladioferens pectinatus*, SC = *Sulcanus conflictus.* Error bars are ± standard error. For G. pectinatus $\delta^{13}C$ n=5 for control, n=3 for treatments and for $\delta^{15}N$ n=2. For S. conflictus only one pooled sample for control and each treatment was measured.

6.5 Discussion

6.5.1 Heterotrophic and autotrophic responses

Bacterial biomass increased significantly in all treatments. Maximum bacterial biomass on day 2 was relative to the concentration of DOC leachate added; supporting our hypothesis that bacterial biomass would increase with increasing DOC additions. We expected DOC additions to increase bacterial biomass as the experiment was conducted following a period of low freshwater inflows with low ambient DOC concentrations and we have previously found bacteria to be carbon limited under these conditions (Hitchcock and Mitrovic, 2013). These results are also consistent with other mesocosm experiments, which have shown that bacterial production can increase with increased additions of DOC (Faithfull et al., 2012; Karlsson et al., 2007).

The link between DOC and bacterial production is also evidenced by the fact that the increase in bacterial biomass was coupled with a rapid reduction in DOC concentrations, with approximately 60% of added leachate DOC utilised in all treatments in the first six days of the experiment. High bacterial utilisation of the added DOC is also supported by the reduced dissolved oxygen concentrations, indicating high levels of respiration during the first days of the experiment (Fig. 6.3A). Taken together, these findings suggest that the added DOC leachate was highly bioavailable. In combination with microbial degradation, photobleaching of humic substances may also have taken place, as demonstrated in other estuaries (Moran et al., 2000), which may in part be responsible for the high bioavailability of the leachate.

There were no significant differences in chlorophyll *a* concentrations between treatments. Chlorophyll *a* declined in the control and all treatments during the initial five days of the experiment (Fig. 6.3C). We suggest this was probably due to bottom-up control through nutrient limitation. Nitrogen and phosphorus declined rapidly in the first 24 hours of the experiment in all mesocosms to concentrations we have previously found limiting in the Bega River (Hitchcock and Mitrovic, 2013). The decrease in chlorophyll *a* was concurrent with a rapid increase in bacterial biomass, (Fig. 6.3B) suggesting that bacteria were likely outcompeting phytoplankton for nitrogen and phosphorous resources. We have previously found some evidence of competition between bacteria and phytoplankton for nitrogen and phosphorous on the

Bega River (Hitchcock and Mitrovic, 2013) and bacteria have been reported to outcompete phytoplankton for nutrients in other systems due to their higher surface area to volume ratios and greater affinity for phosphorus (Currie and Kalff, 1984; Jansson, 1993). Towards the end of the experiment chlorophyll *a* concentrations appeared moderately higher in the +16 treatment (Fig. 6.3C), which might suggest that bacteria played a minor role in nutrient regeneration at this time. As we measured chlorophyll *a* rather than primary production, it is possible that primary production was higher than chlorophyll *a* concentrations imply, especially if both turnover rates and top-down grazing pressure was high. This however seems unlikely due to the low nutrient concentrations from day 1 onwards. Autochthonous carbon contribution to the total carbon pool was likely equally low between treatments.

6.5.2 Zooplankton responses

Zooplankton responses to the DOC leachate additions provide strong evidence of a contribution of this source of DOC into the metazoan food web. The experiment appeared to encompass a full life cycle of copepods with both the control and all treatments showing a relatively similar successional pattern with shifting dominance from nauplii to copepodites, to adults and back to nauplii (Fig. 6.4). While this pattern was consistent through the control and all treatments, the mesocosms receiving higher concentrations of DOC leachate had significantly higher densities of nauplii, copepods and rotifers (Table 6.1). Increases in zooplankton density were preceded by low chlorophyll *a* concentrations and a peak in bacterial biomass, supporting our hypothesis that the DOC additions subsidised the microbial loop to support zooplankton production.

The stable isotope results provide another strong line of evidence that zooplankton were subsidised by the leachate DOC, presumably via heterotrophic bacteria. Specifically, evidence of leachate DOC assimilation was provided by the $\delta^{13}C$ signatures of both *S. conflictus* and *G. pectinatus* in the +16 treatment, which had shifted away from the initial signature (and that of the control) towards the signature of DOC leachate material (Fig. 6.5). This would be expected if the DOC leachate is providing a significant carbon subsidy to the copepods. Similar results have been found in mesocosm studies in Swedish lakes where the carbon signatures of

zooplankton more closely resembled that of glucose in the treatments where higher concentrations of glucose were added (Faithfull et al., 2012; Karlsson et al., 2007).

On the basis of our results we propose three possible, nonexclusive, trophic interactions facilitating the movement of DOC leachate through the food web to zooplankton. Firstly, nauplii and rotifers may have fed directly on bacteria and small bacterivores. Indeed, nauplii of some species have been shown to feed on bacteria directly (Roff et al., 1995) as well as other small protists such as nanoflagellates (Böttjer et al., 2010). The rotifer *Colurella* may feed directly on bacteria or, along with *Asplanchna* may consume small protists (Sanoamuang et al., 2001 and references therein). Nauplii densities remained high in the +16 treatment throughout the experiment whilst rotifer densities increased in this treatment at the end of the experiment. Increases in nauplii (Daniel et al., 2006) and rotifer density (Faithfull et al., 2011) have been witnessed in other mesocosm experiments receiving bioavailable DOC substrates.

The trophic pathways that support nauplii may also be different from those that support mature copepods. Uitto (1996) conducted grazing experiments on micro and mesozooplankton and found that microzooplankton had a greater reliance on nanoprotists, suggesting that juvenile copepods may occupy a lower trophic position than adults. Increased nauplii densities may have resulted in increased survival and maturation into copepodite stages, leading to the higher copepodite and adult densities observed. Arnott et al. (1986) has shown, *G. pectinatus* lays more eggs and has greater maturation success in the presence of more available food resources.

A second possible trophic pathway was that of copepodite and adult copepods consuming ciliates and other protists which had themselves consumed bacteria. Flagellates and ciliates are major consumers of bacteria (Ameryk et al., 2005). In other mesocosm experiments flagellates and ciliates have been found to increase following DOC additions (Bell et al., 1993; Blomqvist et al., 2001). In this study we do not have data for heterotrophic nanoflagellates and ciliates. However, copepods have regularly been found to show a preference for flagellates and ciliates in other estuaries (Bollens and Penry, 2003), and may exert strong top-down control over flagellates and ciliate populations (Zöllner et al., 2009). A potential microzooplankton link between bacteria and copepods is particularly pertinent given that bacteria are not able to produce long

chained polyunsaturated fatty acids which are essential to copepod diets (Ramesh and Demott, 1997). Instead, intermediate organisms, such as rotifers or small protists like ciliates, are needed for their synthesis (Breteler et al., 1999; Lubzens et al., 1985; Martin-Creuzburg et al., 2005).

The third trophic relationship that may have played a role in our experiments was that *S. conflictus* directly consumed the increased numbers of rotifers, nauplii and copepodites. Results from feeding studies have found that the preferred food source of *S. conflictus* is the nauplii of *G. pectinatus*, followed by the nauplii and copepodites of its own species (Ough and Bayly, 1989). Rotifers are also considered a high quality food source for other copepod species, whose density may be controlled by copepod predation (Bollens and Penry, 2003; Kumar and Rao, 2001). In our study, the increased densities of nauplii and copepodites in the +3 and +16 treatments would have provided sufficient food resources for this pathway to occur. Increases in densities of nauplii and rotifer density towards the end of the experiment, when adult copepod numbers had declined, also provides some evidence that nauplii and rotifer populations may have been controlled by copepod predation. Additionally if *S. conflictus* was consuming nauplii and copepodites of *G. pectinatus* and its own species, it may partly explain why densities of both decreased from day 15 onwards.

6.5.3 Microbial loop and terrestrial carbon in estuarine food webs

When these results are viewed in the context of other DOC addition mesocosm experiments, it appears that the quantity of DOC added determines at least in part whether the microbial loop acts as a link (to the metazoan food web) or only a sink. Ducklow et al. (1986) performed a mesocosm experiment on Loch Ewe, Scotland, finding that an addition of 6mCi of labelled glucose (equivalent to 1.32 mg C L^{-1}) was not transferred to zooplankton over 55 days. Their explanation for this finding was due to loss of organic carbon as respiration via the multiple trophic steps in the microbial food web. Berglund et al. (2007) performed mesocosms in the Baltic Sea to test the differences in zooplankton support between an autotrophic food web (stimulated by N and P additions) and a bacterial dominated food web (stimulated by an addition of 0.13 mg L^{-1} per day of glucose plus N and P). They found, over 29 days, that zooplankton densities were significantly lower in the bacteria dominated food web compared to the autotrophic food web. These studies used relatively small DOC

additions, similar to our treatment of +1.5 mg C L^{-1} which also had no significant effects on zooplankton density compared to controls.

In contrast, Karlsson et al. (2007) used a gradient of daily glucose addition treatments, from 0.11 to 3.4 mg L^{-1}, in mesocosms in a sub-arctic lake in Sweden. They found over 9 days that glucose incorporation by zooplankton and zooplankton growth rates increased proportionally across treatments receiving higher glucose additions. Faithfull et al. (2012) performed a longer (44 day) mesocosm experiment in an oligotrophic lake in Sweden, testing zooplankton responses to twice weekly glucose additions of 0.42 and 2.1 mg L^{-1}. They also found that calanoid copepod biomass increased the most in the treatments receiving the highest glucose addition. Similarly, it was only the higher leachate addition treatments in our experiment that showed significant differences in zooplankton biomass. These results suggest that sufficiently large quantities of bioavailable organic carbon can subsidize zooplankton production even if a large proportion of the initial DOC pool is respired by bacteria and smaller protists.

To date, DOC addition experiments have been conducted almost exclusively using glucose, assuming equivalence between glucose and natural dissolved organic material (DOM) in terms of zooplankton response. Mitrovic et al. (2014) recently compared DOC additions of glucose and leaf leachate and showed that whilst both led to significant increases in bacterial production, only the leaf leachate significantly increased density of copepods. One clear difference between glucose and more natural mixtures of DOM is that glucose is highly bioavailable and likely utilised in the mesocosm context within days, whilst natural DOM contains a mixture of compounds of different labilities (Berggren et al., 2010). Wetzel (1995) previously hypothesised that semi-labile allochthonous DOC is important in providing a long-term stable energetic resource in ecosystems. This was evidenced in our experiment by the sustained higher concentrations of bacterial biomass in the +16 treatment (Fig. 6.3B). Whilst natural DOC in the Bega River during peak inflow has a bioavailability of around 30% (Chapter 3), our leachate was at least double that, with around 60% being utilised in the first week. Despite this difference, the leaf leachate still provides a closer representation of natural conditions and DOM inflows compared to studies that use glucose as a source of carbon.

Zooplankton are still generally not considered to be supported by terrestrial carbon, especially via the microbial loop (Fenchel, 2008), despite the fact that there are a considerable number of examples where zooplankton are at least partially subsidised by allochthonous DOC. Notably, these examples are mostly from lentic and estuarine systems which receive large subsidies of allochthonous DOM during hydrological events (Carpenter et al., 2005). In estuaries, freshwater inflows appear to be a driving force behind allochthonous support (Reaugh et al. 2007; Hoffman et al. 2008; Atwood et al. 2011). Our mesocosm experiment provides strong evidence that DOC and bacteria play an important role in zooplankton growth in the Bega River estuary following DOC pulses. Our results have found that whilst both small and large additions of terrestrially-derived DOC can lead to increased bacterial biomass, only the larger additions led to increases in zooplankton density and changes in $\delta^{13}C$ signatures. This suggests that the importance of allochthonous carbon in subsidising higher trophic levels via the microbial loop is in part controlled by the quantity and quality DOC input received.

Chapter 7 General Discussion and Conclusions

7.1 Discussion

The aim of this thesis was to define the role that freshwater inflows have over organic carbon and microbial food web dynamics in south-east Australian estuaries. To do this I conducted a series of monitoring studies and manipulative experiments on the Bega and Clyde River estuaries. Whilst each study focused on specific questions relating to inflows, carbon, bacteria and zooplankton, integrated as a broader thesis the results provide a novel framework for understanding the important role that freshwater inflows have in maintaining ecological processes and productivity within estuaries (Fig 7.1).

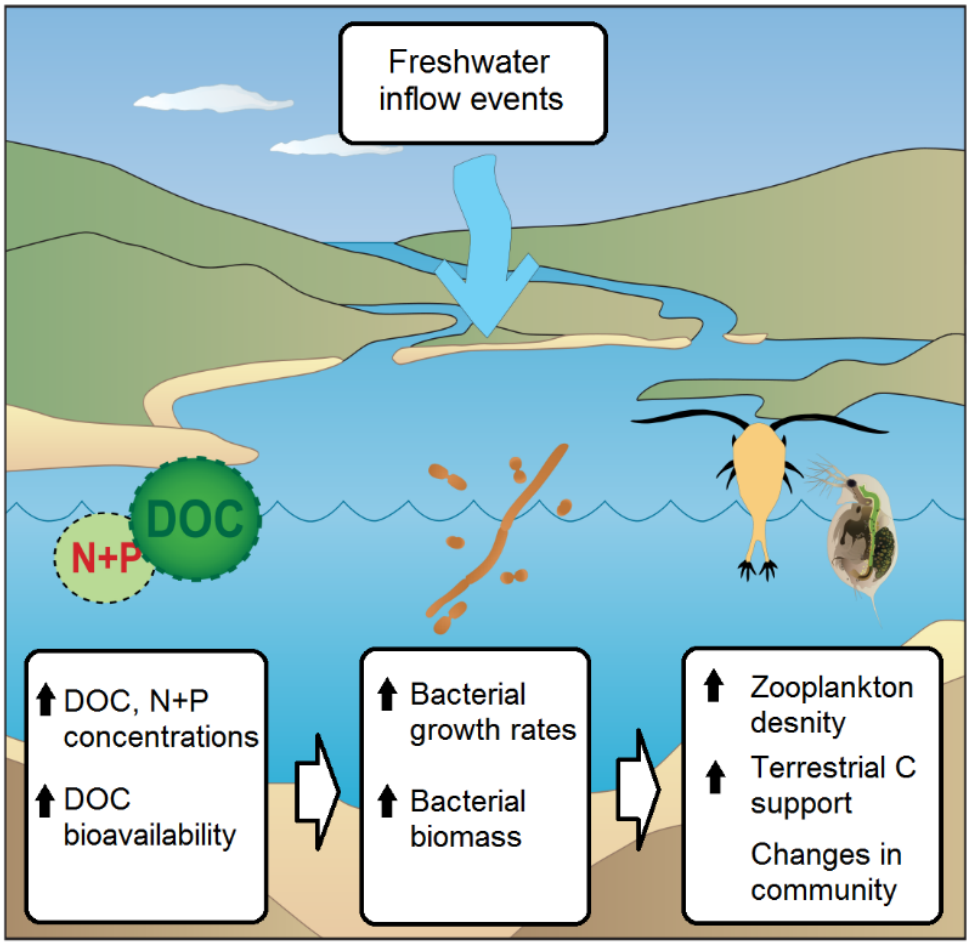

Figure 7.1. Conceptual diagram of the major findings of this thesis.

7.1.1 Freshwater inflows and organic carbon

The major source of organic carbon in estuaries is generally regarded as autochthonous (Bianchi, 2013). As such, DOC is often positively correlated with phytoplankton biomass or chlorophyll *a* (Hansell and Carlson, 2002). On the Bega and Clyde rivers DOC concentrations were always related to discharge and showed no relationship with chlorophyll *a* (Chapter 2). Variation in discharge and associated inputs of allochthonous DOC can cause the relationship between DOC and phytoplankton to become uncoupled. This may occur temporally, where DOC is related to phytoplankton during periods of low flow and related to discharge during periods of high flows (Ochs et al., 2010) or may vary spatially with DOC related to discharge in the upper estuary and related to phytoplankton in the lower more marine estuary (Jassby et al., 1993).

On the Bega and Clyde River estuaries I examined both upper and lower estuary sites, and periods of low and high flow and found DOC concentrations to be closely correlated to discharge. The main reason for this is likely to be the highly episodic nature of the hydrology that characterises these catchments. During this 30 month study, freshwater inflow events occurred on 28 occasions, supplying frequent moderate to large loads of DOC to the Bega and Clyde River estuaries. This may have been enhanced by above average precipitation as a result of Australia being in a La Niña period of southern oscillation during the study period. Primary production may have had a greater influence on DOC dynamics in these estuaries during extended periods of drought and low flow; this is a hypothesis that would need to be tested.

In freshwater systems there have been a handful of studies comparing DOC bioavailability between high and low flow periods finding mixed results (Leff and Meyer, 1991; McLaughlin and Kaplan, 2013; Wiegner et al., 2009). Little information exists on how the bioavailability of DOC in estuaries may vary with freshwater inflows. In Chapter 3, I performed *in-vitro* bioassay experiments to test how the bioavailability of DOC entering the Bega and Clyde estuaries changed during inflows. The results showed the bioavailability of DOC ranged between 2.5-31% and was positively related to discharge. The increases in DOC bioavailability are most likely due to changing hydrologic flow paths during flooding events, bringing younger and diagentically less altered carbon from upper soil horizons into the river. These results

support the findings that DOC bioavailability may increase in upland rivers and streams (Kaplan and Newbold, 1993).

As previous studies of DOC bioavailability in estuaries have been conducted during base flow or unspecified flow conditions, it is possible that estimates of the flux of carbon between estuaries and the atmosphere have been significantly underestimated. This is particularly important as not only is DOC the largest fraction of organic carbon in the aquatic carbon cycle (Battin et al., 2008), but also because the majority of allochthonous DOC is delivered to estuaries during episodic inflow. A quick calculation based on the results of Chapter 2 indicate that during this study on the Bega River, floods dominated hydrological conditions for approximately 7% of the time, but accounted for around 80% of the total DOC load to the estuary. These estimates are consistent with those found in other systems (Buffam et al., 2001; Wiegner et al., 2009).

7.1.2 Differences between Bega and Clyde River estuaries

Though my primary focus in this thesis is on the flow regulated and agriculturally developed Bega River estuary, chapters 2 to 4 also included the Clyde River estuary in order to gain a broader perspective on freshwater inflows across systems with less anthropogenically altered catchments. There were a number of important observations when comparing the two systems. Firstly, there were clear differences in nutrient and turbidity dynamics (Chapter 2). On both estuaries nitrogen and turbidity were positively related to discharge. However, nitrogen and turbidity were substantially higher on the Bega River. These differences may be due to the conversion of forested land to agricultural uses. One of the major sources of nitrogen on the Bega River could be the dairy industry, the main land use for the catchment, and key source of nitrogen in other systems (Harris, 2001)

Secondly, phosphorus was positively related to discharge, and high, on the Bega River, whilst on the Clyde River it remained low at all times and mostly unrelated, or poorly related discharge. These results are consistent with other forested estuaries in Australia which commonly are low in phosphorus (Harris, 2001). Phosphorus concentrations may be related to pollution from sewage treatment plants in the catchment (Davis and Koop, 2006). Higher phosphorus, as well as turbidity, may also be related to erosive

processes, which would be higher in the Bega River catchment due to extensive land clearing, which includes riparian vegetation in much of the middle and lower catchment (Brierley et al., 1999; Tozer et al., 2010).

7.1.3 Bacterial responses to inflows and DOC

Bacteria are the major consumer of organic carbon, play an important role in nutrient remineralisation, and may provide an alternative energy pathway for higher consumers (Day et al., 2013). In estuaries and coastal regions bacterial biomass and production are commonly linked to primary production or chlorophyll *a* due to bacterial reliance on autochthonous DOC (Cole et al., 1988). In fact Gasol and Duarte (2000) suggest *"...the link between bacterial abundance and phytoplankton biomass remains as one the few undisputed patterns in microbial ecology*". On the Bega and Clyde Rivers bacterial biomass was positively related to DOC and phosphorus; bacterial biomass was never related to chlorophyll *a* (Chapter 2). There are similar examples in other estuarine and coastal systems of bacteria being uncoupled from phytoplankton (Hoch and Kirchman, 1993; Ning et al., 2005; Ochs et al., 2010). In these examples the authors suggest this uncoupling is due to the influence of allochthonous inputs. Similarly on the Bega and Clyde Rivers the likely reason bacteria were not related to chlorophyll *a* is that the size and variation of allochthonous inputs were much greater than dissolved autochthonous carbon production during the study.

In Chapter 3, as well as quantifying how DOC bioavailability changes during inflows, I also quantified changes in bacterial growth rates. Growth rates were positively related to DOC bioavailability and discharge. On the Bega River, bacterial growth rates were around four fold higher when grown on water from the peak of a flood event compared to four weeks later when inflows had returned to base flow conditions. The results confirmed patterns found by other authors that DOC quality and chemical composition may play a role in controlling bacterial growth rates (Hopkinson et al., 1998).

When conducting the bioavailability bioassays, I ran the experiments both with and without the addition of nitrogen and phosphorus. This was done in order to account for possible inorganic nutrient limitation, potentially effecting growth rates and carbon usage. In the literature few studies account for nutrient limitation which may be

important as nutrients may be regularly replenished within the natural environment (del Giorgio and Davis, 2003, and references therein). My results showed clear differences on certain occasions, particularly on the Clyde River, where the addition of nutrients led to increased bioavailability and bacterial growth rates, indicating that bacteria were clearly resource limited at certain times.

In Chapter 4 I wanted to understand what resources limit bacterial growth, and how resource limitation may change between high and low FWI. During low inflows bacteria were always carbon limited. Carbon limitation of bacteria appears to be common in many estuaries (Hitchcock et al., 2010; Sala et al., 2002). Limitation in the Bega and Clyde Rivers is probably because DOC concentrations were low during base flows (Chapter 2), but also as the bioavailability of DOC was low during these times (Chapter 3). During high flow conditions bacteria remained primarily carbon limited on the Bega River, but were primarily phosphorus limited on the Clyde River. These results reflect the fact that on the Bega River FWI events brought high loads of both DOC and phosphorus, whilst on the Clyde River phosphorus remained low. Together, the bacteria results from chapters 2 through 4 highlight the importance of FWI as key times when bacterial production is significantly increased.

7.1.4 Inflow events characterised as flood, fresh and base flows

Freshwater inflow events have been conceptualised as key periods facilitating cross-system subsidisation of carbon and nutrients in catchments (Junk and Wantzen, 2004). Characterising all inflow events as essentially the same phenomena does not necessarily reflect the different biogeochemical processes that occur during inflows of differing magnitudes. In Chapter 2 I presented a simple conceptual framework to help develop a more nuanced understanding of the role that freshwater inflow events have on carbon and nutrient dynamics in estuaries. Large flood flows, occurred infrequently, breaking the banks of rivers, whilst smaller fresh flows constituted a rise in discharge that did not break the banks, and occurred more frequently; typically once a month during the study period. In the Bega River, it's been suggested that water regulation most likely reduces smaller to medium fresh flows, whilst having little effect on larger flood flows.

Chapter 2 highlights the potential ecological importance of fresh flows. Whilst floods brought higher loads of DOC to the estuary than fresh flows, DOC delivered to estuaries during floods is thought to move directly through the estuary and be transported to the ocean via plumes (Ford et al., 2005). Concentrations of DOC, as well as nitrogen, entering the estuary were similar under both flood and fresh flow types. Longer residence times associated with smaller inflows allow for longer periods microbial utilisation of DOC (Crump et al., 2004). Bacterial biomass was similarly high during flood and fresh flow events compared to lower concentrations during base flow conditions. Fresh flows also had relatively low turbidity compared to flood flows, and this may be one reason why chlorophyll *a* was higher during fresh flows compared to flood flows on the Bega River. In addition to this, fresh flow occurred almost monthly compared to floods which occurred only once or twice a year. These results imply that special consideration should be given to fresh flows when developing environmental flow rules for the Bega River estuary.

7.1.5 Zooplankton responses to inflows and DOC

There is considerable debate around whether bacteria and the microbial loop act as a 'link' for energy to higher trophic levels or rather solely a 'sink' where the majority of organic carbon is lost as respiration (Brett et al., 2009; Ducklow et al., 1986; Karlsson et al., 2014; Sherr et al., 1987). The most widely accepted view is that the microbial loop does not support zooplankton (Fenchel, 2008). The main reason for this is that more levels in the trophic pathway of microbial food webs are required to transfer DOC to zooplankton, and much of the organic carbon pool is lost through respiration (Ducklow et al., 1986). In chapters 5-6, focusing on the Bega River, I found strong evidence that, contrary to this dominant paradigm, bacteria could act as link, supplying organic carbon from terrestrial sources to zooplankton.

In Chapter 5, I monitored the responses of zooplankton to inflow events and associated organic carbon pulses. Zooplankton ecology in estuaries has been widely studied though relatively few studies have specifically examined zooplankton responses to FWI events. For example, long-term studies on zooplankton dynamics in San Francisco Estuary and Chesapeake Bay, with sampling frequency spanning months, found that freshwater inflows had no bottom-up resource influences on zooplankton (Kimmel and Roman, 2004; Kimmerer, 2002). Where shorter-term studies have

examined zooplankton response to FWI events they have suggested changes in basal resources may lead to increased zooplankton densities on a scale of days to weeks (Hoover et al., 2006). On the Bega River, zooplankton density increased following a flooding event. Redundancy analysis suggested that increasing densities of rotifer, cladocera and copepod taxa were closely linked to bacteria and bacterial consumers.

Estuaries are complex environments. Salinity appeared to be a major determinant of zooplankton assemblage structure. Changes in salinity associated with FWI have been found to have a strong influence over zooplankton assemblage structure in other estuaries (Primo et al., 2009). Shifts in the dominance of freshwater and estuarine species on the Bega River, concurrent with organic matter pulses, made distinguishing the bottom-up effect of resources difficult. I used $\delta^{13}C$ stable isotope analysis to trace the sources of organic carbon supporting copepod and cladocera species; the results showing that allochthonous terrestrial carbon was the dominant source supporting zooplankton. These results agree with other recent stable isotope studies that have shown allochthonous carbon makes a larger contribution to estuarine zooplankton following periods of high FWI (Atwood et al., 2011; Hoffman et al., 2008).

In Chapter 6 I used experimental mesocosms to test whether DOC and the microbial loop could support zooplankton. Mesocosms have been used by a number of authors to trace additions of DOC through the food web (Berglund et al., 2007; Ducklow et al., 1986). On the Bega River I found that additions of DOC led to significant increases in bacterial biomass, and in turn copepod density. Significant increases in copepods only occurred in treatments that received high concentrations of DOC leachate. These results were supported by $\delta^{13}C$ stable isotopes that indicated the DOC additions were incorporated into zooplankton in the treatment receiving the highest subsidy. Most of the studies that have found that bacteria do not support zooplankton have used relatively small subsidies of DOC (Berglund et al., 2007; Ducklow et al., 1986). My results agree with the findings of recent work in Swedish lakes that found zooplankton subsidisation from the microbial loop increases with increasing concentrations of added DOC (Faithfull et al., 2012; Karlsson et al., 2007). The results from chapter 6 are also unique in that I used a leaf leachate made from catchment vegetation which is a more natural source than glucose which most mesocosm studies use as a DOC addition. Recent findings from a mesocosm study conducted on a lowland river

suggest that glucose should not be considered equivalent to more natural DOC, finding that leaf leachate additions led to increased copepod density, but not the addition of glucose at the same concentration (Mitrovic et al., 2014).

Whilst the results presented here refute the widely held contention that allochthonous carbon and bacteria do not support zooplankton; they do not necessarily disagree with the suggestion that much of the organic carbon pool may be lost through respiration. We did not measure transfer efficiency in the food web. However, only large DOC additions in the mesocosm experiment led to significant increases in zooplankton density. This suggests that even if much of the original DOC pool is lost as respiration, if the subsidy is large enough, as occurs during FWI events in the Bega River estuary, then the remaining organic carbon pool may still be sufficient to support zooplankton.

There are also broader implications for our understanding of the food web in the Bega River estuary. Freshwater inflows have been linked to fisheries production in Australian estuaries (Gillson, 2011; Ruello, 1973). Zooplankton are a key food source for juvenile fish in many estuarine and coastal systems. Fish recruitment and survival is in many cases linked to the availability of zooplankton as a food source (Castonguay et al., 2008; Murphy et al., 2012; Newton, 1996; Williams et al., 2013). Stable isotope studies for estuarine fish have found increasing allochthonous support during periods of high inflows (Abrantes and Sheaves, 2010b; Hoffman et al., 2007). It is possible then that the changes happening at the base of the food web in the Bega River following inflows may have far reaching trophic implications.

7.2 Further research

7.2.1 Greater temporal and spatial replication

This study occurred during a period of above average rainfall. There are a range of studies that have found carbon (Jassby et al., 1993), bacteria (Almeida et al., 2007) and zooplankton (Hoffman et al., 2008) dynamics may change between periods of above and below average precipitation and inflows. Understanding how the relationships found here change during periods of extended low FWI will be essential in defining a longer-term view of the Bega and Clyde River estuaries and others in systems in south eastern Australia.

Chapters 5 and 6 focused only on the Bega River estuary. This was due to restrictions in time and labour resources. Conducting monitoring and experimental work on the zooplankton community in the Clyde River will be useful in seeing if the results found on the Bega River are replicated on the Clyde. This may be particularly interesting due to differences in hydrology, nutrient input and volume of the estuary.

There have been limited studies on carbon, bacteria and zooplankton as related to FWI in Australia. There are some examples where at least one of the aforementioned aspects has been studied; the Swan-Caning Estuary, WA (Petrone, 2010); Fitzroy River Estuary, Qld (Ford et al., 2005); Hunter River Estuary, NSW (Hitchcock et al., 2010); Wilson Inlet, WA (Gaughan and Potter, 1995); Bremer River, QLD (Pollard and Ducklow, 2011). Investigating other estuaries, both within south-east Australia and beyond will be crucial in determining how applicable the results and relationships found on these rivers are to other catchments.

7.2.2 Fine scale event sampling

Chapter 2 monitored DOC and nutrient concentrations under a variety of hydrological conditions. I captured a number of inflow events during the study, however this usually encompassed only one to two samples during the peak of the event. Whilst there is little information on how carbon and nutrient loads may vary at shorter time scales during inflow to estuaries, in freshwater systems DOC concentrations can vary greatly over the space of hours (Buffam et al., 2001; Westhorpe et al., 2012). There is a need to capture finer scale changes that may occur during inflow events. Further studies using auto-samplers taking hourly samples during inflow events will be needed to more comprehensively gauge carbon loads exported to estuaries.

7.2.3 Integrating bioavailability and limitation studies

Chapter 4 of this thesis dealt with testing resource limitation for bacteria and phytoplankton. Commonly it is assumed the stoichiometry of resources, that is C:N:P ratios, determine what primarily limits growth. However, as shown in chapter 3, the quantities of a substance do not necessarily reflect the amount biologically available for metabolism. Integrating measures of bioavailability into limitation studies will help to better define when and why certain resources are causing limitation.

7.2.4 Microzooplankton trophic interactions

Whilst Chapters 5 and 6 showed DOC could support copepod production, the particular trophic pathway and microorganisms responsible are unknown. The contributions of microzooplankton to copepod diets remains an area that is poorly understood. Developing better approaches to monitor and experimentally test microzooplankton relations will be crucial to defining microbial food web dynamics.

7.2.5 Expanding methods to trace carbon sources

In chapters 5 and 6 I used stable isotope to differentiate the sources of carbon supporting zooplankton. One of the problems with the method is determining the carbon signatures of phytoplankton when it is hard to isolate them from other organisms or organic materials (Karlsson et al., 2014). In chapter 5 I used benthic algae comprised of the same species that at times dominated phytoplankton assemblages to deal with the issue. Whilst isolating phytoplankton remains a major problem in isotope studies, implementing additional measures such as stable isotope analysis of hydrogen and fatty acid biomarkers are increasingly being used to add additional lines of evidence (Doucett et al., 2007; El-Sabaawi et al., 2009). Using these techniques in future studies will provide a stronger understanding of how source of carbon utilised by zooplankton may vary.

7.2.6 Responses of fish and higher trophic levels

Fisheries production has long been linked to years with high freshwater inflow in estuaries (Gillson, 2011). Stable isotope studies have found fish may be supported by allochthonous DOC (Abrantes and Sheaves, 2010a; Hoffman et al., 2007). Some experimental evidence exists to suggest increased microbial and zooplankton production can support some species of juvenile fish (Faithfull et al., 2012). There is a strong and established link between zooplankton and fish survival and production (Castonguay et al., 2008; Newton, 1996). Monitoring and experimental work is needed to determine if the increased zooplankton production associated with allochthonous carbon pulses witnessed in chapters 5 and 6 can support increased survival and production in juvenile estuarine fish.

7.3 Management recommendations

The hydrology of many coastal catchments in south-east Australia has been altered by the presence of dams. The cumulative stress on the Bega River estuary from the total sum of water extraction is rated high (NOW, 2011b). Whilst the freshwater sections of rivers have received considerable attention in regards to the ecological impacts of river regulation, estuaries have in many cases been ignored. In NSW the Water Management Act (2000) stipulates the need to protect the fundamental health of estuaries. Water Sharing Plans (WSP) are the main tool used to manage water resources in NSW. The Bega River WSP came into operation in 2011. Environmental water requirements for estuaries received little specific attention; the only environmental flow rule present is that pumping must stop when there is no visible flow in the river (NOW, 2011c). Noted in background documents to the plan is concern over a license to construct and operate a sand barrage across the entire Bega River at the start of the estuary (during the period of this study it was never constructed) (NOW, 2011a). Key environmental flow rules in other sections of the river include a first flush rule, which protects the first 24 hours of a flow event after a preceding period of low flow over 30 days, and a transparency rule for Brogo dam (the main dam in the catchment), requiring a portion of inflowing water to be released. There is no guarantee that those flows will reach the estuary.

My research presents a strong case that freshwater inflows play a crucial role in carbon cycling and microbial food webs in the Bega River estuary. Inflow events act as key periods that facilitate cross-system subsides of terrestrial carbon into the estuarine food web. A basic starting point to fulfil the requirements of the Water Act in protecting the "fundamental health" of the estuary is recognising the need for variable freshwater inflow to reach the estuary. The Bega WSP will be reviewed by the Natural Resources Commission between 2016 and 2020. The following are the major recommendations that should be considered arising from this work:

- Establish rules that limit the extraction of medium and high flows to the Bega River estuary.

- Special attention should be paid to small and medium inflow events or fresh flows, as these provide regular loads of DOC to the estuary, and are most at risk from flow regulation.
- Current transparency and first flush rules should be expanded to ensure water reaches the estuary.
- Integrate DOC regressions into existing water modelling to test the effect of different management scenarios on DOC delivery to the estuary.
- Removal of licenses to build or operate any structure that impedes connectivity of the estuary to the river.

Beyond the Bega River, the results presented in this thesis suggest FWI are likely to play an important role in carbon cycling and microbial food webs in other south-east Australia estuaries. This is supportive of previous work carried out in the Hunter River estuary (Hitchcock et al., 2010). Investigating inflow requirements of other NSW estuaries and the importance of carbon in the microbial food web should be investigated as a high priority.

7.4 Conclusions

Freshwater inflows had a major structuring role over carbon, bacteria and zooplankton dynamics. On the Bega and Clyde River estuaries, increasing DOC concentrations were closely coupled with increasing discharge. Whilst phytoplankton production is commonly coupled with DOC in estuaries, it was never related to the variation in DOC concentrations during this study. This appears mainly due to the overwhelming influence of allochthonous DOC delivered during episodic inflow events. Nitrogen, phosphorus and turbidity were also positively related to discharge. Nutrient concentrations were always higher on the Bega River compared to the Clyde River, likely due to agricultural land use in the Bega catchment

The bioavailability of DOC increased during FWI events, and in turn bacterial growth rates were also higher during and immediately following inflow events. Bacterial growth appeared to be carbon limited most of the time, though during high flows on the Clyde River, bacteria became phosphorus limited. Changing availability of DOC and phosphorus during inflow events appeared to be the main reason for shifting

resource limitation. On both rivers bacteria were positively related to increasing DOC and phosphorus concentrations. Unlike many estuaries, bacteria were not related to chlorophyll a, and this appears to be due to the influence of episodic inflows bringing allochthonous carbon and nutrient inputs.

I conceptualised FWI events as large flood flows, and small to medium fresh flows. This showed that differently sized FWI events appeared to result in distinct biogeochemical dynamics in the estuary. DOC, nitrogen and bacteria were all higher under flood and fresh flows compared to lower levels under base flow conditions. POC, phosphorus and turbidity were higher under flood flow conditions compared to fresh flows. This conceptualisation highlighted the likely importance of fresh flows to DOC and bacteria dynamics as they occur much more regularly than flood flows.

Allochthonous carbon and bacteria are generally considered to contribute little to zooplankton production. On the Bega River I found strong evidence that allochthonous carbon and bacteria can in fact support zooplankton production following the input of significant DOC subsidies during FWI events. Zooplankton density increased following a flood event on the Bega River and stable isotope analysis indicated allochthonous terrestrial carbon to be the dominant source of carbon supporting zooplankton. The results were not clear for all taxa, mainly due to the influence of other forces such as salinity which appeared to have a major influence over assemblage structure. Experimental mesocosms confirmed the contention that allochthonous carbon and bacteria can support increased zooplankton production; additions of DOC leachate led to significantly higher densities of zooplankton and changes in stable isotope signatures compared to the control. Significant differences only occurred in treatments receiving the highest DOC subsidy, indicating that the quantity of DOC subsidy may in part control potential microbial support for zooplankton.

Taking Chapters 2 to 6 as separate studies, they all individually contribute new insights to their respective sub-disciplines within aquatic ecology. Viewed together, they present a novel conceptualization of hydrology and freshwater inflows in the coastal carbon cycle and microbial food webs in south-east Australian estuaries. The results provide a strong case to protect freshwater inflow to estuaries.

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