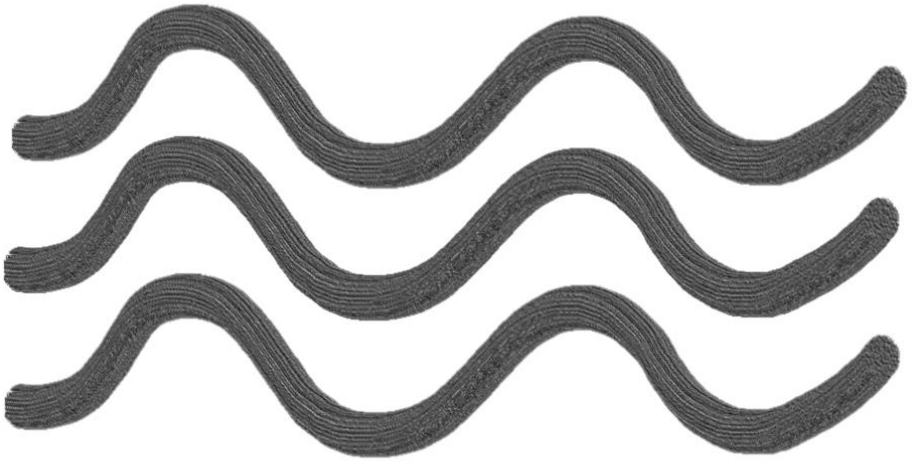
Freshwater inflows to estuaries: Terrestrial resource inputs and planktonic food web responses



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A thesis in fulfilment of the requirements for the degree of

Doctor of Philosophy

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Certificate of original authorship

I certify that the work in this thesis has not previously been submitted for a degree or as part of the requirements for a degree. I also certify that this thesis has been written by me. Any help received throughout the research process and preparation of this thesis has been acknowledged. In, addition, I certify that all information sources and literature used are indicated in the thesis. This research was supported by the Australian Government Research Training Program.

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Place, people, and knowledge are inseparable. In listening we learn.

Preface

This thesis contains 6 chapters. Chapters 2-5 are written as self-contained journal articles in preparation for submission to peer reviewed journals. They are presented in a format similar to independent manuscripts and therefore some repetition may occur between chapters. Where these chapters do cross-reference each other relevant chapter numbers are provided. To reduce further unnecessary repetition a single reference list has been provided at the end of this thesis.

This thesis is a compilation of my own work. I conceptualised the research, obtained and processed samples, conducted data analysis and wrote the chapters contained in this thesis. While guidance, support and assistance were provided by supervisors and co-authors this thesis is written using the singular "I" rather than the plural "we". The titles of each chapter and the contributions of co-authors are listed below.

Chapter 2: Responses of estuarine zooplankton to freshwater inflows: a comparison between a regulated and unregulated estuary

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Chapter 3: Fine scale food web responses to a freshwater inflow event in a small regulated Australian estuary

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Chapter 4: Inflow events drive resource use changes for mesozooplankton in a regulated estuary

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Chapter 5: Can zooplankton fed allochthonous carbon diets support and sustain juvenile fish?

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Roberts, L., Kutay, C., Melbourne-Thomas, J., Petrou, K., Benson, T.M., Fiore, D., Fletcher, P., Johnson, E., Silk, M., Taberner, S. and Filgueira, V.V., 2021. Enabling enduring evidence-based policy for the Southern Ocean through cultural arts practices. *Frontiers in Ecology and Evolution*, *9*, p. 284

Contents

Certificate of original authorship i

Acknowledgements ii

Acknowledgement of Country iv

Preface v

Contents vii

List of Figures xi

List of Tables xvi

List of Abbreviations xviii

Ethics Statement xix

Abstract xx

Chapter 1: Introduction 1

1.1 Research Scope and Significance 1

1.2 Freshwater inflows and estuaries 3

1.3 Estuarine productivity and resource inputs 6

1.4 Basal energy sources and inflows 7

1.5 Consumer responses to inflows 9

1.6 Reducing impacts of river regulation on estuaries 10

1.7 Study Sites and WSPs 11

1.8 Research Aims 15

Chapter 2: Responses of estuarine zooplankton to freshwater inflows: a comparison between a regulated and unregulated estuary 18

2.1 Abstract 18

2.2 Introduction 18

2.3 Methods 21

2.3.1 Site Description 21

2.3.2 Sampling 23
2.3.3 Statistical analysis 24
2.4 Results 25
2.4.1 Hydrology and resource delivery 25
2.4.2 Estuarine zooplankton communities 28
2.4.3 Contrasting responses to environmental drivers 33
2.5 Discussion 35
2.5.1 Contrasting zooplankton communities and drivers 36
2.5.2 Differences in environmental conditions 37
2.5.3 Impacts of river regulation on environmental conditions 39
2.5.4 Management Recommendations 40
2.5.5 Conclusion 41
Chapter 3: Fine scale food web responses to a freshwater inflow event in a regulated Australian estuary 42
3.1 Abstract: 42
3.2 Introduction 42
3.3 Methods 44
3.3.1 Chemical and Chl *a* measurements 47
3.3.2 Bacteria, algal and zooplankton enumeration 47
3.3.3 Statistical analysis 48
3.4 Results 49
3.4.1 Discharge event and weir operation 49
3.4.2 Organic carbon and nutrient delivery 50
3.4.3 Bacterial and Algal Response 53
3.4.4 Zooplankton responses to discharge event 57
3.5 Discussion 60

3.5.1 Basal resources increase with flow 60
3.5.2 Mesozooplankton community responses 62
3.5.3 River Regulation and Management 64
3.5.4 Conclusion 65
Chapter 4: Inflow events drive resource use changes for mesozooplankton in a regulated estuary 66
4.1 Abstract: 66
4.2 Introduction 66
4.3 Methods 68
4.3.1 Site Description 68
4.3.2 Physicochemical, DOC and chlorophyll *a* data 69
4.3.3 Stable Isotope Analysis 70
4.3.4 Stable Isotope Endmembers 71
4.3.5 Statistical Analyses 73
4.4 Results 75
4.5 Discussion 83
4.5.1 Allochthony following inflows 83
4.5.2 Allochthony and relationships to sPOM $\delta^{13}C$ 85
4.5.3 Prolonged allochthony 87
4.5.4 Limitations 88
4.5.5 Conclusions 89
Chapter 5: Can zooplankton fed allochthonous carbon diets support and sustain juvenile fish? 90
5.1 Abstract 90
5.2 Introduction 91
5.3 Methods 93

5.3.1 Experimental Setup and Acclimation 93
5.3.2 *Artemia* and Feeding 94
5.3.3 Fish Sampling 96
5.3.4 POM and Tissue analyses 96
5.3.5 Statistical analyses 97
5.4 Results 98
5.5 Discussion 104
5.5.1 tDOM influence on growth 104
5.5.2 tDOM, inflows and recruitment 107
5.5.3 Limitations 109
5.5.4 Conclusion 110
Chapter 6: General Discussion and Conclusions 111
6.1 General Discussion 111
6.1.1 Planktonic responses in regulated systems 111
6.1.2 Role of terrestrial carbon 113
6.2 Further Research Directions 116
6.2.1 Temporal and spatial replication 116
6.2.2 Terrestrial carbon resources and co-metabolism 117
6.2.3 The role of mixotrophs 117
6.2.4 Responses of higher trophic levels to terrestrial inputs and heterotrophy 118
6.3 Management Recommendations 118
6.4 Conclusions 120
7. References 121
8. Appendices 155

List of Figures

Figure 1.1: A conceptual model of freshwater movement through an estuary. Freshwater enters the estuary from the upper catchment via rivers and tributaries and mixes with marine water to create a brackish environment before it is exported to the ocean. Variations in the magnitude of flows may cause flooding and lateral connections to the catchment, which may transport organic material and nutrient resources from catchment to the estuary. Image taken from OzCoasts 2015. 4

Figure 1.2: The algal grazer chain and microbial loop food webs. The grazer chain is a simpler and shorter food web, utilising primary producers as its basal resource, with zooplankton grazers passing energy onto higher trophic levels. The microbial loop may be subsidised by freshwater inputs of allochthonous dissolved organic carbon, transferring energy to higher order consumers through a combination of heterotrophic microbes, flagellates and ciliates. Energy transfers of autochthonous material are highlighted with green arrows, while allochthonous additions are indicated by black arrows. 8

Figure 1.3: The position of the Manning and Williams estuaries (black dots) and their catchments (highlighted red) on the NSW coast. 13

Figure 1.4: The Williams River transition from freshwater (left) to estuary (right) through Seaham Weir (centre) (Hunter Water 2011). 14

Figure 1.5: A view of the Seaham Weir gates from the wall. Freshwater (left) is prevented from entering the estuary (right). Photo courtesy of Prof. Simon Mitrovic. 14

Figure 2.1: Site and discharge gauge positions for the Manning River. Sites are denoted by black circles on the river channel. Site 1 is the most upstream point, with site numbers increasing downstream to Site 3 near Taree. WaterNSW gauge 208004 (Manning @ Killawarra) is indicated by the red square. Position of the study site along the NSW coast is indicated by the black circle in the inset image. 22

Figure 2.2: Site, weir and discharge gauge positions for the Williams River. Sites are denoted by black circles on the river channel. Site 1 is the most upstream point (near Seaham) with site numbers increasing downstream to Site 3 (closer to Raymond Terrace). Seaham Weir is denoted by the red triangle upstream of Seaham. WaterNSW gauge 210010 (Williams @ Glen Martin) is indicated by the red square upstream of Clarence Town. Position of the study site along the NSW coast is indicated by the black circle in the inset image. 22

Figure 2.3: Discharge (ML/Day – shaded grey area) of the Manning (A) and Williams (B) River estuary with salinity (ppt- points and lines) from the three sampling sites in each, Site 1 being most upstream and Site 3 the most downstream. Figures C and D show temperature (ºC – thick grey line) and Chl a concentration (µg/L – points and lines) for the Manning and Williams estuaries respectively at the same three stations. Discharge data was taken from gauges 208004 (Manning @Killawarra) and 210010 (Williams @Glen Martin) as shown in Figure 2.1 and 2.2. Temperature is displayed as an average between the three sites of each estuary as there was little variation between sites. Chl a at Williams Site 2 in March 2018 was 82.5 µg/L.....26

Figure 2.4: Comparisons of A) dissolved organic carbon (DOC (mg/L)), B) turbidity (NTU)), C) nitrates and nitrites (NO_x (mg/L)), D) filtered reactive phosphorous (FRP (mg/L)), E) Chlorophyll a (Chl a (µg/L)) and F) dissolved oxygen (DO (%)) concentrations between 30-day antecedent flow class (Q30 Class) conditions (low, moderate (Mod), fresh and flood) for the Manning (M) and Williams (W) estuaries. Error bars represent standard error of the mean. Number of samples taken within each flow class for Manning n = 41, 25, 10, 16 respectively and for Williams n = 29, 21, 18, 27. 28

Figure 2.5: Multidimensional scaling (MDS) ordination of the zooplankton community data for the Manning (M – open triangles) and Williams (W – closed triangles) estuaries. Ellipses represent 95% confidence interval. The stress of the model is indicated in the bottom right-hand corner.....30

Figure 2.6: Density (Ind/L=individuals/L) of major zooplankton community groups (nauplii, copepodites and adults) for sites 1,2 and 3 in the Manning (A, C and E respectively) and the Williams (B, D and F) between January 2015 and June 2019. Other represents a combination of the remainder of the zooplankton from Table 2.1. 32

Figure 2.7: Distance-based redundancy analysis of the copepod communities of the Manning (A) and Williams (B) River estuaries for samples collected between January 2015 and June 2019. Species abbreviations in red correspond to those provided in Table 2.1. Environmental variable abbreviations are as follows: Q30 = 30-day antecedent discharge (ML/Day), Q60 = 60-day antecedent discharge (ML/Day), Temp = temperature (oC), NO_x = nitrate and nitrite concentrations (mg/L), FRP = filtered reactive phosphorous (mg/L), DOC = dissolved organic carbon (mg/L), Sal = salinity (ppt), DO = dissolved oxygen saturation (%), Turb = turbidity (NTU).....34

Figure 3.1: Site, weir and discharge gauge positions for the Williams River. Study sites are denoted by black circles with Site 1 being the most upstream site, north of Clarence Town and Site 4 being the most downstream site, south of Seaham. Seaham Weir is denoted by the red triangle upstream of Seaham. WaterNSW gauge 210010 (Williams at Glen Martin) is indicated by the red square upstream of Clarence Town. 46

Figure 3.2: Discharge (ML/Day) for the Williams River and the salinity (ppt) and temperature (°C) of the two sites in the Williams River estuary between September 26 and December 12, 2018. Discharge was taken from NSW Water gauge 210010 - Glen Martin (grey shading). The sections of the hydrograph shaded black are the periods when the Seaham Weir gates were open (black) allowing flow downstream to sites 3 and 4. 50

Figure 3.3: Discharge (ML/Day) and concentrations of dissolved organic carbon (DOC), dissolved inorganic nitrogen (NO_x) and filtered reactive phosphorous (FRP) of 4 sites from the Williams River and estuary between the September 26 and December 12, 2018. Figures A, C and E were taken during the flow period and are insets of figures B, D and F. The dark bar on the x-axis of B, D and F indicates the period represented by A, C and E. Fine scale sampling during the inflow event was taken at sites Site 1 and 2, and Site 4, while post- flow samples were only taken at Site 3 and 4. Error bars represent standard error. 53

Figure 3.4: Discharge (ML/Day) and concentrations of bacterial cells (cells/L) in the Williams River estuary during (A) and following (B) the inflow event. Site 3 was not sampled intensively through the first flow peak. The dark bar on the x-axis of B indicates the period encompassing A. Samples for Site 3 on November 8 and 14 were compromised during analysis and hence not included. Error bars represent standard error of the mean. 54

Figure 3.5: Discharge (ML/Day) and, chlorophyll a (A, Chl a, µg/L) total algal biovolume (B, mm^3/L) in the Williams River at Site 3 and 4 prior and following the inflow events. Figures C and D outline the algal community composition as a percentage of biovolume at Site 3 and 4, respectively. Genera comprising these 7 major groups can be found in Appendix 4. 56

Figure 3.6: Discharge (ML/Day) and abundances of nauplii (A), copepodite (B), adult copepod (C) and cladoceran (D) population concentrations (ind/L = individuals/L) across the October flow peaks. Site 2 was sampled with higher temporal resolution after the first peak to investigate fine scale changes in response to discharge. Error bars represent the standard error of the mean (SEM). 58

Figure 3.7: Distance-based redundancy analysis of the mesozooplankton community of the

Williams River Estuary across an inflow event in 2018. “Pre-Flow” samples (red) are those taken on September 26, 2018, 10 days before the inflow event. “Flow” samples (green) are those from October 12 to 30, 2018, while weir gates were still being actively operated. “Post-Flow” samples (blue) are those from the November 2 to December 12, 2018, during which weir gates were almost exclusively closed. Significant environmental variables ($p < 0.05$) constraining the model are presented. 60

Figure 4.1: Site, weir and discharge gauge positions for the Williams River. Estuarine sites are denoted by black circles on the river channel. Site 1 is the most upstream black circle (near Seaham) with Site 2 being the downstream black circle nearer to Raymond Terrace. Seaham Weir is denoted by the red triangle upstream of Seaham. WaterNSW gauge 210010 (Williams at Glen Martin) is indicated by the red square upstream of Clarence Town. The red square between Site 1 and 2 is where the nutrient amendment experiment site. 69

Figure 4.2: A) Discharge (shaded peaks) and salinity, B) chlorophyl a (Chl a), C) dissolved organic carbon (DOC) D) turbidity and F) $\delta^{13}C$ of suspended particulate organic matter (sPOM) through the study period, January 2018 to August 2018, at Sites 1 and 2 in the Williams River estuary. Error bars represent the standard error of the mean. 77

Figure 4.3: Stable isotopic signatures of carbon ($\delta^{13}C$, ‰) and nitrogen ($\delta^{15}N$, ‰) for mesozooplankton consumers from the Williams River estuary tidal freshwater zone during pre- and post-flow periods between January and August 2018. June samples were assigned their own group “flowing” due to their temporal proximity to an inflow in June. Mean endmember source signatures (± SE) are also shown; Est. Alg = estuarine algae, Sed = sediment organic matter, Lom = Lomandra longifolia, Cas = Casuarina glauca. 79

Figure 4.4: Mean percentage contributions of algae (estuarine algae) and terrestrial (Lomandra longifolia) food web resources to mesozooplankton diets between months where zooplankton δ13C was enriched (January, March, May and June) and depleted (April, July and August) between January 2018 to August 2018 in the Williams River estuary as determined by Bayesian mixing model. Error bars represent standard error of the mean. 82

Figure 5.1: Bacterial cell concentrations (cells/µL, n=9)) for the 3 DOC addition treatments 0 mg/L, 5 mg/L and 10 mg/L in the Artemia on-growing chambers during the bio-encapsulation period. 0 hr measurements were taken minutes after the addition of leachate and Artemia to freshly prepared on-growing tanks. 40 hr measurements were taken from pre-prepared on-growing tanks to be used for feeding that day, approximately 40 hours after the

addition of leachate and Artemia. Error bars represent standard error of the mean (n =9). 99

Figure 5.2: $\delta^{13}C$ (13C) and $\delta^{15}N$ (15N) stable isotope ratios for fish tissues at the beginning (Initial) and conclusion of the experiment for all 3 treatments (Fish 0 mg/L, Fish 5 mg/L and Fish 10 mg/L). Isotopic ratios are also displayed for particulate organic matter samples from the Artemia on-growing tanks (POM 0 mg/L, 5 mg/L and 10 mg/L) and Artemia (Artemia 0 mg/L, 5 mg/L and 10 mg/L) from the 3 treatments. Error bars represent standard error of the mean for each sample.....100

Figure 5.3: Weight (A), standard length (B), fork length (C) and total length (D) of juvenile Australian bass for experimental carbon treatments of 0 mg/L, 5 mg/L and 10 mg/L across the study period. Measurements of fish when acquired are presented as “Initial” measurements. Error bars represent standard error of the mean (n = 30). Time points refer to T0 = start of experiment, T1 = 2 weeks, T2 = 4 weeks and T3 =6 weeks into the experiment. T3 was the final point of the experiment 101

Figure 5.4: Principal component analysis of truss-based morphology for juvenile Australian bass from treatment A (control – black circles), B (5 mg/L DOC addition – blue triangles) and C (10 mg/L DOC addition – red squares). PC1 was characterised by lengths of X2, X6 and X10 while PC2 represented X13, X8 and X12 (Appendix 13). X2 = length between the anterior tip of the upper snout and the origin of spinous dorsal fin, X6 = the length between the origin of spinous dorsal fin and the origin of pelvic fin, X10 = the length between the origin of spinous dorsal fin and the origin of anal fin, X13 = the length between the origin and insertion of the anal fin and X12 = the length between the origin of the anal fin and the insertion of the 2nd dorsal fin 103

Figure 5.5: Highly unsaturated fatty acid concentration of homogenised fish tissues for initial measurements and the control (0 mg/L), 5 mg/L and 10 mg/L treatments over the 6-week experimental period. A = docosahexaenoic acid (DHA, 22:6n-3), B = eicosapentaenoic acid (EPA, 20:5n-3) and C = arachidonic acid (ARA, 20:4n-6). Error bars represent standard error of the mean (n= 3). Time points refer to T0 = start of experiment, T1 = 2 weeks, T2 = 4 weeks and T3 =6 weeks into the experiment. T3 was the final point of the experiment.....104

List of Tables

Table 1.1: Summary of the role of freshwater inflows in estuaries and the potential negative impacts of their reduction (adapted from Drinkwater and Frank 1994; Gillanders and Kingsford 2002 and Pierson et al., 2002). 6

Table 2.1: Mean, standard error and range for the density of most common zooplankton in the Manning and Williams estuaries between January 2015 and June 2019. Sample % represents the percentage of samples in which the zooplankton was found. Abbreviation in parentheses corresponds with usage in Figure 2.7. 29

Table 2.2: Results of multivariate abundance analysis ("mvabund" function) for factors River (n=2), Site (n= 3) and Flow Class (30-day antecedent flow class) n= 4) within the study. Q30 flow classes were determined using historic flow percentile curves. LRT = likelihood ratio test. Df = degrees of freedom.....31

Table 2.3: Significant constraining variables in the Manning and Williams dbRDA models (Figure 2.7) as determined by Monte-Carlo permutational analysis (999 permutations).....35

Table 4.1: Stable isotope ratios (‰) of organic matter sources for the Williams River estuary for carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) with standard deviation (±SD), and relevant C:N elemental ratios. The estuarine algal signature of $\delta^{13}C$ is derived from the treatment samples of the amendment experiment while $\delta^{15}N$ or estuarine algae is from the control samples of the amendment experiment, due to the effects of additional nutrients on the $\delta^{15}N$ of treatment samples.....73

Table 4.2: Dominant mesozooplankton taxa in the Williams River estuary sorted by relative enrichment or depletion of $\delta^{13}C_Z$ through January to August of 2018, including where and when they were sampled, average $\delta^{13}C_Z$, $\delta^{13}C$ minus sPOM ($\delta^{13}C_Z - \delta^{13}C_{POM}$), average $\delta^{15}N$, $\delta^{15}N$ minus sPOM ($\delta^{15}N_Z - \delta^{15}N_{POM}$), expected TEF (trophic enrichment factor) range based on their trophic level (TL), and the primary, secondary and tertiary sources utilised by each group as identified by Bayesian mixing model (50% confidence interval). ± symbols indicate standard error, GP = Gladioferens pectinatus, OS = Oithona spp., BM = Bosmina meridonalis, SC = Sulcanus conflictus, Est. Alg = estuarine algae, Sed = sediment organic matter, Lom = Lomandra longifolia and Cas = Casuarina glauca. 81

Table 5.1:Two-way ANOVA results for bacterial concentrations in the bio-encapsulation tanks for treatments (0 mg/L, 5 mg/L, 10 mg/L) and bioencapsulation time (Bio-Time –

0hours, 40 hours). Df = degrees of freedom, Sum Sq = sum of squares, Mean sq = mean of squares.....98

List of Abbreviations

DOC	Dissolved organic carbon
FRP	Filtered reactive phosphorous
FWI	Freshwater inflow
OM	Organic Matter
NO_x	Nitrates and nitrites
NSW	New South Wales
Q30	30-day antecedent discharge
Q60	60-day antecedent discharge
tDOM	Terrestrial dissolved organic matter
tOM	Terrestrial organic matter
WSP	Water sharing plans

Ethics Statement

A study within this thesis involved the use of animals (Chapter 5). This research was approved by the University of Technology Sydney Animal Care and Ethics Committee under permit ETH19-3895. The research was conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (8th Edition 2013), the NSW Animal Research Act (1985) and Regulations (2010).

Abstract

Freshwater inflows are crucial to estuarine processes, regulating habitats and delivering important resources. However, river regulation has substantially reduced freshwater inflows to estuaries, affecting them negatively. This thesis aimed to enhance understanding of 1) how estuarine planktonic food webs responded to inflows in a regulated estuary; and 2) the importance of terrestrial carbon resources to estuarine food webs. These are important questions in estuarine flow-ecology and water resource management. To achieve this a suite of monitoring and manipulative studies were carried out in the Williams and Manning River estuaries.

A 4.5-year monitoring study comparing the unregulated Manning with the regulated Williams demonstrated the potential impacts of river regulation with results from the Williams contrasting strongly with the Manning. The Williams estuary was characterised by high concentrations of DOC and nutrient, regardless of flow conditions, with zooplankton communities consistently dominated by copepod nauplii, displaying no seasonal trends and responding positively to inflows and inputs of terrestrial DOC. Fine-scale monitoring of an inflow on the Williams estuary showed the importance of inflows in delivering terrestrial carbon and phosphorus resources and in stimulating bacterial and algal productivity. However, this did not translate to increased zooplankton production, relative to pre-flow periods.

Strong evidence was found for the importance of terrestrial carbon resources delivered to estuarine food webs by inflows. Analysis of zooplankton stable isotopes across a variable flow period showed that terrestrial carbon resources were being utilised by zooplankton following inflows, suggesting its importance in upper estuarine areas. Experimental results also indicated that terrestrial carbon in mesozooplankton food webs could contribute to the growth of juvenile fish of an endemic estuarine species. Together these results highlighted the importance and role of terrestrial carbon in estuarine food webs and productivity.

Individually my studies contribute new knowledge to important ecological and management questions in estuaries. Combined as a thesis they provide strong evidence for the importance of FWIs to estuaries and their food webs, highlighting the importance of terrestrial carbon as an energetic resource and contributor to estuarine productivity. This knowledge contributes to the wider literature recognising the importance of freshwater inflows to estuaries and supports their protection.

Chapter 1: Introduction

1.1 Research Scope and Significance

Estuaries are semi-enclosed bodies of water connected to the ocean; an interface between freshwater catchments and the open ocean and are characterised by tidal action and freshwater mixing (Cameron and Pritchard 1963; Fairbridge 1980). They are highly valued for their ecological functions and biodiversity, remaining sites of great social, economic, and cultural value with many global population centres built around them (Pierson et al. 2002). Unfortunately, many estuaries worldwide have become degraded due to a range of factors, including pollution by industrial and urban wastes, reclamation of adjoining wetlands and the dredging of channels (Edgar et al. 2000). Estuaries have also been greatly affected by the diversion and extraction of freshwater for human purposes, known as river regulation, and the subsequent reductions in the discharge of freshwater to estuaries this process results in (Gillanders and Kingsford 2002; Adams 2014).

Freshwater inflows (FWIs) are inputs of freshwater to estuarine environments that can occur as instream or riverine flow, catchment runoff, snowmelts, groundwater return flows and urban stormwater or sewage. FWIs are an integral part of estuaries, key to the physical, chemical, and biological processes that maintain estuarine function and productivity (Alber 2002; Chilton et al. 2021). Consequently, any alteration, reduction or removal of FWIs can cause significant ecological harm to estuaries (Bunn and Arthington 2002; van Niekirk et al. 2012; Kiwango et al. 2015). Disruption to the natural FWI patterns of estuaries can negatively influence natural processes regulating salinity, habitat availability and connectivity, and carbon and nutrient cycling (Morais et al. 2009; Gillson 2011; Scharler et al. 2020). Alterations in natural flow regimes can also impact the reproduction and dispersal of many estuarine biota including socially valued species of fish, birds, and plants (Drinkwater and Frank 1994; Gillanders and Kingsford 2002). Within Australia, an increasing population and a growing demand for freshwater has led to increased river regulation. This, combined with changing precipitation patterns due to climate change, suggests it is critical to develop the understanding of the role of FWIs to estuaries and impacts from their reduction (Gillanders et al. 2011; Davies et al. 2014; Arthington et al. 2018a).

Assessing the importance of FWIs to estuaries was paramount to water management

authorities and scientists in Australian in the early 2000s (Gillanders and Kingsford 2002; Pierson et al. 2002; Scheltinga et al. 2006). During this period legislative provisions were made at national and state levels regarding the importance of FWIs to estuaries, with a strong emphasis on adaptive management. The federal government's *National Water Initiative* specified that states and territories were to address the "impacts… on the environment that [river regulation activities] may have downstream (including estuaries) …" (COAG 2004) while at a state level in New South Wales (NSW) the Water Management Act 2000 recognised "the fundamental health of our rivers and … estuaries have to be protected". While these actions have led to the successful implementation of environmental flow protections across Australia, such as Water Sharing Plans (WSP) in NSW, consistent monitoring, evaluation and revision of these strategies is required, yet is often lacking (Davies et al. 2014).

Two key areas of scientific information needed to assist water resource managers and policy makers in developing and evaluating FWI protections for estuaries are; 1) the response of estuarine biota to altered flow regimes, including through changes to habitats and resource availability and 2) how estuarine productivity changes in relation to FWIs, addressing whether changes in primary productivity (producers) translate to changes in secondary productivity (consumers) (Scheltinga et al. 2006; Adams 2014; Davies et al. 2014). Similar questions remain within the broader scientific community regarding flow-ecology relationships in estuaries, with the impact of reduced inflows and the importance of terrestrial resource inputs to estuarine productivity central to ongoing work (Morais et al. 2009; Hitchcock and Mitrovic 2015a; Meunier et al. 2017; Roelke et al. 2017; Voss et al. 2020).

This thesis addresses two key knowledge gaps: 1) How do estuarine biota respond to inflows in regulated estuarine systems? 2) How important are terrestrial resource inputs to estuarine systems, in particular terrestrial carbon? These questions are addressed through a longer term (4 year) field monitoring study in two South Eastern Australian estuaries, the regulated Williams River and unregulated Manning River, as well as two shorter term monitoring studies (lasting 2 and 8 months, respectively) focused on the Williams River observing changes in water quality and estuarine food webs to freshwater inflows. These are supplemented with a manipulative laboratory-based experiment examining the role of terrestrial carbon resources in providing energy to juvenile fish. My research was focussed on the upper, freshwater-tidal to mesohaline section of the regulated Williams River estuary as changes in FWI regimes are most likely to influence the upper reaches of estuaries (Bate et

al. 2002; Pierson et al. 2002) and the existing WSP covering this system (*WSP for the Hunter and Alluvial Water Sources 2009)* was due for evaluation.

Zooplankton food webs were a focus in this thesis as they are highly responsive to changing water quality and environmental conditions across short and long-term time scales (Paul et al. 2016; Oghenekaro et al. 2018) and are key element of estuarine food webs, transferring energy from basal resources to higher trophic levels (Tan et al. 2004; Buyukates and Roelke 2005; Hitchcock et al. 2016b). The importance of FWIs and their associated resource inputs to estuarine productivity, ecology and zooplankton food webs are elaborated on in the following sections.

1.2 Freshwater inflows and estuaries

FWIs are critical processes in estuarine environments, capable of shaping the physical, biogeochemical and ecological character of estuaries and coastal systems (Alber 2002; Chilton et al. 2021). Generally, we conceive of FWIs as instream flow from a river to an estuary (Figure 1.1). However, catchment runoff and groundwater return flow can make significant contributions to FWIs following rainfall activity. The timing, quantity and quality of FWIs to estuaries are also known as an estuary's flow regime. These flow regimes play a central role in the physical and biological functioning of estuaries (Poff and Zimmerman 2010). FWIs can maintain base flows, or low flow conditions, which can be important in maintaining longitudinal connectivity to upstream areas, estuarine habitats and salinity gradients (Drinkwater and Frank 1994). Larger pulses of freshwater discharge during or following storm and rainfall events, such as freshes and floods, are also important to estuarine processes (Scharler et al. 2020). Freshes, defined as pulsed discharge events that remain within riverbanks, are important in delivering resources from the upstream terrestrial catchment and tributaries to estuaries as well as flushing upstream areas of estuaries. Floods, which are rarer events that break riverbanks and establish flood plain connectivity, are also important in flushing estuaries, including the lower more saline reaches, but can also deliver resources to estuaries from the wider flood plain as water returns to the main channel (Pierson et al. 2002).

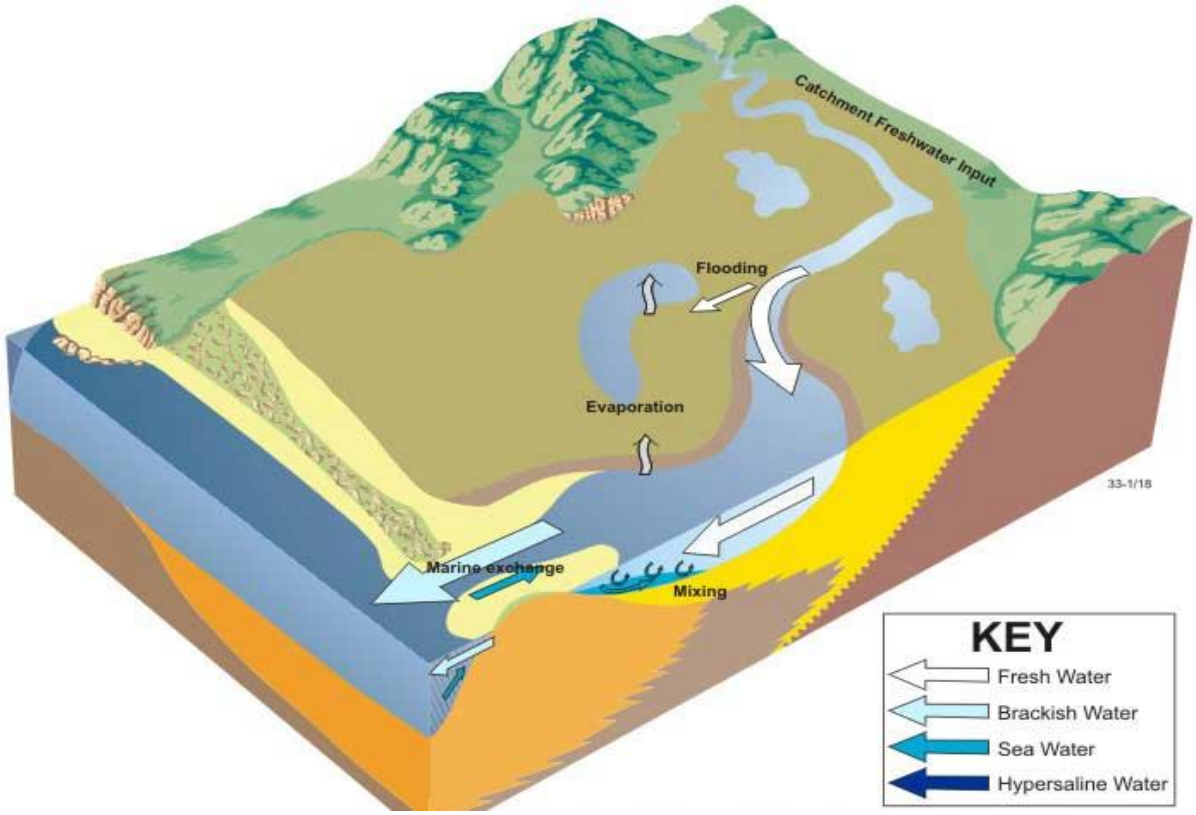

Figure 1.1: A conceptual model of freshwater movement through an estuary. Freshwater enters the estuary from the upper catchment via rivers and tributaries and mixes with marine water to create a brackish environment before it is exported to the ocean. Variations in the magnitude of flows may cause flooding and lateral connections to the catchment, which may transport organic material and nutrient resources from catchment to the estuary. Image taken from OzCoasts 2015.

The characteristics of flow regimes can vary greatly between catchments, both spatially and temporally. For example, catchments in higher latitudes may experience continual low flows year-round and significant seasonal inflows following snow or glacial melting, while equatorial catchments may experience higher inflows year-round due to the nature of rainfall in the tropics, with increased rainfall and discharge in monsoon seasons (Walker 1985). In contrast to these patterns, FWI's in temperate eastern Australia are considered highly variable (Finlayson and McMahon 1988), often tied to local or large-scale climatic patterns of rainfall like Australian East Coast Lows, developing with warm sea surface temperatures, or the El Niño and La Niña Southern Oscillation (Simpson et al. 1993; Piechota et al. 1998; Callaghan et al. 2014). These temporal and spatial variations in FWI characteristics make generalisations about their influence on estuarine processes and ecology difficult to apply uniformly (Adams 2014; Davies et al. 2014).

Estuarine ecosystems have evolved to be resilient to dynamic hydrological and environmental conditions and rely on this natural variation and connectivity for many processes (Table 1.1, Pierson et al., 2002, Chilton et al., 2021). However, major disruptions to natural flow regimes

through river regulation processes (such as damming and freshwater extractions) are likely to impact ecological functioning of systems by altering estuarine conditions, and in turn, biota (Table 1). FWI reductions can increase water residence time in estuaries (Wolanski and Elliott 2016) and that of sediments, nutrients, phytoplankton and pollutants (Clark and O'Connor 2019; Fonseca et al. 2020), which can aggravate issues of stratification, eutrophication and pollution (Chilton et al. 2021). FWI reductions can also increase the salinity of estuaries reducing the extent of the river-estuary interface zone (Pierson et al. 2002), an important estuarine habitat (Bate et al. 2002). Habitat changes like this can lead to changes in the distribution and abundance of endemic estuarine species and may support invasive species becoming more prevalent (Sheaves 1998). Reduced inputs of terrestrial resources associated with inflows can also negatively impact the primary and secondary productivity of estuaries (Drinkwater and Frank 1994; Gillanders and Kingsford 2002; Vinagre et al. 2011). This is particularly relevant in the upstream, low salinity areas of estuaries where ecological processes and water quality conditions are predominantly governed by freshwater inflows (Fairbridge 1980; Pierson et al. 2002). It is therefore important to develop a more thorough understanding of relationships between inflows and estuarine ecology, and the impacts reduced FWIs can have on individual estuaries given the complex and varied nature of responses (Roelke et al. 2013; Arthington et al. 2018b).

Table 1.1: Summary of the role of freshwater inflows in estuaries and the potential negative impacts of their reduction (adapted from Drinkwater and Frank 1994; Gillanders and Kingsford 2002 and Pierson et al., 2002).

Roles of FWIs in estuarine processes	**Possible impacts of FWI reduction on estuaries processes**
• **Connectivity to freshwater ecosystems** • **Regulation of salinity gradients** • **Reduction in water column stratification** • **Resuspension of sediments** • **Delivery of organic material and nutrients** • **Flushing of pollutants and particulates** • **Dispersal of organisms**	**at Low Flow** • **Decreased connectivity with freshwater ecosystems** • **Increased salinity and duration of high salinity periods in upper estuary** • **Increased risk of depleted oxygen at depth and stratification in upper estuary** • **Aggravation of pollution problems/increased water residence time** • **Reduced dispersal of eggs and larvae** • **Increased risk of invasive species** • **Reduced secondary productivity** **at Medium-High Flows** • **Reduced flushing of deeper sections of estuaries** • **Reduced connectivity to adjacent ecosystems (wetlands/floodplains)** • **Reduced inputs of organic material and nutrients**

1.3 Estuarine productivity and resource inputs

Estuaries are considered highly productive systems (Gillanders and Kingsford 2002), yet at the same time can be net heterotrophic, consuming more material than they produce (Findlay et al. 1991). To sustain this high productivity estuaries therefore rely on the input of resources from the surrounding catchment (Pierson et al. 2002; Montagna et al. 2002), with FWIs playing a vital role in transferring these resources from catchment to estuary (Puckridge et al. 1998; McKee et al. 2000; Ford et al. 2005). During storm events, rainfall in the upper catchment and floodplain can mobilise terrestrial organic matter, containing carbon and nutrients, from catchment surfaces and soil horizons transporting them longitudinally and laterally through the catchment to downstream areas like lowland rivers and estuaries (Aitkenhead-Peterson et al. 2003). Though material transported by FWIs can be exported to near shore coastal areas by larger inflows (Bouwman et al. 2005; Schlacher et al. 2009; st. Pierre et al. 2020), resources remaining in the estuary can stimulate high productivity periods and the production of key food web resources such as phytoplankton, benthic and microbial

communities (Buyukates and Roelke 2005; Kim et al. 2014; Carney et al. 2015; Hitchcock and Mitrovic 2015a). This process is similar to the "Flood Pulse Concept" in riverine ecosystems (Junk et al. 1989) which highlights the role of large flow events in transporting resources longitudinally and laterally to downstream areas, resulting in pulses of increased biomass production. However, given the complex hydrology and responses of estuarine systems to FWIs, the specific mechanisms linking inflows, terrestrial resource inputs and estuarine productivity need further research.

Years with higher freshwater inflows are frequently linked to increases in the productivity of commercially valued estuarine species such as fish and crustaceans (Gillanders and Kingsford 2002; Arthington 2012; Saeck et al. 2013). Reductions in inflows and riverine discharge into estuaries have also been linked with declining fish stocks in estuaries and near shore coastal systems (Kashef 1981; Gillanders and Kingsford 2002; Morais et al. 2009). Mechanisms that potentially link inflows and secondary productivity in estuaries include inflows increasing habitat availability and connectivity for estuarine species and inflows delivering basal resources that promote the productivity of food web resources like phytoplankton and zooplankton (Gillson 2011 *and references therein*). As freshwater inflows are reduced by anthropogenic river regulation (Adams 2014; Arthington et al. 2018b) understanding how freshwater inflows and resource inputs stimulate productivity in estuaries is a key question in estuarine ecology (Scheltinga et al. 2006; Adams 2014; Davies et al. 2014).

1.4 Basal energy sources and inflows

Ecologists conceptualise two distinct basal sources of carbon in aquatic systems, allochthonous and autochthonous carbon (Cole et al. 2002). Autochthonous carbon production encompasses in-channel primary producers like phytoplankton, benthic algae and seagrasses (Correll 1978) while allochthonous carbon is produced outside the river channel system, e.g., terrestrial primary production, and transported in. Both sources of carbon have been linked to increased productivity in aquatic systems (Tan et al. 2004; Hoffman et al. 2008), though autochthonous primary production and the algal grazer chain are considered the primary energetic pathway (Figure 1.2, Jassby et al. 2003; Brett et al. 2009; Cloern et al. 2014). However, FWIs can have a significant influence on the availability of these two basal carbon sources to estuarine food webs (Schlacher et al. 2009; Bruesewitz et al. 2013; Dorado et al. 2015).

Freshwater inflows can influence autochthonous primary production in a number of ways. FWIs can deliver nutrients such as nitrogen and phosphorus, stimulating increased primary productivity (Mallin et al. 1993; Kim et al. 2014; Dorado et al. 2015). Larger inflows may reduce phytoplankton growth and abundance in estuaries by advecting populations or through increased turbidity and hence reduced light availability for phytoplankton (Dorado et al. 2015; Andersson et al. 2018). During larger flood events this can mean initial reductions in primary production during the event, and potential increases in primary production as flows subside and conditions suitable for algal growth return (Dorado et al. 2015).

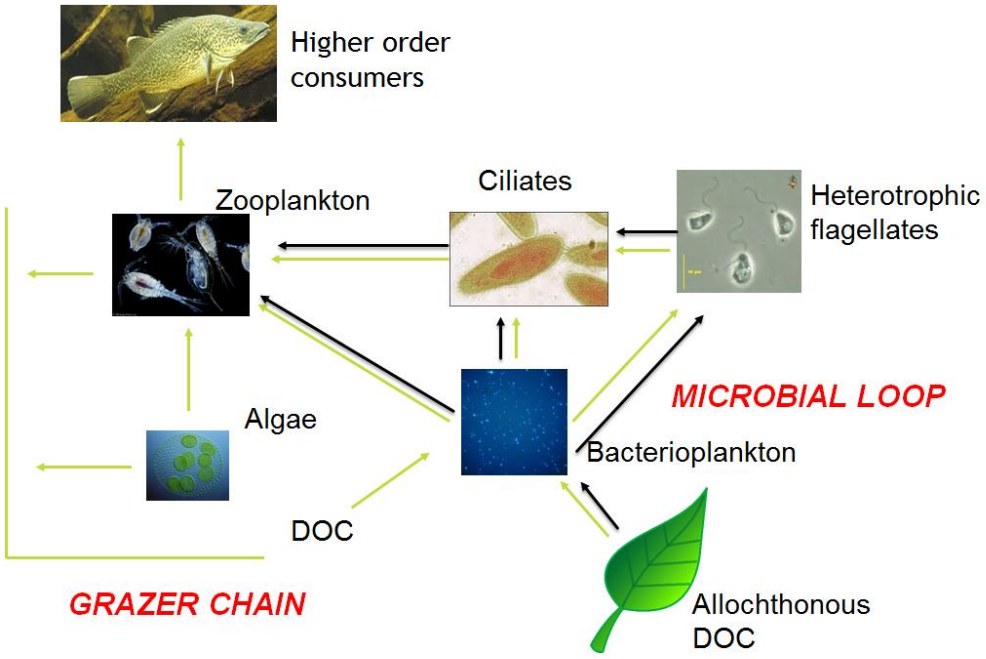

Figure 1.2: The algal grazer chain and microbial loop food webs. The grazer chain is a simpler and shorter food web, utilising primary producers as its basal resource, with zooplankton grazers passing energy onto higher trophic levels. The microbial loop may be subsidised by freshwater inputs of allochthonous dissolved organic carbon, transferring energy to higher order consumers through a combination of heterotrophic microbes, flagellates and ciliates. Energy transfers of autochthonous material are highlighted with green arrows, while allochthonous additions are indicated by black arrows.

Allochthonous carbon is derived from terrestrial sources such as leaf litter, soil horizons and detritus transported from the wider catchment to aquatic systems during rain and flood events (Thurman 1985). Inputs of terrestrial organic matter can stimulate microbial energy pathways in estuaries during and shortly after high flow periods (Hitchcock and Mitrovic 2015a; Meunier et al. 2017; Andersson et al. 2018). The microbial loop (Figure 1.2) first assimilates organic carbon, often in its dissolved form (DOC), via bacterioplankton. Bacteria assimilating terrestrial resources can be consumed by a combination of heterotrophic flagellates, ciliates

and microzooplankton such as rotifers and copepod nauplii, all of which can also directly utilise larger particulate organic carbon (Vogt et al. 2013; Mitra et al. 2014a; Trochine et al. 2015).

1.5 Consumer responses to inflows

Changes in food web resource availability following resource inputs by FWIs can influence estuarine food web productivity, favouring certain species and altering community structure (Nicolle et al. 2011; Winder and Jassby 2011; Paczkowska et al. 2017). Estuarine meso-zooplankton (0.2-20 mm), particularly crustaceous varieties like copepods and cladocerans, are important links in estuarine food webs, passing energy from available basal resources to higher order consumers such as fish (Newton 1996; Tan et al. 2004; Castonguay et al. 2008; Zöllner et al. 2009). These zooplankton are capable of utilising both autochthonous primary production and allochthonous microbial carbon pathways (Figure 1.2), both of which can be stimulated by inflows (Hoover et al. 2006; Hitchcock et al. 2016a; b; Choi et al. 2020). Understanding how secondary producers such as zooplankton respond to FWIs is therefore critical to informing how the broader health and productivity of estuarine food webs.

An important debate in aquatic ecology is the role terrestrial carbon resources and the microbial loop may play in contributing to the productivity of higher order consumers, such as fish, following inflows. Evidence of terrestrial carbon contributing to higher order aquatic consumers has been found through chemical tracing techniques such as stable isotope analysis (Pace et al. 2004; Abrantes and Sheaves 2010; Cole et al. 2011; Berggren et al. 2018). However, the topic remains debated for two key reasons; 1) although terrestrial carbon is easily assimilated by the microbial loop it is considered an inefficient pathway, with most of the energy it provides respired by bacteria, passing little energy up the food web (Ducklow et al. 1986); and 2) terrestrial carbon is considered a poor quality food resource, in comparison to algae, containing few of the essential fatty acids necessary for consumer growth and survival (Brett et al. 2009, 2017; Taipale et al. 2014). While some authors contend that the flux of terrestrial carbon from inflows can support zooplankton populations when in large supply (Pace et al. 2004; Carpenter et al. 2005; Cole et al. 2011), others suggest that it is primarily used for energetic demands and does not contribute to the growth of higher order consumers in aquatic food webs (Brett et al. 2009, 2017; Taipale et al. 2014). While this question remains more debated within freshwater systems, there seems to be greater acceptance that terrestrial carbon can contribute to planktonic food web production in

estuaries (Hoffman et al. 2008; Atwood et al. 2012; Hitchcock et al. 2016b; Berggren et al. 2018).

Several authors have related increased freshwater discharge, terrestrial carbon availability and its assimilation by juvenile fish with increased fish populations in estuaries (Darnaude et al. 2004; Hoffman et al. 2007; Wai et al. 2012). There is evidence the production of both planktivorous and benthic feeding taxa can be supported by terrestrial organic matter (Darnaude et al. 2004; Hoffman et al. 2007; Kostecki et al. 2010; von Biela et al. 2013). These results differ to those from freshwater systems, perhaps because of; 1) the quantity of terrestrial organic matter transported to estuaries following inflow events may be greater than lakes or upland rivers given the position of estuaries in the catchment; and 2) the quality of terrestrial organic matter entering estuaries during inflow events is increased substantially compared to low flow periods (Lennon and Pfaff 2005; Asmala et al. 2014b; Hitchcock and Mitrovic 2015b). Regardless of the precise mechanism involved, it does appear that terrestrial carbon resources may be a more important resource in estuaries than is currently appreciated (Abrantes and Sheaves 2010; Abrantes et al. 2013) and hence further study is required to understand its role in food webs.

1.6 Reducing impacts of river regulation on estuaries

River regulation refers to the modification of riverine flow through dams, weirs, channel modification and freshwater extraction (Arthington et al. 2018a). This is one of the main processes reducing the amount and timing of freshwater flowing to estuaries. Globally, there is a renewed period of dam building and river regulation, particularly in semi-arid regions such as Australia (Arthington et al. 2018a). Understanding and recognising the impacts of this processes on estuaries and FWIs is a key step in ensuring the continued ecological health and productivity of estuaries (Adams 2014; Arthington et al. 2018b).

In the early 2000's there was a large push to address and reduce the impacts of river regulation and FWI reduction on estuarine environments (Alber 2002; Gillanders and Kingsford 2002; Pierson et al. 2002; Montagna et al. 2002; Scheltinga et al. 2006). This work was largely focussed in three countries, the USA, South Africa and Australia, with a combination of scientific research and policy development leading to the establishment of frameworks and legislation for protecting freshwater inflows to rivers and estuaries (Alber 2002; Adams 2014; Arthington et al. 2018a). Many of these initiatives embraced the use of environmental flow provisions to achieve favourable environmental outcomes (Chilton et al.

2021). Environmental flows are defined as "the quantity, timing, and quality of water flows required to sustain freshwater and estuarine ecosystems and the human livelihoods and well-being that depend on these ecosystems" (Arthington et al. 2018a). Environmental flow provisions proposed by protective frameworks can take many forms including water allocations for environmental purposes, targeted and timed dam releases or limits to water extraction (Arthington et al. 2018b). The success of the many frameworks providing environmental flow provisions to estuaries relies on regular reviews of their ecological outcomes to ensure the effectiveness of current rules (Davies et al. 2014). Although there has been a great deal of advancement in, and implementation of, environmental flow protections for rivers and estuaries, there remains a lack of evaluation of existing management strategies (Davies et al. 2014; Arthington et al. 2018a).

Within the state of New South Wales in Australia, the main legislative instrument developed to protect FWIs to estuaries (and other freshwater dependant ecosystems) are Water Sharing Plans (WSP). These plans specify when, and how much, water can be extracted from systems by dependent stakeholders (NOW 2009a). The rules of WSPs are defined on a per catchment basis, implementing a combination of environmental flow provisions (including end-of-system flows, dam releases and environmental water allocations) and flow protections (including extraction limits and flow related pumping restrictions) to protect or maintain natural flow variability in riverine and estuarine systems. The effective implementation of WSPs relies on the input of new scientific data and reviews of the outcomes of existing management rules. Subsequently WPSs are reviewed every 10 years. This thesis contributes to the assessment and evaluation of the *WSP for the Hunter Alluvial Water Sources 2009*, addressing knowledge gaps surrounding the effectiveness of existing environmental flow protocols for the Williams River estuary (Davies et al. 2014).

1.7 Study Sites and WSPs

The flow rules within WSPs seek to "protect a proportion of natural river flows for fundamental ecological needs from increases in long-term water extraction" with changes in "water quality…and ecological condition of… water sources" to be used as indicators to measure the success of the implemented strategies (NOW 2009a). Part of this thesis was devoted to an ecological assessment of the Williams River estuary, which is the estuary most effected by river regulation within the aforementioned WSP (NOW 2009a). While the influence of inflows and river regulation on estuaries are broadly understood (Drinkwater and

Frank 1994), smaller scale, local assessments are key to the active management of local systems and a detailed understanding of the effect of river regulation on flow-ecology relationships in estuaries (Davies et al. 2014; Arthington et al. 2018a).

Williams River

The Williams River is a perennial river on the southeast coast of Australia, located north of the city of Newcastle, NSW (Figure 1.3). The Williams catchment is relatively small at 974 km^2 and is comprised of 80% agricultural land, 14% national park and has 6% held by Hunter Water Corporation, a local water utility (Hunter Water, 2011). The river flows 142 km from the southern side of the Barrington Tops region, joining the Hunter River estuary near Raymond Terrace. The flow of the Williams is highly variable but larger inflows due to storm activity mostly occur between February and May (Callaghan et al. 2014). The Williams River is a major source of drinking water for the Newcastle area (Hunter Water, 2011). Freshwater discharge is regulated between the river and the estuary by Seaham Weir (Figure 1.4 and 1.5), an approximately 10,000 ML volume weir pool on the Williams River operated by Hunter Water Corporation (Hunter Water, 2011). Seaham Weir imposes a tidal limit on the Williams River, preventing brackish water from entering the weir pool (Figure 1.5) and allowing extraction of freshwater to nearby Grahamstown Dam (~180,000 ML) for water storage. Downstream of the weir the estuarine section of the Williams River extends for 15 km to its confluence with the Hunter. The Williams River estuary is a frequently utilised both recreationally and commercially by the local community. As a tributary to the Hunter River, the majority of the remaining saltmarsh and mangroves associated with the system are downstream of the confluence of the two rivers, although there are small stands of mangroves throughout the Williams River up to Seaham Weir.

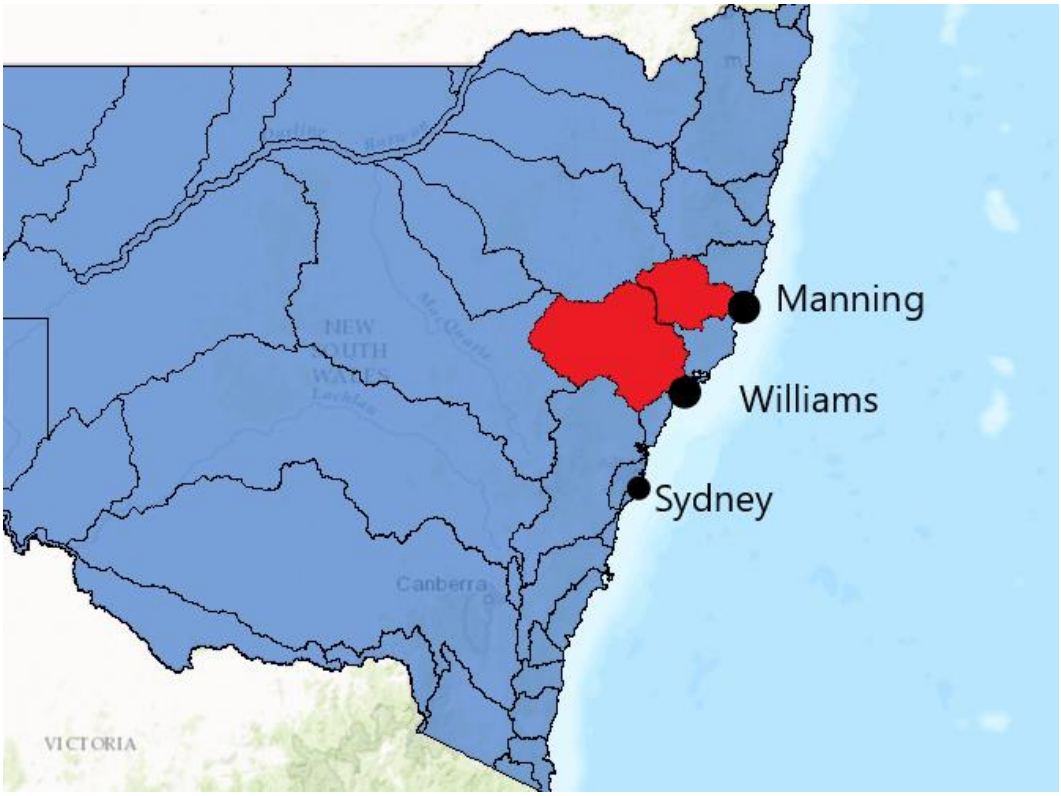

Figure 1.3: The position of the Manning and Williams estuaries (black dots) and their catchments (highlighted red) on the NSW coast.

As a major source of drinking water to the region the Williams River has had its natural flow regime significantly altered. Estimates of the yearly extraction of freshwater from the Williams River are approximately 189,000 ML/year from Seaham Weir (via Balickera Canal) and 50,000 ML/Year from the Chichester River, an upstream tributary, at Chichester Dam, a combined extraction of ~ 240, 000 ML/Year from the river (NOW 2009a). With significant extraction and a regulatory structure (Seaham Weir) enforcing a tidal limit (Figure 1.4 and 1.5) there are likely to be ecological impacts in the estuary due to these changes. The *Water Sharing Plan for the Hunter and Alluvial Water Sources 2009* specifies rules protecting a portion of inflows on the Williams River from extraction by dependent stakeholders to hopefully ameliorate some of the ecological impacts on the Williams estuary. Freshwater extraction from the Williams River and the Seaham Weir Management Zone (weir pool) are prevented under Low (15 ML/Day) and Very Low (6 ML/Day) flow conditions as measured at the NSW water gauge 210010 @ Glen Martin, 12 km upstream of the start of the weir pool (NOW 2009a). However, no daily releases are currently specified in the WSP from Seaham Weir to the estuary and there can be long periods (months) where hydrological connectivity is lost between the riverine weir pool and the downstream estuary (Figure 1.4).

Figure 1.4: The Williams River transition from freshwater (left) to estuary (right) through Seaham Weir (centre) (Hunter Water 2011).

Figure 1.5: A view of the Seaham Weir gates from the wall. Freshwater (left) is prevented from entering the estuary (right). Photo courtesy of Prof. Simon Mitrovic.

Manning River

To compare the influence of river regulation and the effectiveness of the WSP rules on the Williams River estuary a control system, which is unregulated, was used. The Manning River was selected as it remains one of the few largely unregulated rivers in NSW and is relatively close to the Williams, being ~100 km northeast along the NSW coast (Figure 1.3). While it is a larger catchment than the Williams, the Manning experiences similar magnitudes of freshwater discharge during storm events and, due to its geographical proximity, similar

seasonal timings in larger freshwater inflows (Callaghan et al. 2014). The Manning travels 261 km from the northern area of the Barrington Tops region (the same headwater region as the Williams) and meets the ocean through a double delta at both Harrington and Old Bar. Its catchment is less agriculturally developed than the Williams catchment with approximately 60% of its 8,100 km^2 classified as non-agricultural or forested land (BMT WMB 2016). Like the Williams, the majority of estuarine vegetation such as salt marsh and mangroves are located in the lower estuary although small stands of mangroves are present in the upper reaches. Cease to pump rules are enforced during Very Low Class flows on the Manning (137 ML/Day on a rising river and 98 ML/Day on a falling river) but there is little extraction compared to the Williams (NOW 2009a; b). Approximately 16,000 ML/Year is extracted from the catchment by local water utilities and domestic stock licenses, approximately 7% of the water extracted from the Williams. There are no weirs or dams along the main channel of the Manning River(NOW 2009b).

1.8 Research Aims

The overall aim of this thesis was to understand the influence of freshwater inflow events on estuarine food webs. This work focuses on how basal resources and planktonic communities respond to freshwater inflow events, and whether secondary production of zooplankton and juvenile fish is supported by terrestrial organic matter. To answer these questions, I conducted a combination of short and long-term observational studies, and a manipulative experiment to help reveal these fundamental flow-ecology relationships. Below is a summary of the following chapters which detail this work:

Chapter 2: In this chapter I investigate the potential influence of river regulation on water quality and zooplankton communities of the upper Williams River estuary and compare to a nearby unregulated Manning estuary. This study was conducted in the upper reaches of both estuaries over 4 years with 37 sampling trips conducted on each river. This study is important with comparisons between regulated and unregulated estuaries lacking within the scientific literature, particularly over multiple years and variable flow conditions. The aim of this study was to:

- Assess the influence of river regulation on the water quality and mesozooplankton community of the upper Williams estuary.

Chapter 3: Building on the results of Chapter 2 which showed different zooplankton communities between a regulated and unregulated estuary, Chapter 3 focuses on the short-term response of the lower estuarine food web to an inflow event in the regulated Williams. This chapter used fine scale monitoring of water quality and multiple trophic levels across an inflow event and the following 2 months to investigate short-term ecological responses to inflows that may have been missed by the monthly monitoring of Chapter 2. Specifically, it aimed to:

- Investigate the delivery of terrestrial resources to the Williams estuary across an inflow period.
- Investigate the responses of basal food web to an inflow, namely bacterial and algal communities.
- Investigate responses of mesozooplankton communities to an inflow event and changing resource availabilities.

Chapter 4: Building on preliminary results in Chapter 2, which positively linked inflows and DOC concentrations in the Williams estuary, this Chapter investigates if there is a switch in basal resource use by the mesozooplankton community following the delivery of terrestrial carbon by inflows. It uses stable isotope analysis during a subset of the longer-term sampling in Chapter 2, tracing resource use of mesozooplankton across a period of hydrological variability. While most stable isotopes studies in estuaries following inflows focus on freshwater plumes in the near coast or in larger estuarine bays, this is one of the first to investigate changing resource use in the upper reaches of a highly regulated estuary. The specific aims were:

- Investigate if terrestrial resources delivered to the upper Williams estuary by inflows were utilised by the mesozooplankton community.

Chapter 5: This chapter builds on the findings of Chapters 2-4 that suggest juvenile mesozooplankton may play a substantial role in the transfer of terrestrial dissolved organic matter (tDOM) to higher order consumers. As a manipulative study, I altered the availability of tDOM to cultured zooplankton nauplii populations, which were then fed to juvenile Australian bass (*Macquaria novemculeata*), investigating the effect of tDOM concentrations in prey resources on juvenile fish growth. This is one of few studies to investigate the transfer of terrestrial carbon to juvenile fish through a planktonic food web and the first to investigate terrestrial carbon transfer using a single source diet. It aimed to:

- Examine if terrestrial carbon resources could contribute to the somatic growth of juveniles from an endemic fish species.

Chapter 6: In this chapter the findings of Chapters 2-6 are discussed with overall conclusions drawn and recommendations for future research made. Management recommendations regarding relevant WSPs are also provided and discussed in a broader context.

Chapter 2: Responses of estuarine zooplankton to freshwater inflows: a comparison between a regulated and unregulated estuary

2.1 Abstract

Freshwater inflows play an important role in the health and productivity of estuaries. However, changes to freshwater inflow regimes can alter estuarine water quality and food webs. One area that requires better understanding is how zooplankton populations may respond to altered freshwater inflow regimes. In this study water quality conditions and concentrations of estuarine zooplankton, predominantly copepods, were monitored over 4 years in the upper reaches of two estuaries, one regulated by a weir at the estuary-river interface with significant water extractions, the other unregulated. Higher concentrations of dissolved organic carbon (DOC), nitrate/nitrite (NO_x), reactive phosphorous (FRP) concentrations, turbidity and chlorophyll *a* (Chl a) were present in the regulated Williams River compared to the unregulated Manning. The Williams also demonstrated substantial reductions in dissolved oxygen and salinity in response to freshwater inflows in comparison to the Manning. Differences in the zooplankton community between the two estuaries were apparent, driven by higher adult copepod concentrations in the Manning during warmer months and by increased concentrations of nauplii in the Williams. Redundancy analysis indicated that the zooplankton community in the Manning were significantly influenced by water temperature (positively), 30-day antecedent flow (negatively). This contrasted with the Williams where increased zooplankton concentrations were positively related to 60-day antecedent flow and negatively to DO and salinity, with temperature a secondary driver of community abundance. The contrasting zooplankton community structures and responses to inflows between the two estuaries may be due to the environmental conditions in the Williams, which are influenced by river regulation and reduced freshwater inflow.

2.2 Introduction

Freshwater inflows are integral to the health of estuarine ecosystems (Alber 2002; Pierson et al. 2002). They regulate and facilitate ecological processes, such as organic matter and nutrient cycling, ensuring the productivity and continuation of estuarine species (Gillanders and Kingsford 2002; Pierson et al. 2002). However, renewed increases in dam building and river regulation to satisfy the global demand for freshwater have resulted in inflows to many estuaries being reduced (Arthington et al. 2018a). Given the complexity of estuarine systems

and their responses to freshwater inflows (Roelke et al. 2013), understanding the impact of river regulation on individual estuaries and the effectiveness of existing management protocols are key steps in adaptive management (Adams 2014; Davies et al. 2014).

River regulation alters the natural flow regime of estuaries, which can have negative impacts on the water quality, habitat characteristics and biota of estuaries (Drinkwater and Frank 1994; Poff et al. 1997; Gillanders and Kingsford 2002). Reduced inflows can salinise estuaries, reducing the size of the freshwater-estuarine interface or gradient (Drinkwater and Frank 1994), an important habitat for fish and invertebrate species (Bate et al. 2002). Inflow reductions may also increase the likelihood of water column stratification and the residence time of nutrients, organic matter, sediments and pollutants in estuaries (Pierson et al. 2002; Uncles et al. 2003), which can lead to issues of eutrophication, reduced dissolved oxygen saturation or heavy metal and chemical pollution (Connell et al. 1981; Drinkwater and Frank 1994). River regulation, along with changing land use practices, can also alter the delivery of terrestrial carbon and nutrient resources to estuaries disrupting ecological processes and resulting in reduced primary and secondary productivity (Harris 2001; Gillanders and Kingsford 2002). Assessing the impact of river regulation through changes to estuarine water quality, environmental characteristics and determining how key estuarine biota respond to these changes are an important step in managing the health of estuaries and their freshwater inflow requirements (Scheltinga et al. 2006; Adams 2014; Davies et al. 2014).

Estuarine mesozooplankton are a key component of estuarine food webs and ecology, linking primary and lower food web production pathways to higher trophic levels (Kobayashi et al. 1996; Winder and Jassby 2011). Their assemblages, generally dominated by crustaceous copepods, can vary between estuarine, marine, and freshwater taxa with relative abundances determined by environmental factors, predominantly temperature, salinity, flow conditions, and resource availabilities (Mallin 1991; Hirst and Bunker 2003; Paul et al. 2016; David et al. 2016). Furthermore, the abundance of estuarine zooplankton is often a major factor in the recruitment, growth and survival rates of estuarine fish populations (Newton 1996; Hamilton et al. 2020). Their quick responses to changing environmental conditions make estuarine mesozooplankton useful indicators for the ecological health of estuaries in response to river regulation (Ferdous and Muktadir 2009; Drira et al. 2018; Hamilton et al. 2020).

Within estuaries freshwater inflows can play a significant role in shaping mesozooplankton communities (Muha et al. 2012). Inflows can regulate habitats (Drinkwater and Frank 1994), provide various food resources (Buyukates and Roelke 2005; Hoover et al. 2006; Hitchcock

et al. 2016b), physically advect or import populations (Kobayashi et al. 1998; Kimmerer et al. 2018) and trigger hatching events after prolonged periods of stratification (Nielsen et al. 2012; Broman et al. 2015). Therefore, reducing freshwater inflows to estuaries through river regulation can significantly alter zooplankton community structure and abundance (Buyukates and Roelke 2005; Miller et al. 2008; Kimmerer et al. 2018). This may result in further ecological impacts such as reduced phytoplankton grazing or reduced recruitment of juvenile fish (Newton 1996; Tan et al. 2004; Hamilton et al. 2020). A sound understanding of how mesozooplankton populations respond to river regulation and inflows is an important element in the adaptive management of regulated estuaries (Scheltinga et al. 2006; Adams 2014; Hamilton et al. 2020).

The aim of this study was to assess the impact of freshwater inflows on the environmental conditions and mesozooplankton community of a regulated estuary. I conducted a 4-year monitoring study on the south-east coast of Australia, comparing two nearby estuaries, one largely unregulated and the other regulated with a weir at the estuary interface, an upstream dam and water extractions during inflows, to investigate the possible impacts of river regulation. The length of this study is due to its origins as a part of a WaterNSW monitoring program which I have been involved in, collecting data for this study throughout my Honour's candidature (2015/16). For the purposes of this study, I focussed on changes in water quality and the mesozooplankton community in the upper reaches of these estuaries, as the impacts of regulation and reduced inflows are more likely to occur here than at the mouth of the estuary (Bate et al. 2002; Pierson et al. 2002). I hypothesised that there would be 1) differences in the water quality of each estuary in response to similar sized inflow events, e.g., concentrations of nutrients, organic matter, and dissolved oxygen, due to the regulation of freshwater inflows in one system, and 2) that there would be differences in the abundance and community structure of mesozooplankton between the two estuaries, which could be attributed to the impact of river regulation. In addition, as river regulation generally results in negative impacts on estuarine biota (Drinkwater and Frank 1994) , I expected, 3) on average, a greater concentration of mesozooplankton in the unregulated estuary compared to the regulated.

2.3 Methods

2.3.1 Site Description

Two estuaries in New South Wales, Australia, were sampled in this study: The Manning (Figure 2.1) and the Williams River (Figure 2.2). The two estuaries were selected for their geographic similarity and, importantly, their varying degrees of river regulation. The Manning River remains one of the few unregulated rivers in NSW and 60% of its 8400 km^2 catchment is classified as non-agricultural (forested) (BMT-WMB 2016). There is approximately 4.83 and 0.13 km^2 of mangrove and saltmarsh cover respectively throughout the estuary. The Williams, while a smaller catchment at 974 km^2, is 80% agricultural land, 14% national park and has 6% held by Hunter Water, a local water corporation (Hunter Water 2011). Flow in the Williams River is regulated by Seaham Weir, operated by Hunter Water, allowing extraction to nearby Grahamstown Dam, providing a source of drinking water for the area (NOW 2009a). The weir imposes a tidal limit, preventing brackish water from entering the upstream freshwater weir pool. As a tributary to the Hunter River, the majority of saltmarsh and mangroves associated with the estuary are downstream of the confluence of the two rivers, although there are small stands of mangroves throughout the Williams.

For each estuary, three sites were selected to characterise a similar position in the catchment that encompassed the upper reaches of each estuary, 30 - 50 km upstream of the estuarine mouth, along an approximate 15-20 km longitudinal distance, with sites being between 5 and 7 km apart (Figure 2.1 and 2.2). These sites were originally selected as part of a WaterNSW monitoring program beginning in 2014 to characterise the upper portion of each estuary toward the extend of its tidal limit; being artificially enforced in the Williams and naturally occurring in the Manning. The most upstream site on the Williams was 500m downstream of Seaham Weir, 47 km from the river mouth (Figure 2.1), while the most upstream on the Manning was at Wingham approximately 1 km downstream of the tidal limit, approximately 42 km from the mouth (Figure 2.2), where the main channel is no longer navigable by boat. Sampling occurred between February 2015 and June 2019. During this period, both estuaries experienced a range of flow conditions including substantial freshwater inflows.

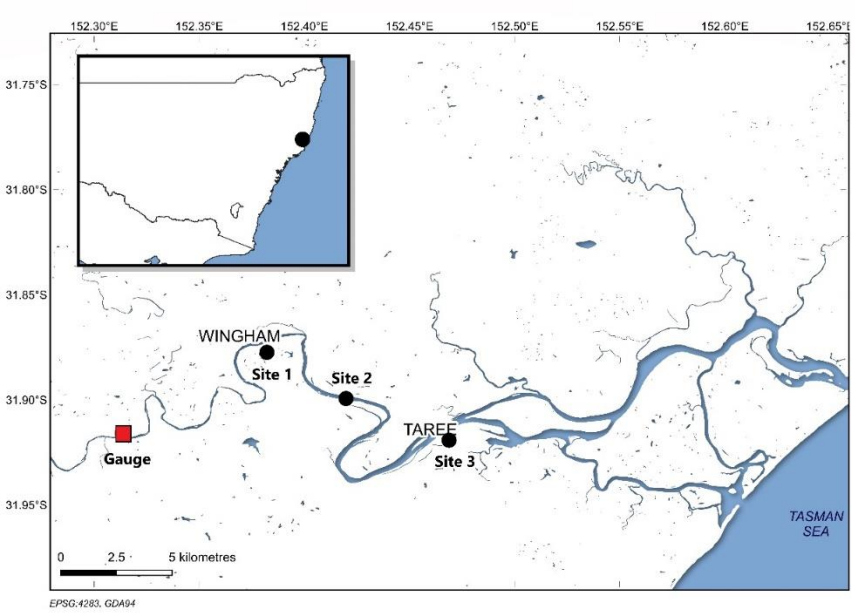

Figure 2.1: Site and discharge gauge positions for the Manning River. Sites are denoted by black circles on the river channel. Site 1 is the most upstream point, with site numbers increasing downstream to Site 3 near Taree. WaterNSW gauge 208004 (Manning @ Killawarra) is indicated by the red square. Position of the study site along the NSW coast is indicated by the black circle in the inset image.

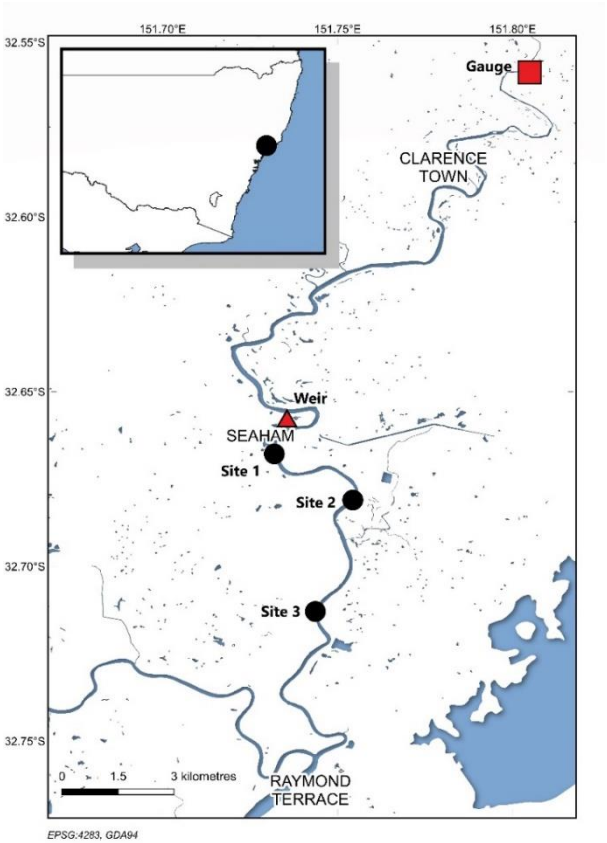

Figure 2.2: Site, weir and discharge gauge positions for the Williams River. Sites are denoted by black circles on the river channel. Site 1 is the most upstream point (near Seaham) with site numbers increasing downstream to Site 3 (closer to Raymond Terrace). Seaham Weir is denoted by the red triangle upstream of Seaham. WaterNSW gauge 210010 (Williams @ Glen Martin) is indicated by the red square upstream of Clarence Town. Position of the study site along the NSW coast is indicated by the black circle in the inset image.

2.3.2 Sampling

Sampling occurred monthly and included *in-situ* surface measures of physicochemical water quality parameters salinity, dissolved oxygen (DO), temperature, as well as of dissolved organic carbon (DOC), dissolved nitrates/nitrites (NO_x), filtered reactive phosphorous (FRP) and Chl *a* concentration. Sampling was conducted starting from the most downstream site on each estuary (Site 3) at the ebb tide, moving upstream on the rising tide. Sampling was completed within 0-2 hours of high tide. DOC and nutrient samples (NO_x and FRP) were taken in duplicate (due to the high external costs of long-term monitoring), filtered through 0.45 μm syringe tip filters (Minisart, 16533Q), and collected in sample rinsed 250 mL PET bottles. Samples were frozen as soon as possible (within 4 hours). DOC samples were analysed by the High Temperature Combustion Method (Sugimura and Suzuki 1988). Chlorophyll *a* (Chl *a*) samples, taken in duplicate, were collected by filtering 500 mL of water onto GF/C filter papers (Whatman, 47 mm). Filter papers were frozen until Chl *a* was determined using the grinding technique and an ethanol solute with corrections for phaeophytin (APHA et al. 2005). Chl *a* concentration was analysed with a detection limit of 1 μg/L. Nutrient samples (NO_x and FRP) were analysed using flow injection analysis on Lachat Quikchem 8500 Series II using standard methods (APHA 2005). Physicochemical measurements were taken using a Hydrolab field hand-meter Surveyor 4 and MS5 sonde probe, measuring water temperature, dissolved oxygen saturation (DO), salinity, and pH. Turbidity was measured using a Hach 2100P turbidimeter.

Zooplankton samples were collected using a 12 L Schindler trap. To provide a depth profile, samples were taken from the surface, 1 m and 2 m depths and combined at each sampling point. Integrated depth samples were taken in triplicate with each replicate consisting of a 36 L integrated depth sample. Samples were filtered through a 35 μm net and then made up to 25 mL with 96% ethanol to preserve. Samples were homogenised by inverting sample bottles before counting. Aliquots were taken using an Eppendorf automatic pipette and transferred to S-trays. Disposable pipette tips used were cut allowing an opening of 5 mm in diameter preventing the under representation of larger zooplankton in samples. Between 3-10 mL of sample was enumerated by light microscopy (Olympus, SZX12) depending on the density of zooplankton. Mesozooplankton taxa were identified to the order level with most identified to genus, however, as copepods were the dominant mesozooplankton other orders of rarer mesozooplankton in the study (e.g., Cladocera and polychaeta) were removed from analysis. To allow general comparisons of copepod concentration between estuaries, copepods were

grouped as nauplii (NII – NVI), copepodites (CI-CV) or adults.

2.3.3 Statistical analysis

Statistical analysis was conducted in R (4.1.1) (R Development Core Team 2020). Three-way ANOAVAs were conducted on water quality variables (DOC, turbidity, NO_x, FRP, Chl *a* and DO) to test for the interaction of site (upstream, middle or downstream site), Q30 flow categories (low, moderate, fresh and flood) and River (Manning or Williams) using base R with data transformed (log(x+1) for all variables except NO_x and FRP for which a 4^{th} root was used) to satisfy assumptions of normality and homoscedasticity.

Q30 flow categories were created for analysis using discharge values obtained from Water NSW's Real-Time Data gauges 208004 (Manning, Figure 2.1) and 210010 (Williams, Figure 2.2). Percentile flow curves were constructed from long term discharge data (50 years). Q30 flow categories were constructed using historic flow curve percentiles for each estuary, with each category defined as "Low" (flow magnitudes occuring > 80% of the time), "Moderate" (occuring between 80% and 30% of the time), "Fresh" (between 30% and 10%) or "Flood" flows (> than 10% of the time). These allowed a comparisson of similar sized events between each river. Q10 and Q60 (10 and 60 day antecedent flow) were also calculated for each sampling point and used in analysis, though not as flow categories. Correlation analysis was conducted using Pearson's R (presented as R^2) through the "Hmisc" package (Harrel 2015).

Multivariate abundance analysis to identify the differences in zooplankton communities between rivers, sites and flow conditions was conducted using "mvabund" from the "mvabund" package (Wang et al. 2012). Negative binomial distributions were used with log(x+1) transformed data to ensure an appropriate fit of residuals which were visually inspected to ensure correct fit (Appendix 1, Wang et al. 2012). Pairwise comparisons indicated differences between factors ($p < 0.05$). "Simper" from the "vegan" package was used to determine the contribution of copepod groups to differences shown by multivariate general linear models. Distance-based redundancy analysis (dbRDA) was used to identify significant ($p < 0.05$) environmental drivers influencing copepod community concentration in each estuary using the "capscale" functions in the "vegan" package (Oksanen et al. 2013). Mesozooplankton community concentrations were transformed (log(x+1)) to reduce the influence of the high abundance groups, particularly nauplii. Relevant water quality and environmental vectors were selected and fitted to the ordination models using the "envfit"

function from “vegan”, ensuring that the variable inflation factor remained under 3. While the antecedent flow measurements for 10, 30, 60 and 90 days all correlated it was found that Q30 and Q60 were the best explanatory flow variables for the Manning and Williams zooplankton communities respectively. Significant variables in the models were identified using Monte-Carlo permutations through the “anova.cca” function in “vegan”. All figures were constructed using “ggplot2” (Wickham 2016).

2.4 Results

2.4.1 Hydrology and resource delivery

Flow conditions were variable in both estuaries during the study period. Larger inflows tended to occur toward the earlier months of the year between January and April with the largest flows through the Manning River in March 2017 (85,000 ML/Day) and April 2018 (51,000 ML/Day), while in the Williams the largest flows were in February 2015 (98,000 ML/Day) and January 2016 (78,000 ML/Day) (Figure 2.3a and b). Discharge correlated negatively with salinity at all sites across the study (Appendix 2). In the Manning there was evidence of an estuarine salinity gradient with all sites being significantly different in salinity throughout the study (Figure 2.3a, ANOVA, $F_{2,89} = 27.6$, $p < 0.001$). In contrast there was no longitudinal difference in salinity in the Williams estuary (Figure 2.3b, ANOVA, $F_{2, 92} = 0.68$, $p = 0.51$). Salinities between the rivers were comparable, except for Site 3 in the Manning, which was consistently more saline than the Williams sites, particularly during low flow periods toward the end of the study (>20 PSU).

Temperature ranges were similar between estuaries throughout seasons (10- 30^{o}C, Figure 2.3c and d) with minimal differences between sites within each estuary. Chl *a* concentrations were often greater in the Williams than in the Manning. The maximum concentration in the Manning was 20 µg/L at Site 2 in May 2015, which was exceeded on more than 20 occasions in the Williams (Figure 2.3c and d). Chl *a* concentration was positively correlated to temperature in the Manning at Site 1 and 3 ($R^2 = 0.19$ and 0.33, $p < 0.001$ for both) however, there were no significant relationships between Chl *a* and temperature in the Williams.

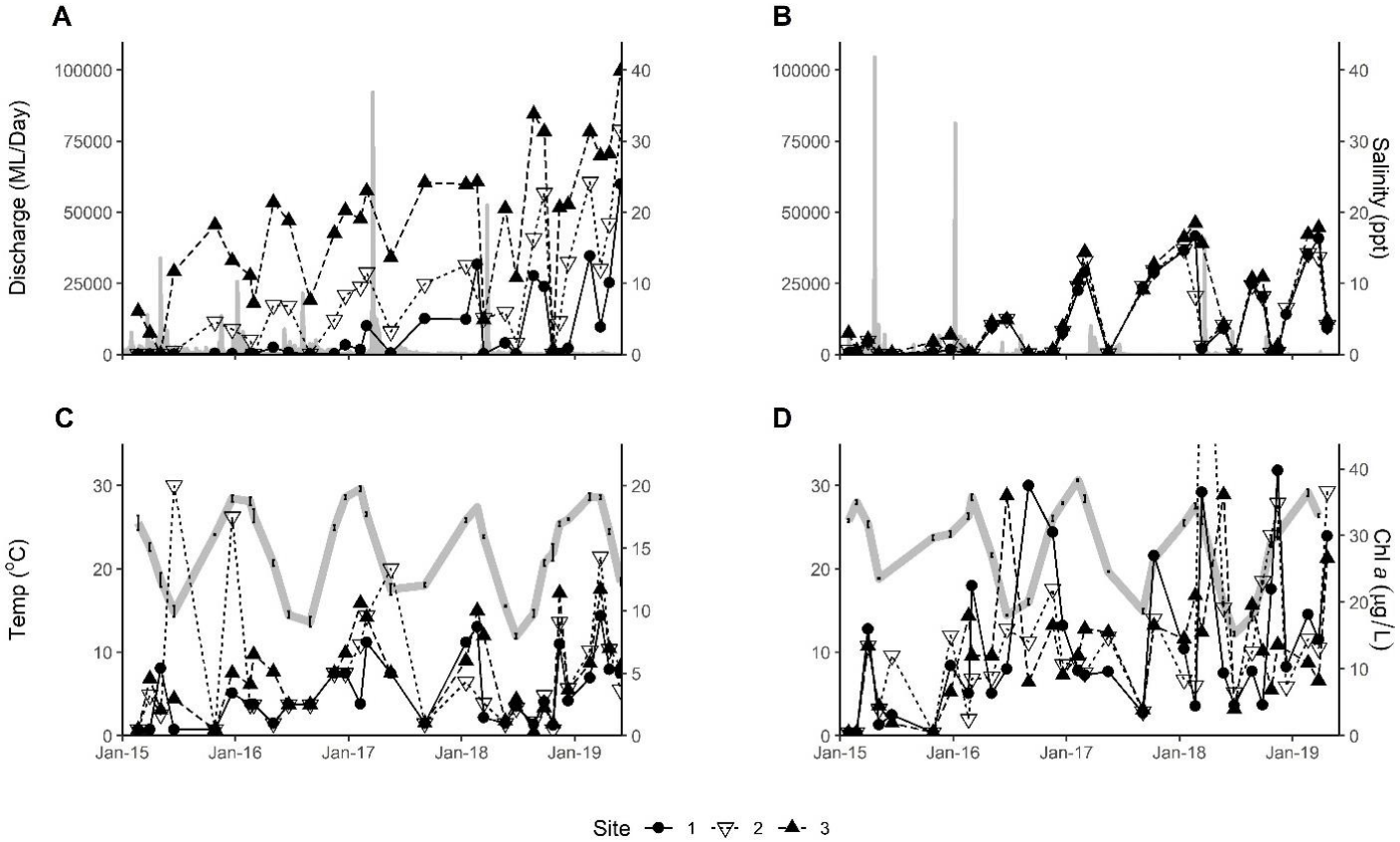

Figure 2.3: Discharge (ML/Day – shaded grey area) of the Manning (A) and Williams (B) River estuary with salinity (ppt- points and lines) from the three sampling sites in each, Site 1 being most upstream and Site 3 the most downstream. Figures C and D show temperature (°C – thick grey line) and Chl a concentration (µg/L – points and lines) for the Manning and Williams estuaries respectively at the same three stations. Discharge data was taken from gauges 208004 (Manning @Killawarra) and 210010 (Williams @Glen Martin) as shown in Figure 2.1 and 2.2. Temperature is displayed as an average between the three sites of each estuary as there was little variation between sites. Chl a at Williams Site 2 in March 2018 was 82.5 µg/L.

Three-way ANOVAs comparing River*Site*Q30 flow classes showed no significant interaction between the factors for any water quality variable, with significant differences present for all variables between rivers (Appendix 3). DOC concentrations (7 – 9mg/L) and turbidity (15 – 21 NTU) were higher in the Williams than the Manning (DOC; 3 – 5 mg/L, turbidity; 4 – 8 NTU) regardless of flow class (Figure 2.4a and b, DOC; $F_{1, 163} = 45.7$, $p < 0.001$, Turbidity; $F_{1, 163} = 44.3$, $p < 0.001$). In the Manning, DOC concentration decreased with higher magnitude flow conditions (from ~5 mg/L to <3 mg/L), while turbidity was increased (Figure 2.4a and b). DOC and turbidity were statistically similar under all flow classes in the Williams though greatest during "Fresh" conditions. There were significant positive correlations between antecedent discharge and DOC at Site 2 and 3 in the Williams, but none in the Manning ($p < 0.05$, Appendix 2). Turbidity correlated positively with all antecedent discharge metrics at all sites in the Williams ($p < 0.05$, Appendix 2) and at Site 2 (Q30) and 3 (Q10) in the Manning ($p < 0.05$, Appendix 2).

Concentrations of NO_x were highly variable in each estuary but were significantly higher in the Williams than the Manning (i.e., >0.075 mg/L, Figure 2.4c, 2-ANOVA, $F_{1, 163} = 11.8$, $p < 0.001$). NO_x increased under higher magnitude flow classes in the Manning, being highest and most variable under "Fresh" conditions with significant positive correlations between antecedent discharge and NO_x present in the Manning ($p < 0.05$, Appendix 2). In contrast NO_x was highest, and most variable, under low flow conditions in the Williams (0.011 mg/L ± 0.43). There was no relationship between NO_x and antecedent flow in the Williams and the lowest average concentrations of NO_x in the Williams were under "Flood" conditions. FRP concentrations were greater in the Williams than in the Manning under all flow conditions (i.e,, >0.04mg/L, Figure 2.4d, 2-ANOVA, $F_{1,163} = 46.3$, $p < 0.001$). Increased magnitude of flow class had no impact on FRP concentrations in the Manning but increased concentrations in the Williams (Figure 2.4d). Despite this pattern in the Williams, there was no significant relationship between antecedent discharge and FRP at any site in either river across the study (Appendix 2).

Chl *a* concentration was significantly higher in the Williams than in the Manning regardless of flow conditions (i.e., >10µg/L, Figure 2.4e, 2-ANOVA, $F_{1,163} = 56.1$, $p < 0.001$). Average Chl *a* in the Manning did not change between flow classes though it was negatively correlated to antecedent discharge (Appendix 2). Chl *a* in the Williams was similar under all flow conditions although under Fresh flow conditions it was increased. DO was largely unaffected by flow classes in the Manning remaining above 90% saturation throughout the

study and increasing slightly under flood conditions. This was significantly higher than the average DO concentration in the Williams (Figure 2.4e, 2-ANOVA, $F_{1,163} = 4.9$, $p = 0.028$) where decreased under fresh and flood conditions. DO was negatively correlated to antecedent discharge in the Williams throughout the study (Appendix 2).

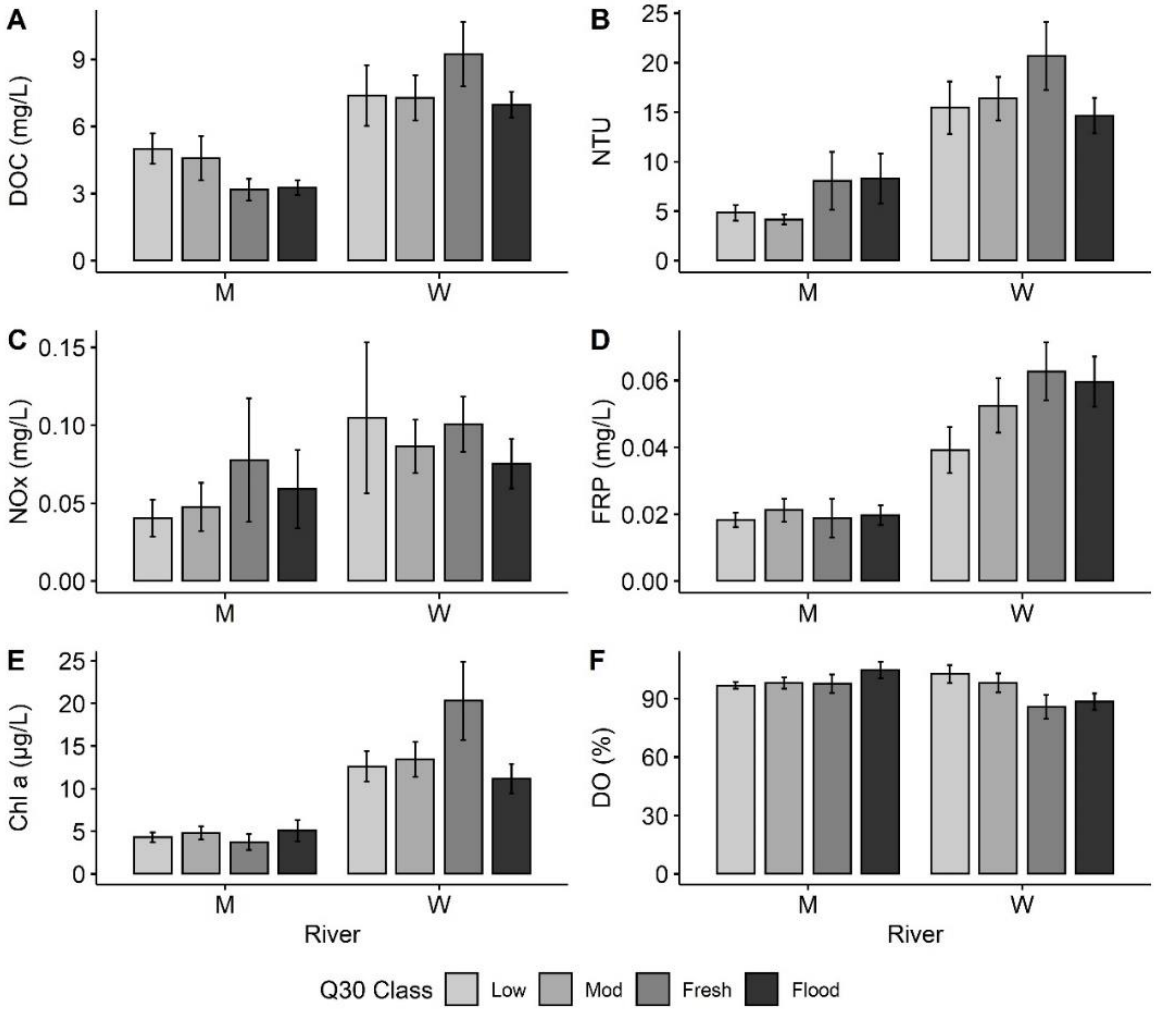

Figure 2.4: Comparisons of A) dissolved organic carbon (DOC (mg/L)), B) turbidity (NTU)), C) nitrates and nitrites (NO_x (mg/L)), D) filtered reactive phosphorous (FRP (mg/L)), E) Chlorophyll a (Chl a (µg/L)) and F) dissolved oxygen (DO (%)) concentrations between 30-day antecedent flow class (Q30 Class) conditions (low, moderate (Mod), fresh and flood) for the Manning (M) and Williams (W) estuaries. Error bars represent standard error of the mean. Number of samples taken within each flow class for Manning n = 41, 25, 10, 16 respectively and for Williams n = 29, 21, 18, 27.

2.4.2 Estuarine zooplankton communities

Eleven mesozooplankton species/groups were found in > 5% of samples within each estuary during the study. Copepod nauplii were the most dominant group found in 100% of samples in each estuary, with juvenile copepodites also found in most samples (Table 2.1). Four major adult copepods were identified; *Sulcanus conflictus, Oithona* spp., *Gladioferens pectinatus*

and *Acartia* sp.. Of these, *Oithona* spp. and *S. conflictus* were the most dominant adult species in the Manning and Williams estuaries, respectively. Two cladocerans species, *Bosmina meridonalis* and *Sididae* sp., were found in both estuaries but more frequently and in higher average concentrations in the Williams estuary (Table 2.1). Larval polychaetes, crab zoea and harpacticoids were also found in the study in both estuaries though infrequently and at low densities.

Table 2.1: Mean, standard error and range for the density of most common zooplankton in the Manning and Williams estuaries between January 2015 and June 2019. Sample % represents the percentage of samples in which the zooplankton was found. Abbreviation in parentheses corresponds with usage in Figure 2.7.

	Manning				Williams			
Zooplankton	**Mean**	**SE**	**Range**	**Sample %**	**Mean**	**SE**	**Range**	**Sample %**
Nauplii (NP)	1081.4	159.0	19.4 - 9027.8	100	2754.2	344.3	25.9 - 19,680.6	100
Copepodites (CD)	253.6	49.2	0 - 2069.4	92.4	275.2	39.6	0 - 1916.67	95.8
Sulcanus conflictus (SC)	36.1	7.0	0 - 305.6	57.6	146.4	39.1	0 - 2824.1	71.6
Oithona spp. (OT)	487.7	146.1	0 - 8333.3	62.0	32.6	9.1	0 - 555.6	45
Gladioferens pectinatus (GP)	26.2	12.0	0 - 726.1	16.3	26.9	10.7	0 - 708.3	18.9
Sididae sp. (SD)	0.5	0.5	0 - 41.6	1.1	27.88	19.8	0 - 1861.1	12.6
*Bosmina meridonalis (*BM)	0.5	0.4	0 - 37.0	3.2	26.4	9.7	0 - 722.2	21
Acartia sp. (AC)	0.6	0.6	0 - 55.6	2.2	6.1	3.3	0 - 247.9	6.3
Polychaeta (PO)	1.7	0.5	0 - 27.7	17.4	1.1	0.6	0 - 41.6	7.4
Zoea (Z)	1.4	0.7	0 - 41.6	7.6	0.1	0.1	0 - 6.9	2.1
Harpacticoid (HT)	0.2	0.1	0 - 4.62	5.4	0.5	0.2	0 - 13.9	7.3

Multidimensional scaling of the zooplankton community from each estuary showed some difference between the estuaries (Figure 2.5) which was confirmed by multivariate abundance analysis. Three-way multivariate abundance analysis of the zooplankton community (River*Site*Q30 Flow Class) through generalised linear models (GLMs by “mvabund”) revealed there was a significant difference in community structure between the two rivers (LRT = 45.35, p = 0.001), despite the overlap in Figure 2.5, but not between sites or flow

classes (Table 2.2).

SIMPER analysis of the mesozooplankton community demonstrated that the four major zooplankton driving the community difference between the two rivers were nauplii (69.5 %), copepodites (11.7 %), *S. conflictus* (10.1 %) and *Oithona* spp. (6.1%), with greater abundances of nauplii, copepodites and *S, conflictus* found in the Williams and *Oithona* spp. being more abundant in the Manning.

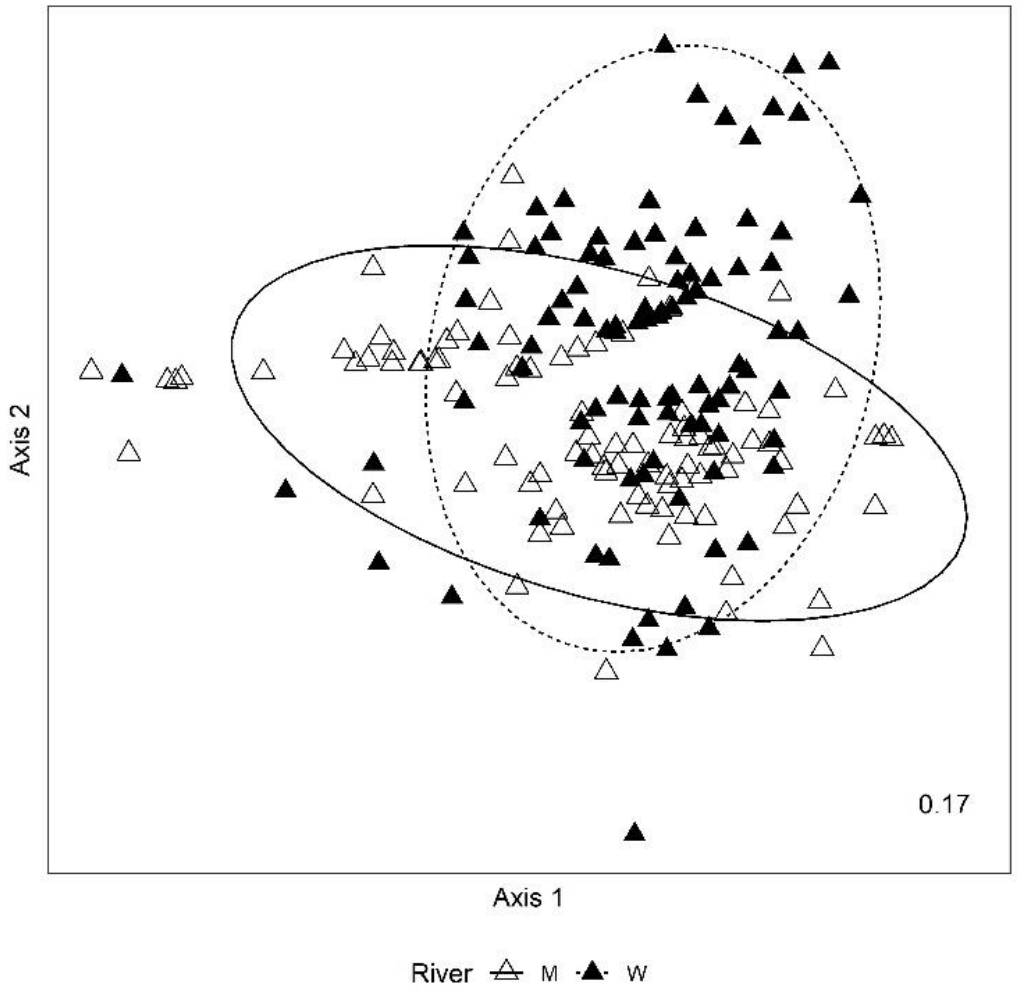

Figure 2.5: Multidimensional scaling (MDS) ordination of the zooplankton community data for the Manning (M – open triangles) and Williams (W – closed triangles) estuaries. Ellipses represent 95% confidence interval. The stress of the model is indicated in the bottom right-hand corner.

Table 2.2: Results of multivariate abundance analysis ("mvabund" function) for factors River (n=2), Site (n= 3) and Flow Class (30-day antecedent flow class) n= 4) within the study. Q30 flow classes were determined using historic flow percentile curves. LRT = likelihood ratio test. Df = degrees of freedom

Factor	Residual Df	Df. Diff	LRT	p-value
River	185	1	45.35	0.001*
Site	183	2	14.96	0.648
Flow Class	180	3	24.95	0.732
River*Site	178	2	25.84	0.161
River*Flow Class	175	3	20.59	0.711
Site*Flow Class	169	6	77.59	0.144
River *Site*Flow Class	163	6	45.77	0.089

Seasonal patterns of increased zooplankton concentrations were evident in the Manning during the austral summers (December-February) of 2016/17, 2017/18 and 2018/19 (Figure 2.6 a, c and e). These peaks in total zooplankton concentration in the Manning often coincided with an increase in the proportion of copepodites or *Oithona* spp. in the community (Figure 2.6a, c and e). At Site 1 (Figure 2.6a) increases in total zooplankton concentration in February 2018 to above 10,000 ind/L (individuals per litre) and February 2019 to above 7,000 ind/L coincided with increases in the proportion of *Oithona* spp. copepods to above 50% of the total community. Similarly, total copepod density increases in Site 2 (Figure 2.6c) in February 2017, January 2018 and February 2019, to between 8,000 and 12,000 ind/L also coincided with increases in the proportion of *Oithona* spp. in the community to levels above 50%. Though weaker, this pattern was still evident in concentration peaks in site 3 (Figure 2.6e) for January 2018 and 2019, where *Oithona* spp. represented 48% and 25% of the population, respectively, although in 2019 contributions of copepodites (50%) were larger than in other peaks of total copepod density.

In contrast, substantial increases in the total zooplankton concentration in the Williams were driven almost exclusively by high concentrations of copepod nauplii (Figure 2.6b, d and f). Populations of copepodites and adult copepods did not show the same seasonal trends in the Williams as in the Manning. At Site 1, the three highest peaks in total copepod concentration,

in February and December 2016 and November 2018, were all comprised of at least 90% nauplii (Figure 2.6b). The two largest peaks for total copepod concentration in the study during December 2016 and May 2017 at Site 2 (both above 15,000 ind/L, Figure 2.6d) were also comprised of at least 90% nauplii, as was the largest peak at Site 3 in May 2017 (Figure 2.6f, approximately 5,600 ind/L). Although there were occasions in the Williams where adult copepod and copepodite concentrations did exceed 50% of the total population, these occurred at relatively low total zooplankton population concentrations (e.g., January and December 2017 at Site 1, September 2018 at Site 2, and September 2018 at Site 3).

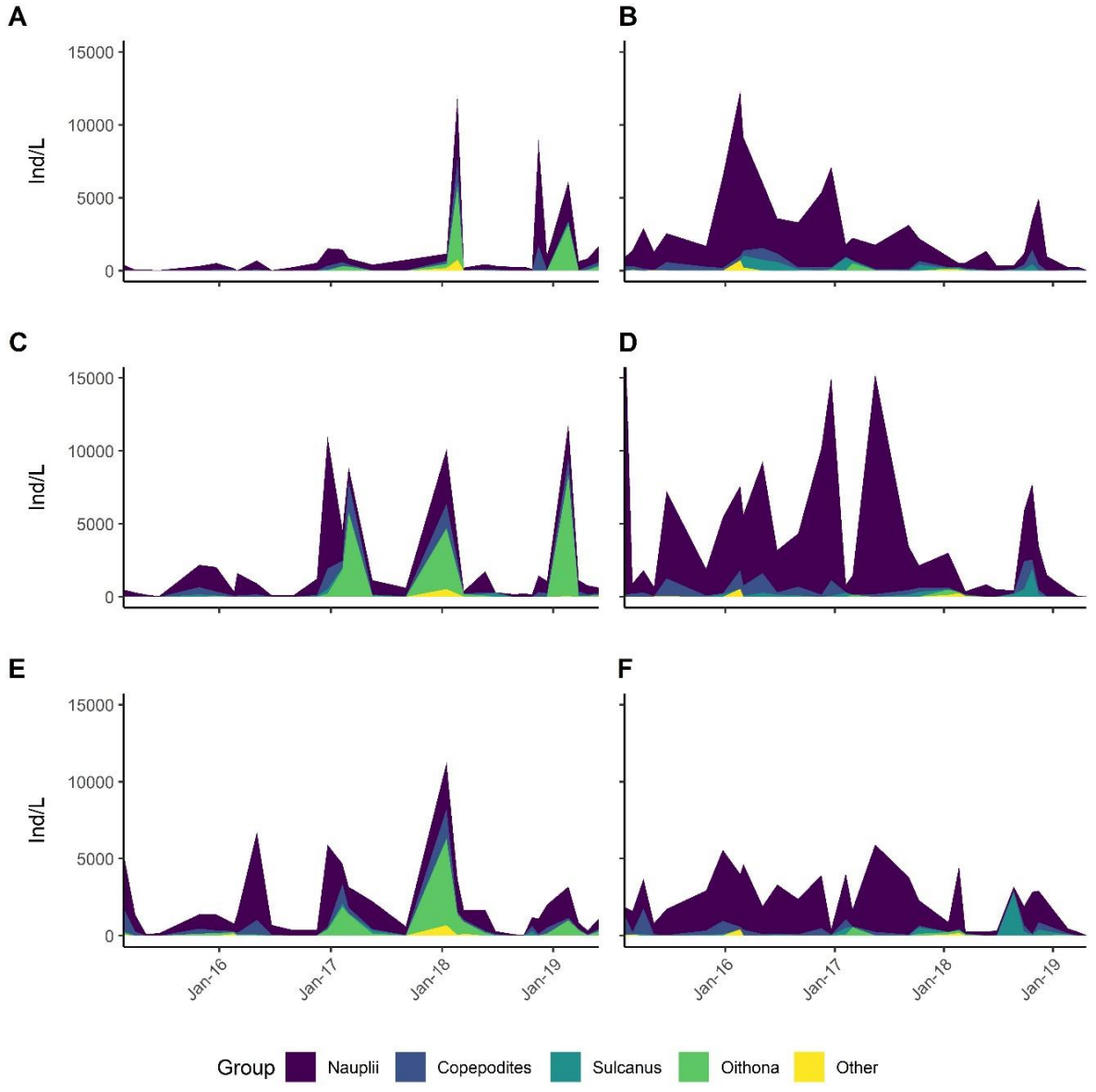

Figure 2.6: Density (Ind/L=individuals/L) of major zooplankton community groups (nauplii, copepodites and adults) for sites 1,2 and 3 in the Manning (A, C and E respectively) and the Williams (B, D and F) between January 2015 and June 2019. Other represents a combination of the remainder of the zooplankton from Table 2.1.

2.4.3 Contrasting responses to environmental drivers

Distance-based redundancy analysis (dbRDA) of the zooplankton community and environmental variables in each estuary showed how zooplankton communities responded differently to environmental conditions in each estuary. Within the Manning, dbRDA explained 43.6% of variation in the community (Figure 2.7a, Table 2.3, $F_{10, 81} = 4.46$, $p = 0.001$) with Q30, water temperature, salinity and DO being significant explanatory variables in the model (Table 2.3). The primary axis (Axis 1) was the only significant axis within the model ($F_{1, 81} = 35.7$, $p = 0.001$) explaining 80.1% of the constrained ordination within the community. This axis was characterised by increased temperature, salinity and Chl *a*, which the dominant zooplankton in the Manning (Table 2.1; nauplii, copepodites and *Oithona* spp.) were positively related to, and increasing Q30 which they were negatively related to.

In the Williams, dbRDA explained 41.1% of variation in the zooplankton community (Figure 2.7b, Table 2.3, $F_{10, 84} = 3.85$, $p = 0.001$**)** with temperature, salinity, Q60, FRP, Chl *a,* turbidity and DOC all being significant explanatory variables in the model (Figure 2.7b, Table 2.3, $F = 9.4$, $p = 0.001$). Three axes were significant in the Williams model. Axis 1 ($F_{1,84} = 17.88$, $p = 0.001$, 46.5% of constrained ordination) was characterised by salinity, DO and Q60. Nauplii, copepodites, *G. pectinatus*, *Sididae* sp. and *B. meridonalis* were all positively related to increasing Q60 while *S. conflictus* was positively related to salinity, DO and Chl *a*. Axis 2 ($F_{1,84} = 9.42$, $p = 0.001$, 24.5 % of constrained ordination) was defined by temperature, salinity, turbidity and DOC with *Oithona* spp. and *G. pectinatus* positively related to increased salinity and temperature and negatively to DOC. Axis 3 ($F_{1,84} = 5.32$, $p = 0.028$, 13.8 % of constrained ordination), which is not shown in Figure 2.7b, was characterised by DOC, turbidity, Q60 and FRP which were all positively related. Nauplii, copepodites and *S. conflictus* were all positively related to these variables in Axis 3.

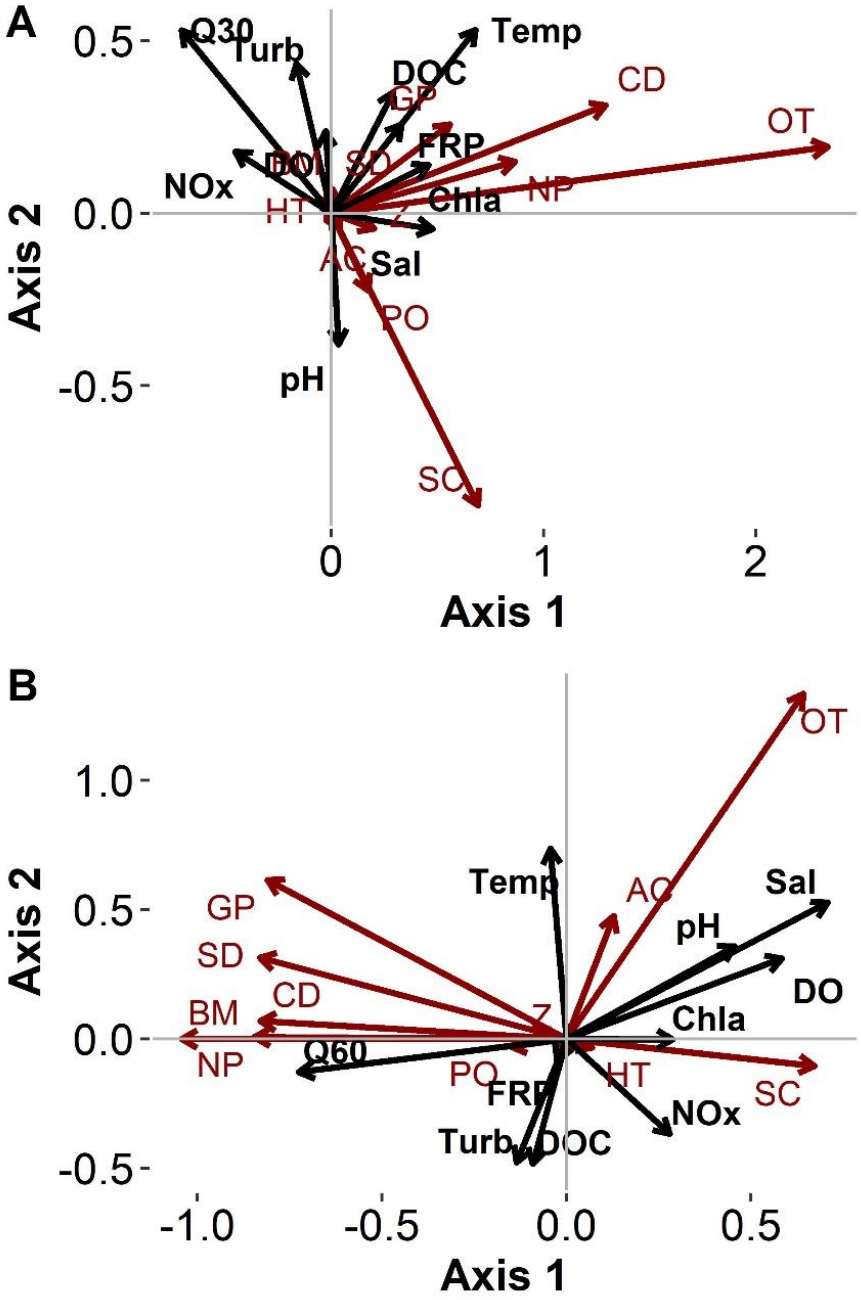

Figure 2.7: Distance-based redundancy analysis of the copepod communities of the Manning (A) and Williams (B) River estuaries for samples collected between January 2015 and June 2019. Species abbreviations in red correspond to those provided in Table 2.1. Environmental variable abbreviations are as follows: Q30 = 30-day antecedent discharge (ML/Day), Q60 = 60-day antecedent discharge (ML/Day), Temp = temperature (oC), NO_x = nitrate and nitrite concentrations (mg/L), FRP = filtered reactive phosphorous (mg/L), DOC = dissolved organic carbon (mg/L), Sal = salinity (ppt), DO = dissolved oxygen saturation (%), Turb = turbidity (NTU).

Table 2.3: Significant constraining variables in the Manning and Williams dbRDA models (Figure 2.7) as determined by Monte-Carlo permutational analysis (999 permutations).

dbRDA Model	Manning		Williams	
Constraining variable	**$F_{1,81}$**	**p-value**	**$F_{1,84}$**	**p-value**
Temperature	17.8	0.001	5.80	0.001
Salinity	9.1	0.001	11.25	0.001
Flow	7.3 (Q30)	0.001	5.4 (Q60)	0.001
DO	3.6	0.012	3.1	0.004
Chl *a*			1.9	0.04
FRP			2.7	0.006
DOC			2.4	0.031
Turb			2.2	0.036

2.5 Discussion

This study examined the impact of freshwater inflows on the environmental conditions and mesozooplankton community of a regulated estuary (Williams) and a nearby unregulated estuary (Manning). The Williams estuary had higher average concentrations of DOC and inorganic nutrients than the Manning regardless of flow conditions; higher concentrations of Chl *a* than the Manning throughout the study (Figure 2.3 and 2.4, Appendix 3); homogenous salinity across the freshwater-estuary interface, compared to a salinity gradient in the Manning (Figure 2.3); and decreased DO following "Fresh" and "Flood" conditions (Figure 2.4, Appendix 2). All of these have been previously associated with negative impacts of river regulation and reduced inflow to an estuary (Drinkwater and Frank 1994; Pierson et al. 2002). I also found contrasting zooplankton community structures between the two estuaries with different environmental factors shaping these communities and while nauplii dominated the zooplankton assemblage in each estuary, their concentrations were also driven by contrasting factors between the two estuaries. In the Manning, temperature, salinity, DO and Q30 were the dominant factors influencing the zooplankton communities in the Manning River estuary (Figure 2.7a, Table 2.3) with high seasonal concentrations of *Oithona* spp. present during the

austral summers when inflows were low (Figure 2.6a, c and e). In contrast, the zooplankton community composition of the Williams was dominated by copepod nauplii throughout the study (Table 2.1, Figure 2.6b, d and f), which responded positively to discharge (Q60) and inputs of DOC (Figure 2.7b, Table 2.3), with a lower abundance adults (mostly *S. conflictus*) present throughout the study (Figure 2.6b, d and f).

2.5.1 Contrasting zooplankton communities and drivers

Temperature is a major driver of copepod growth (Huntley and Lopez 1992; Horne et al. 2016) so the pattern of increased zooplankton biomass, most notably nauplii, copepodites and *Oithona* sp., during warm periods and their positive relationship to temperature in the Manning (Figure 2.6a, c, e and 7a) aligns with patterns commonly reported for estuarine and marine copepods (Mallin 1991; Tan et al. 2004). Indeed, although seasonal growth of estuarine copepod populations can be linked to seasonal increases of phytoplankton and Chl *a*, temperature is often a stronger limiting factor (Leandro et al. 2007). Likewise, negative relationships between discharge and most zooplankton in the Manning (Figure 2.7a), are expected patterns for estuarine zooplankton, particularly copepods, as a short-term response to inflows (Pace et al. 1992; Abramova and Tuschling 2005; Hitchcock et al. 2016a).

In contrast to the Manning, nauplii and copepodites in the Williams shared positive relationships to Q60 antecedent discharge (Table 2.1, Figure 2.5) and to turbidity and DOC concentrations (Table 2.1, Figure 2.7b). Nauplii shared no relationships with shorter term antecedent flow measurements in the Williams (in contrast to the freshwater cladocerans), which suggests they were not advected to the estuary from upstream areas as has been seen previously with other copepods (Kimmerer et al. 2018). Nauplii concentrations may have increased in the Williams due to the increased fecundity of adults following resource inputs to the estuary (Uriarte et al. 2005; Zhang et al. 2015) or through newly hatched individuals from estuarine sediment egg-banks. This may have led to a delayed or "lagging" relationship between inflows and their stimulation of nauplii hatching and subsequently increased concentrations, resulting in a stronger relationship with the Q60 metric compared to the shorter time frame Q5, Q10 and Q30 metrics. Hatching events can constitute a large pool of potential recruits to planktonic populations in estuaries (Marcus 1991) and are generally triggered by seasonal temperature increases (Gyllström and Hansson 2004). The main copepod species present in the study (*Sulcanus conflictus, Gladioferens pectinatus and Oithona* spp.) all have eggs that are known to respond to temperature cues and generally hatch following seasonal increases in temperature (Sabatini and Kiørboe 1994; Newton and

Mitchell 1999; Hemraj et al. 2017a), as seen in the Manning (Figure 2.6 a, c and e, 2.7a). However, nauplii in the Williams did not display a positive relationship with temperature, but instead with Q60 flow (Figure 2.7b). This may indicate that factors related to inflows such as DO levels and organic enrichment might be important as triggers for hatching periods in the Williams (Broman et al. 2015; Uriarte et al. 2005).

Reduced water column DO, seen to occur after inflows to the Williams (Figure 4f), and anoxic conditions at depth and in sediments due to stratification, which can also occur at low flow periods in the upper Williams estuary (*unpublished data*), may prevent seasonal hatching of copepod nauplii, arresting the metabolic respiration of egg cysts in sediment (Clegg 1997; Jørgensen et al. 2019). In the Williams it may be that inflows stimulate the hatching of nauplii by oxygenating sediments and bottom waters (Broman et al. 2015), resulting in increased nauplii concentrations after inflows (Figure 2.7), before they re-stratify in the following weeks (*unpublished data*). Any subsequent nauplii populations may have been supported by resource inputs during inflows such as DOC, to which nauplii and copepodites were related in the Williams. Terrestrial inputs by inflows have been shown to provide detrital and microbial food web resources for juvenile copepods which may become an important food web resource following inflows (Roff et al. 1995; Vogt et al. 2013; Hitchcock et al. 2016b).

Despite higher concentrations of nauplii in the Williams compared to the Manning after inflows and throughout the study, this did not translate into increased adult copepod concentrations in warmer periods (Table 2.1, Figure 2.6) as it did in the Manning. Adult copepod populations were on average relatively low (< 50 ind/L) throughout the study in the Williams even during warmer periods, except for the predatory *S. conflictus* (Table 2.1), which favours nauplii as its prey (Ough and Bayly 1989). For this species, the increased nauplii availability in the Williams may be a reason for its increased concentration, potentially reducing herbivorous adult copepod concentrations by consuming their nauplii. The consistently lower concentrations of larger bodied adult copepods from the Williams estuary may have further reaching ecological effects in the system, impacting on the control of phytoplankton (Tan et al. 2004) and the recruitment of juvenile fish populations (Castonguay et al. 2008; Adams et al. 2015; van Deurs et al. 2015).

2.5.2 Differences in environmental conditions

Contrasting environmental conditions between the two estuaries, some symptomatic of river

regulation, may have contributed to the lower densities of adult copepods in the Williams. Compared to the Manning, the Williams estuary had increased DOC, nutrient and Chl *a* concentrations, regardless of flow conditions, and increased turbidity and reduced DO following inflows (Figure 2.4). Increased organic carbon loading to estuaries can reduce oxygen saturation in the water column (Wang et al. 2018), negatively influencing copepod nauplii survival and development (Stalder and Marcus 1997; Uriarte et al. 2005). As DOC concentrations were increased by inflows in the upper Williams, and DO reduced (Figure 2.4, Appendix 2), this may have hindered the development and recruitment of juvenile copepods into adults following inflows (Uriarte et al. 2005). While DO in the Williams following inflows did not decrease at the surface to levels that have been observed to induce mortality in adults in previous studies (< 2.0 mg/L, Stalder and Marcus 1997) it was decreased at times to ranges that have previously impacted development and survival of juvenile copepods (~6.0 mg/L, Uriarte et al. 2005). The negative effects of decreased DO on copepods have also been recorded in eutrophic systems where nutrient loading is high and algal blooms occur (Lawrence et al. 2004; Marcus 2004; Plew et al. 2020), although eutrophication can hinder copepod growth through other mechanisms.

High concentrations of nutrients and Chl *a* (Figure 2.4c, d and e), as well as the presence of cyanobacterial blooms in the Williams study sites (*unpublished* data) could be considered evidence of eutrophication and local ecosystem degradation (Cloern et al. 1983; Paerl 1988). Eutrophication and cyanobacterial blooms can result in poorer food quality for copepods (Lehman et al. 2013; Schmidt and Jónasdóttir 1997 *and references therein*) and impede their growth through sublethal effects (Lehman et al. 2013; Marcus 2004; Perbiche-Neves et al. 2016), which can lead to shifts in community structure (Uye 1994; Chew and Chong 2016). Estuarine *Oithona* spp. are known to be regulated strongly by food availability and quality in summer warmer periods (Sabatini and Kiørboe 1994; Santhanam and Perumal 2012). Their absence from the Williams in summer periods may be due to inadequate food resources caused by eutrophic conditions and longer water residence times due to the impacts of river regulation. Previous studies have highlighted the importance of freshwater inflows in establishing phytoplankton community diversity, and the importance of this to zooplankton populations (Buyukates and Roelke 2005; Miller et al. 2008).

While the measurement of top-down control was outside the scope of this study, planktivores such as juvenile fish may have contributed to the reduced adult copepod concentrations in the Williams. Previous work in the Murray River estuary found that low adult numbers despite

higher concentrations of nauplii and copepodites suggested top down (predation) pressures during lower flow periods (Furst et al. 2019; Geddes et al. 2016). Top-down control has been shown to structure estuarine copepod communities and give stability to estuarine food webs (Maes et al. 2005; Wasserman et al. 2013), although excess predation by fish may remove copepod species (Kimmerer and McKinnon 1989). However, the influence of predatory effects in aquatic ecosystems usually requires an existing bottom-up influence on zooplankton populations (Fox et al. 2012; Gruner et al. 2008; Wagner et al. 2004) and it is likely that future management of the Williams estuary should focus on these issues.

2.5.3 Impacts of river regulation on environmental conditions

Reductions in discharge to the Williams estuary through river regulation may have contributed to the contrasting water quality conditions between the two estuaries, and therefore the different zooplankton communities. Seaham Weir substantially reduces inflows from the freshwater portion of the catchment to the Williams estuary, diverting approximately 189,000 ML/year of freshwater to Grahamstown Dam via Balickera Canal (NOW 2009a). Moreover, in low flow periods it reduces the connectivity between the river and the estuary. For example, the Seaham Weir gates were opened for a cumulative 1 hour and 8 minutes between late December 2017 and March 1, 2018 during a low rainfall/discharge period, eliminating any riverine connectivity for nearly 3 months (*unpublished data*). By reducing inflows, the export of water, particulates, and nutrients to the ocean is lessened and can result in increased residence time of nutrients and organic carbon in the upper reaches of estuaries (Connell et al. 1981; Drinkwater and Frank 1994; Plew et al. 2020). The higher concentrations of DOC and nutrients in the Williams, compared to the Manning, may indicate increased residence time of freshwater and reduced flushing of the estuary with export of materials to downstream areas reduced (Connell et al. 1981; Pierson et al. 2002). Reduced inflows and turbulence due to river regulation, combined with the high nutrient concentrations in the Williams, may have contributed to the higher concentrations of Chl *a* in the upper Williams compared to the Manning by creating low flow conditions preferable to algal productivity (Cerco and Cole 1993; Kimmerer 2002; Mitrovic et al. 2006).

River regulation may also account for the decrease in DO following inflows in the Williams (Figure 2.4, Appendix 2). Organic carbon loading can reduce DO in estuaries due to the oxygen demands of microbial processing (Ford et al. 2005; Wang et al. 2018). Increased concentrations of DOC and reduced water column DO downstream of weirs and barrages

following inflows to estuaries have previously been linked to river regulation (Connell et al. 1981; Hemraj et al. 2017b). The increased concentration and retention of DOC in the upper estuary, due to river regulation and reduced fluvial export, can lead to increased microbial processing in the upper estuary, further reducing DO following inflows (Connell et al. 1981). Issues of reduced DO can also be exacerbated under eutrophic conditions, when lysed cells following phytoplankton blooms can be respired by bacterial communities creating hypoxic conditions (Howarth et al. 2011; Wang et al. 2017). Reduced connectivity and movement of water during low flow periods, and following inflows, can also contribute to reductions in estuarine salinity gradients (Figure 2.3), a further symptom of freshwater inflow reduction (Drinkwater and Frank 1994; Pierson et al. 2002). While this study did not focus on the stratification of the water column in the upper Williams estuary, extended periods of oxygen, temperature and salinity stratification can occur in the Williams during low flow periods and most prevalent in summer *(unpublished data*). Stratification is a known indicator of river regulation that negatively impacts estuarine ecology, and in combination with reduced salinity gradients, can reduce the area of available habitats for estuarine species which prefer less saline water. This potentially impacts the community structure of zooplankton and other estuarine species (Drinkwater and Frank 1994; Gillanders and Kingsford 2002; Pierson et al. 2002).

2.5.4 Management Recommendations

Increased concentrations of DOC, turbidity, nutrients and Chl *a,* along with a reduced DO saturation following inflows and a reduced salinity gradient in the upper Williams compared to the Manning, may all be symptoms of river regulation (Drinkwater and Frank 1994; Pierson et al. 2002) and may have contributed to the contrasting zooplankton communities between each estuary. If reductions in inflows can negatively influence adult copepod concentration by creating more eutrophic conditions or reducing oxygen saturation in estuaries there may be long term ecological effects to higher trophic species like juvenile fish (Adams et al. 2015; van Deurs et al. 2015; Hamilton et al. 2020). While management protocols are in place on the Williams River estuary through NSW government WSPs (NOW 2009a) it is evident that these are currently not alleviating all issues of river regulation, particularly in regard to the residence time of water and flushing in the estuary during low flow periods (Pierson et al. 2002). Management decisions for the Williams should focus on reducing the water residence time of the upper Williams estuary during low flow periods, possibly by ensuring daily releases similar to natural flows and maintenance of hydrological

connectivity to the riverine sections of the system. This may help reduce the residence time of nutrients, phytoplankton, and particulates, and the occurrence of water column stratification in this area, potentially alleviating some of the ecological issues that are presenting themselves in the zooplankton community (Gillanders and Kingsford 2002; Pierson et al. 2002). Previous studies have demonstrated how increased freshwater inflow can positively impact zooplankton concentrations and diversity by regulating phytoplankton diversity, ensuring better quality algal prey (Buyukates and Roelke 2005; Hoover et al. 2006; Miller et al. 2008) or provisioning allochthonous resources (Hitchcock et al. 2016a; Hoffman et al. 2008; Schlacher et al. 2008).

2.5.5 Conclusion

Higher adult copepod concentrations were found in the unregulated Manning estuary, with the zooplankton community increasing in concentration during warmer seasons, correlating positively to temperature and negatively to discharge. In the Williams, this seasonal pattern was not present with nauplii dominating the copepod community throughout the study, and juvenile copepod groups correlating positively to discharge and to associated DOC inputs, rather than temperature. Compared to the unregulated Manning, the regulated Williams had higher concentrations of nutrients and Chl *a*, representative of more eutrophic conditions and reduced DO after inflows, which may have been due to increased residence time of DOC in the upper Williams. These conditions, symptomatic of river regulation, may have contributed to the different zooplankton communities in each estuary and suppressed the development of nauplii into adult copepods in the regulated estuary.

Chapter 3: Fine scale food web responses to a freshwater inflow event in a regulated Australian estuary

3.1 Abstract:

Changes in water quality and subsequent ecosystem responses to freshwater inflow events can occur rapidly in estuaries. Despite this, most flow-response studies are typically sampled at weekly to monthly intervals. This leaves a knowledge gap around the rapid changes that occur in ecological communities in response to flow events. To address this, I conducted a flow-response study that monitored for changes to dissolved organic carbon, nutrients and bacterial cell concentrations every 4 hours for the first four days of a flow event followed by additional monitoring of algae and zooplankton responses every 3-5 days for six weeks. This was performed in the Williams River estuary, NSW, Australia, upstream and downstream of a weir which controls flow between freshwater and mesohaline zones. I observed rapid increases in dissolved organic carbon and phosphorous concentrations during the inflow which were tightly coupled with increases in bacterial concentrations. Following the inflow event, while the weir remained open, increased algal concentrations occurred, dominated by euglenoids. In the same period juvenile copepod populations, which had initially been reduced by the inflow, began to increase in concentration, as did freshwater cladocerans probably advected to the estuary. However, after the closure of the weir gates the mesozooplankton community was substantially reduced in concentration relative to the preceding community while the gates were open and the pre-flow community. Fine-scale monitoring suggested that inflows are key in stimulating basal resources for planktonic food webs and that rapid changes to flow conditions can quickly shift the composition of mesozooplankton communities. By understanding these rapid changes to planktonic food webs in a regulated system water resource managers may be able to develop flow protocols to better support estuarine food web productivity.

3.2 Introduction

Freshwater inflows are critical to the health of many estuarine ecosystems, regulating physical and ecological processes (Alber 2002; Pierson et al. 2002). Rainfall induced flow events can deliver the majority of the annual carbon, nitrogen and phosphorous budget to estuaries over short time scales (McKee et al. 2000; Wilson 2008). This delivery of resource subsidies from the surrounding catchment can stimulate estuarine food web productivity, with evidence from

microbial (Hitchcock and Mitrovic 2015a; Meunier et al. 2017) to ichthyofaunal communities (Robins et al. 2005; Hoffman et al. 2007; Stoessel et al. 2018). However, inflows and the delivery of resource subsidies can be disrupted by river regulation, which can have implications for food web productivity in estuaries (Gillanders and Kingsford 2002; Sabo et al. 2018; Ru et al. 2020). Understanding the response of food webs to freshwater inflows in regulated estuaries is therefore critical in addressing and managing the impacts of river regulation (Adams 2014).

Resources transported to estuaries by inflows can stimulate basal productivity through two distinct energy pathways. The first is through the delivery of inorganic nutrients by inflows, potentially stimulating phytoplankton productivity (Mallin et al. 1993; Burford et al. 2012) which may be more apparent in warmer seasons (Murrell et al. 2007; Paczkowska et al. 2019). The second is the delivery of organic matter (OM), in particulate (POM) or dissolved (DOM) forms, which can stimulate microbial and heterotrophic protist communities in estuaries (Wetzel 1995; Hitchcock and Mitrovic 2015b) and even some zooplankton communities (Carrasco and Perissinotto 2011; Carrasco et al. 2013). After high flow periods both energy pathways can provide food resources to higher trophic levels, such as zooplankton and juvenile fish (Grange and Allanson 1995; Hoffman et al. 2007; Hitchcock et al. 2016b).

Mesozooplankton are an important trophic link in estuarine systems, linking primary producers and fish (Winder and Jassby 2011; Bice et al. 2016; Paul et al. 2016). Zooplankton communities can be strongly influenced by inflow pulses, with populations of some species decreased via advective forcing (Pace et al. 1992; Lawrence et al. 2004) while others increase through advection to the estuary from upstream areas (Kimmerer et al. 2018), egg hatching events (Nielsen et al. 2012; Broman et al. 2015), or populations capitalising on increased resource availability following inflows (Buyukates and Roelke 2005; Hitchcock et al. 2016b). While mesozooplankton community responses to flows have been assessed in many settings (Wooldridge and Callahan 2000; Lara-Lopez and Neira 2008; Geddes et al. 2016; Hitchcock et al. 2016a), the collective response of estuarine mesozooplankton and their supporting food web (e.g., bacteria, phytoplankton and basal nutrient resources) to inflows are less often evaluated and hence poorly understood.

Freshwater inflow event studies generally utilise low sampling frequencies (weekly, fortnightly or monthly resolutions (e.g., Connolly et al. 2009; Cook et al. 2010; Hitchcock and Mitrovic 2015a; Kimmerer et al. 2018; Varkitzi et al. 2018; Possamai et al. 2020). These may miss fine scale temporal responses to freshwater pulses (Wilson 2008). The time period of many flow events is short, occurring on a scale of hours to days, with terrestrial carbon and nutrient

concentrations sometimes tightly coupled to discharge events (McKee et al. 2000; Wilson 2008). Nutrient uptake by phytoplankton or macroalgae can also reduce concentrations of nutrients within hours to days (Ferguson et al. 2004; Moore et al. 2006) and microbial and algal populations can also fluctuate within hours to days in response to resource additions (Hitchcock et al. 2010). Although some studies have employed higher resolution sampling regimes to monitor both basal resource availability and zooplankton community responses to flow events in inland rivers and coastal bays (Hoover et al. 2006; Westhorpe and Mitrovic 2012), relatively few have been conducted in upper estuarine areas (e.g. Wilson, 2008).

This study investigates fine scale temporal changes in organic carbon, nutrient, microbial, phytoplankton and mesozooplankton concentrations over a two-month period during and after a freshwater inflow pulse in an estuary in south-eastern Australia. It is one of few studies to investigate resource delivery to an estuary during an inflow pulse using a sub-weekly (e.g., 2 to 3 sampling occasions per week) temporal sampling resolution (e.g. Hoover et al., 2006; Wilson, 2008). Furthermore, it integrates upstream monitoring to outline the timing and delivery of DOC and nutrients from freshwater reaches, through a weir, to an estuary. The inclusion of bacterial, phytoplankton and mesozooplankton community responses to the inflow provides one of the first temporally resolved estuarine food web response studies for a mesozooplankton community in Australia. I hypothesised that terrestrial DOC and nutrients would be mobilised by inflows, increasing their concentrations in the estuary, leading to short term increases in microbial and algal concentrations in the estuary in direct response to resource inputs. I believed if microbial and algal resources were increased following the inflow, their increased availability would translate to increased concentrations of mesozooplankton in the estuary post-flow (following the inflow), relative to the pre-flow concentrations (sampled in a trip prior to the inflow).

3.3 Methods

The Williams River is a regulated river in south-eastern NSW near the city of Newcastle. It is a tributary to the larger Hunter River with a confluence at Raymond Terrace (Figure 3.1). The Williams River is regulated by Seaham Weir (Figure 3.1) imposing a tidal limit on brackish water, allowing the extraction of freshwater to nearby Grahamstown Dam for drinking water purposes by Hunter Water (Hunter Water, 2011). The resultant weir pool has an approximate volume of 10,000 ML. Seaham Weir itself is comprised of a rock weir and two control gates, which can be actively operated during high flow periods as measured at the upstream gauge

of Glen Martin (WaterNSW gauge 210010, NOW 2009). There are significant correlations between the discharge at the Glen Martin gauge and the Weir gates ($R^2 = 0.97$) for the period between 2010 and 2017. These relationships allowed us to conservatively estimate bulk volumes of discharge through the weir during the study using the discharge at Glen Martin (210010) and gate opening and closing times. The conservatively estimated discharge hydrograph arising from this method was used in the analysis of all sites and variables sampled below Seaham Weir.

Two freshwater and two estuarine sites were sampled from October to December 2018, in response to a freshwater inflow event on the Williams River. The most upstream freshwater site, Site 1, is approximately 30 km upstream from Seaham Weir and is characterised by continually flowing clear shallow water (0.5-1.5 m deep) across a rocky substrate during low flow conditions. The site lies in a constrained channel with good riparian vegetation cover and macrophyte growth present. The second freshwater site, Site 2, is 500 m upstream from Seaham Weir, situated in the weir pool. This site is up to 8 m deep and generally experiences low flows, stratifying during summer periods with low rainfall. In the estuary the most upstream site, Site 3, is 500 m downstream of the weir wall and is characterised by deep pockets of water (7-9 m) which also experience thermal and oxygen stratification in the summer due to low discharge through the site. There is some mangrove vegetation present (left bank) as well as urban development (right bank). The final site, Site 4, is 3.5km downstream of the weir. While not as deep (4-6 m) it too experiences stratification during low flows, mostly during summer. This site has a wooden reinforced left bank and open cattle pasture with eroding banks on the right.

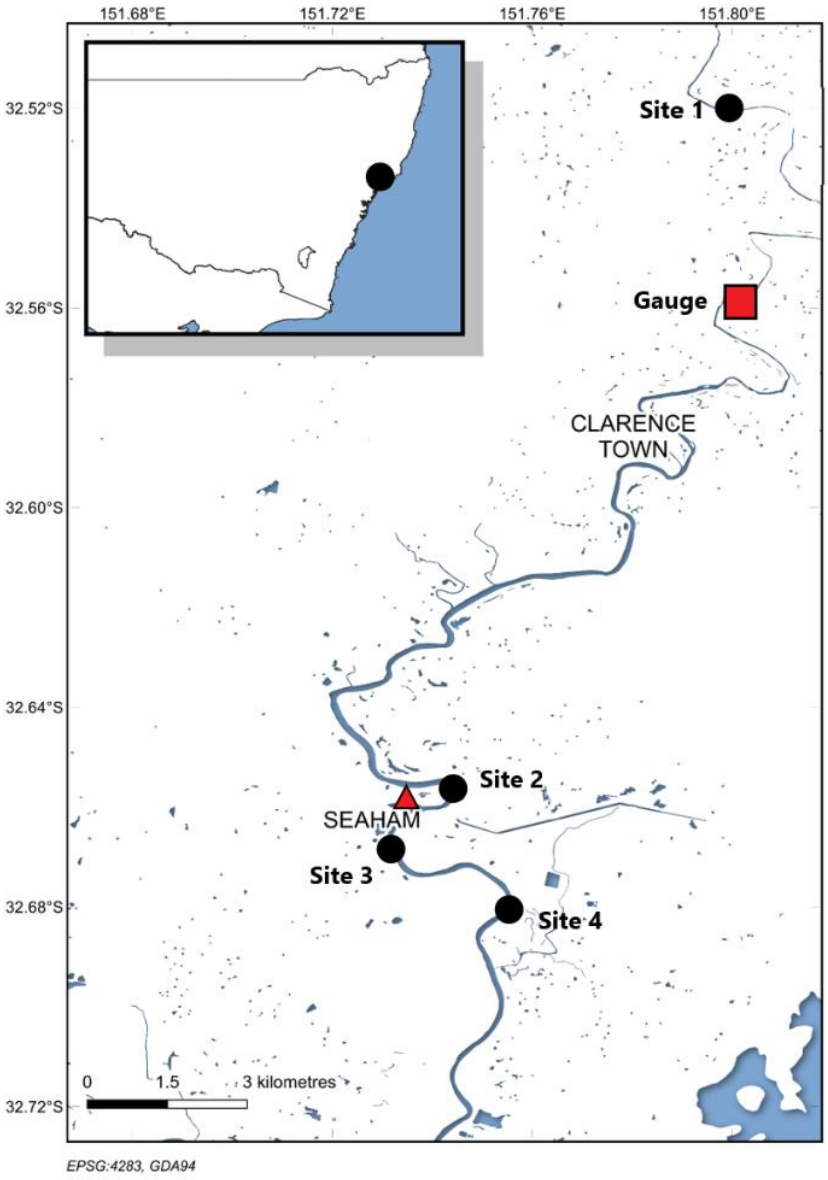

Figure 3.1: Site, weir and discharge gauge positions for the Williams River. Study sites are denoted by black circles with Site 1 being the most upstream site, north of Clarence Town and Site 4 being the most downstream site, south of Seaham. Seaham Weir is denoted by the red triangle upstream of Seaham. WaterNSW gauge 210010 (Williams at Glen Martin) is indicated by the red square upstream of Clarence Town.

The monitoring period was designed to capture an inflow event in high resolution, including the delivery of resources to the system and the response of the planktonic food web up to the mesozooplankton community. Intensive sampling of water quality components such as carbon, nitrogen and phosphorous concentrations as well as bacterial cell counts were conducted across an inflow peak three times a day from October 6 to 10, 2018, at Site 1, 2 and 4. No sampling occurred during this period at Site 3 as it was inaccessible due to river height. Estuarine algae and zooplankton were not sampled during the intensive sampling period as I wanted to assess their response to resource delivery by inflows rather than the

direct physical impact of flow. Fortunately, the estuarine sites (site 3 and 4) were also sampled on the September 26, a fortnight prior to the October inflow, providing pre-flow background values for the study.

A second inflow peak occurred on October 11, 2018. Water quality sampling continued at all sites, including Site 3, after this inflow with samples collected sub-weekly from October 12 through to November 14, 2018, with a final sample taken on December 12, 2018, giving 10 post-flow sampling occasions spanning a period of two months. These sampling occasions included algal and zooplankton samples at Site 3 and 4, to explore their responses and recovery following the flow events that occurred in October.

3.3.1 Chemical and Chl *a* measurements

Filtered surface water samples were taken in duplicate at each site, using 0.45 µm filters, stored in polyethylene bottles and frozen as soon as possible in portable fridge/freezers. Duplicate samples were used due to the high financial costs of external analysis. Samples were analysed for inorganic nitrogen (nitrates and nitrites), filtered reactive phosphorus and DOC. Nutrient samples were analysed using flow injection analysis with a Lachat Quikchem 8500 Series II using standard methods (APHA et al. 2005). DOC samples were analysed using the High Temperature Combustion Method (Sugimura and Suzuki 1988).

Chl *a* concentration was identified by filtration of 500 mL of surface water through a 0.7 µm pore-sized glass fibre filter using a Mitivac hand pump, with filters wrapped in foil, frozen and then stored for analysis. Chl *a* samples were taken in duplicate. Chl *a* concentration was determined by using the grinding technique and an acetone solute with corrections for phaeophytin (APHA 2005). Physicochemical measurements (temperature, dissolved oxygen, salinity and pH) were taken using a Hydrolab field hand-meter Surveyor and MS5 minisonde probe at the surface. Turbidity was measured using a Hach 2100P turbidimeter.

3.3.2 Bacteria, algal and zooplankton enumeration

Bacteria were collected in triplicate after pre-filtering 1 mL samples through a fine mesh (approx. 150 µm) net into vials pre-loaded with glutaraldehyde for preservation. Samples where snap frozen in liquid nitrogen and then kept at -80 ºC. To process, bacteria samples were thawed in hot water for 1-2 minutes, with 100 µl of sample then pipetted into 1.5 mL Eppendorf tubes for analysis. A CytoFLEX LX flow cytometer was used to enumerate the microbial community. Cells were differentiated by preparing a protocol on the CytExpert application, specific channels were adjusted depending on the type of cells visualised.

Phytoplankton and virus cells were differentiated according to forward scatter (FSC), side scatter (SSC) and red fluorescence (chlorophyll) and bacterial cells were specifically discriminated using side scatter (SSC) and green fluorescence (SYBR-Green). All samples were run at flow rates of 25 µl/min for 1 minute. Bacterial community visualisation required SYBR-Green staining prior to flow cytometry analysis, therefore, an additional 100 µl of sample was taken from the initial 1.6 mL cryovials and stained with 5 µL of SYBR-Green prepared at a 1:500 ratio these incubated at 4 ^{o}C for 20 minutes then run on the flow cytometry machine.

Phytoplankton surface water samples (250 mL) taken and were preserved with Lugol's iodine for identification and enumeration of phytoplankton. Sample identification and enumeration was conducted at 200x magnification using light microscopy (Olympus BX41) and a Sedgwick-Rafter counting chamber. Where necessary, samples were concentrated 5× prior to counting, settling in 50 mL measuring cylinders for 24 h. The upper 40 mL was removed after checking all phytoplankton had settled and were no longer present in the upper layer. Phytoplankton taxa were identified to genus using identification literature by Prescott and Prescott (1978). Counting precision was performed to ± 10 % with at least 100 units of the dominant taxa counted following Hötzel and Croome (1999). Biovolumes were calculated using the most appropriate conversion factors from Newcombe (2012) and Olenina (2006).

Zooplankton were sampled using a 12 L Schindler trap and integrated depth samples from 0, 1 and 2 metres, giving a total sample volume of 36 L. Samples were taken in triplicate and preserved immediately with >80% ethanol and later identified and enumerated by light microscopy using an Olympus SZX12. Copepod juveniles were considered nauplii (stages NII to NVI) and copepodites (stages CI to CV), with all other taxa identified to species.

3.3.3 Statistical analysis

Statistical analysis was undertaken in R 4.1.1 (R Development Core Team 2020). Correlation matrices were constructed and analysed using Pearson's R for all variables in the study (presented as R^2) through the "Hmisc" package (Harrel 2015) in R (v4.1.1, R Core Team 2016). The adjusted hydrograph based on the weir closing and opening times was used for sites downstream of the weir in comparison to the an-altered hydrograph for sites upstream. Two subsections of these hydrographs were also used to find if there were relationships between variables and discharge in certain periods, between the first and second flow (October 6 to 10) and after the second inflow (12th of October onwards). The specific period

used for correlation analysis is presented in the text below. All figures were constructed using "ggplot2" (Wickham 2016). Distance-based redundancy analysis (dbRDA) was used to determine the community distribution of the mesozooplankton community relative to environmental variables throughout the study. This analysis was conducted in R (4.1.1) using the "vegan" package (Oksanen et al. 2013) and the "capscale" function. Distance matrixes were constructed using transformed mesozooplankton community data (log(x+1)) and Bray-Curtis dissimilarity. Significant water quality and environmental vectors were fitted to the ordination models using the "envfit" function with multi-step analysis determining the significant drivers of mesozooplankton distribution through "ordistep". Algal biovolumes were included as constraining variables as zooplankton may have consumed them as a food source. Non-significant co-variables were removed ($p > 0.05$) and the variable inflation factor of the remaining variables was less than 3. The significance of the dbRDA model was tested using the "anova.cca" function from vegan which performs Monte-Carlo permutation testing (999 permutations). Multivariate abundance analysis was conducted to identify differences in community structure across the flow using generalised linear models (GLM) through the "mvabund" package (Wang et al. 2012). Transformed community data (log(x+1)) was inputted to a GLM with a negative binomial distribution ensuring the correct fit of residuals, which were inspected visually (Wang et al., 2012). The significance of the model was tested using the "anova.manyglm()" function from "mvabund" with pairwise comparisons indicating difference between flow periods identified in the dbRDA model, and univariate GLM analysis indicating which species had significantly different concentrations between flow periods.

3.4 Results

3.4.1 Discharge event and weir operation

An inflow event occurred on the Williams River between October 6 and 15, 2018. The flow event had two distinct peaks (Figure 3.2), one of 7,829 ML/Day (October 7) and the second of 7,197 ML/Day (October 11). The weir gates were closed until the start of this inflow event and were then intermittently opened and closed throughout the flow period allowing freshwater to enter the estuary (Figure 3.2). Our conservative estimates of discharge volume through the weir gates indicate that the two peaks in discharge released approximately 12,000 and 5,500 ML respectively. The volume of water during the first peak was large enough to have completely replaced the weir pool volume (~10,000 ML). All releases from the weir after the second inflow peak were less than ~300 ML, decreasing as inflows through the

upstream gauge fell (Figure 3.2). Salinity decreased from ~8 ppt at both estuarine sites prior to the flow and remained below < 1 ppt throughout the study inclusive of the penultimate sampling trip in mid-November (Figure 3.2a). Salinity in mid-December, the final sampling point, was 5.6 and 6.4 ppt at Sites 3 and 4, respectively (Figure 3.2a). Temperature rose in the estuary across the study period concurrent with season patterns, from ~18 ºC at both sites prior to the inflow to 25 ºC at both sites by the end of the study (Figure 3.2b).

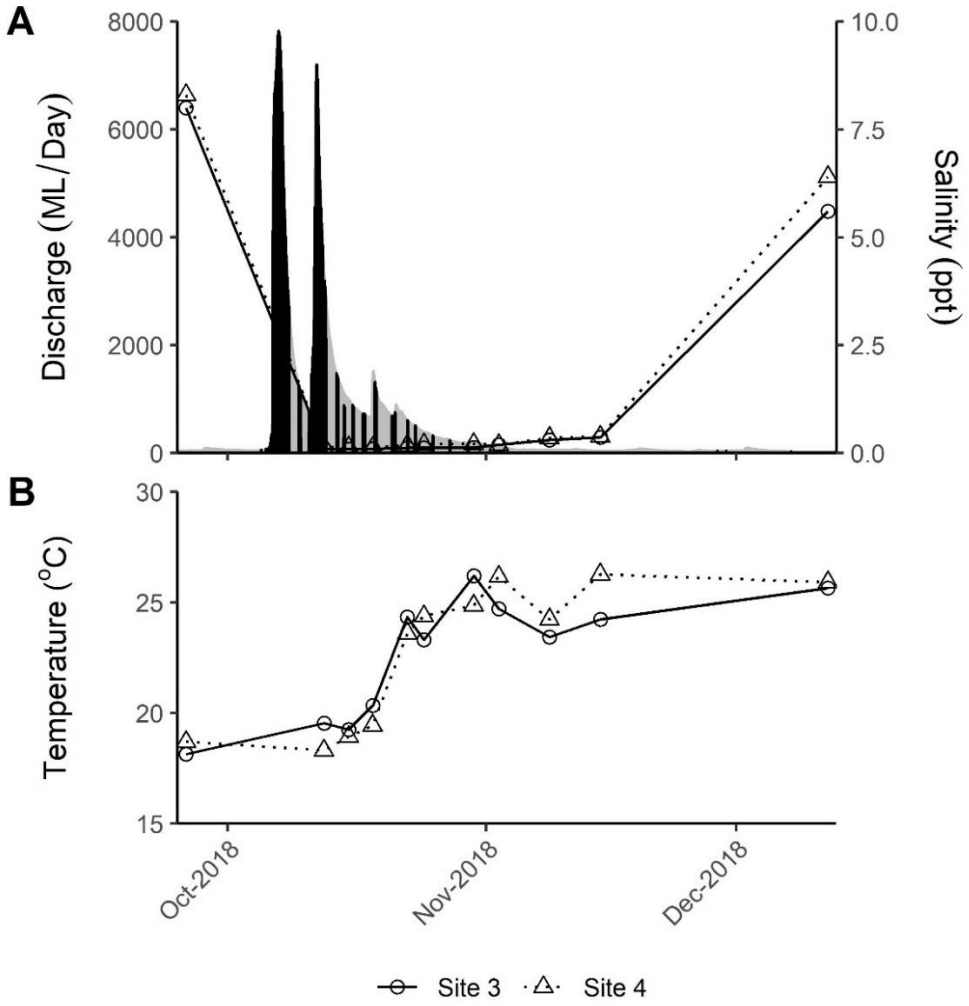

Figure 3.2: Discharge (ML/Day) for the Williams River and the salinity (ppt) and temperature (ºC) of the two sites in the Williams River estuary between September 26 and December 12, 2018. Discharge was taken from NSW Water gauge 210010 - Glen Martin (grey shading). The sections of the hydrograph shaded black are the periods when the Seaham Weir gates were open (black) allowing flow downstream to sites 3 and 4.

3.4.2 Organic carbon and nutrient delivery

Intensive water sampling of the first flow peak at Sites 1, 2, and 4 showed the fine scale fluctuations of DOC and inorganic nutrients (Figure 3.3a, c and e). No prior DOC concentrations were available for Site 1, but DOC concentrations peaked at 21.5 (± 0.9) mg/L

on the rising limb of the flow on the afternoon of October 6, decreasing with the falling limb to 4.8 (± 0.2) mg/L on October 10 (Figure 3.3a). A day later, on October 7, DOC concentrations peaked at Site 2 in the morning, increased from pre-flow event concentrations of 2.5 (± 0.4) mg/L to 19.8 (± 1.1) mg/L before decreasing to 10.1 (± 0.1) mg/L on October 10 (Figure 3.3a). Similarly, on the evening of October 7, DOC concentrations peaked in the estuary at Site 4 increased from pre-flow event concentrations of 3.5 (± 0.01) mg/L to 19.6 (± 0.4) mg/L before decreasing to 9.0 (± 0.1) mg/L on October 10 (Figure 3.3a). DOC concentrations were correlated with discharge between October 6 and 10 (Site 1, $R^2 = 0.63$, $p < 0.01$; Site 2, $R^2 = 0.54$, $p < 0.01$; Site 4, $R^2 = 0.68$, $p < 0.01$). Overall, DOC concentrations in the estuary increased in response to flow, from pre-flow levels of 2.5 (± 0.4) and 3.5 (± 0.01) mg/L, at Site 3 and 4 respectively, to 9.8 (± 1.4) and 11.1 (± 0.4) mg/L on October 12 before falling to 6.8 (± 1.2) and 8.5 (± 0.1) mg/L on October 18 (Figure 3.3b). Concentrations then remained between 7 and 9 mg/L at both sites for the remainder of the study, except for on November 2, when DOC at Site 1 fell to 5.5 (± 0.2) mg/L. Concentrations of DOC were generally higher at the more downstream Site 4 site, compared to Site 3 (Figure 3.3b).

NO_x concentrations increased at Site 1 during the first inflow peak from 0.14 (± 0.01) mg/L at midday on October 6, peaking at 0.29 (± 0.03) mg/L at midday on October 9, 2018, before declining to 0.07 (± 0.011) mg/L on October 12 after the second inflow (Figure 3.3c). At Site 2, NO_x declined from pre-flow concentrations of 0.28 (± 0.01) mg/L on September 26, to 0.08 (± 0.01) mg/L on October 8, before rising again to 0.17 (± 0.001) mg/L following the second inflow on October 12 (Figure 3.3c). NO_x also declined at Site 4 during the first flow peak from 0.16 (± 0.01) mg/L pre-flow to 0.07 (± 0.01) mg/L on the morning of October 8 before increasing to 0.17 (± 0.01) mg/L on October 12 following the second inflow, similar to pre-flow levels at this site (Figure 3.3c). Across the flow event, NO_x concentrations increased at Site 3 from 0.12 (± 0.02) to 0.16 (± 0.04) mg/L on October 12 (Figure 3.3d). NO_x was variable at both estuarine sites following the flow event, increasing to 0.33 (± 0.01) mg/L at Site 1 on November 8, and 0.25 (± 0.03) mg/L at Site 2 on the October 30, then decreasing to 0.02 (± 0.003) and 0.04 (± 0.001) mg/L on November 14 before falling to < 0.01 mg/L at both sites in December (Figure 3.3d).

Filtered reactive phosphorous (FRP) concentrations followed similar patterns to DOC at Site 1 during the first flow peak, peaking at 0.08 (± 0.001) mg/L on the afternoon of October 6, before decreasing to 0.03 (± 0.001) mg/L on October 8 (Figure 3.3e). FRP concentrations

peaked a day later at Site 2 increasing from 0.03 (± 0.001) mg/L pre-flow to 0.12 (± 0.001) mg/L on October 7, before decreasing to 0.07 (± 0.001) mg/L on October 12 (Figure 3.3e). FRP concentrations at Site 4 quickly increased from 0.01 (± 0.001) mg/L pre-flow to 0.10 (± 0.001) mg/L at midday on October 6 on the rising limb of the first peak (Figure 3.3e). This then decreased to 0.06 (± 0.01) mg/L on the morning of October 7, increased to 0.14 (± 0.005) mg/L on the afternoon of October 9 and decreased to 0.08 (± 0.001) mg/L on October 12, following the second inflow peak (Figure 3.3e). Both estuarine sites had increased FRP concentrations relative to their pre-flow levels, with Site 3 also increasing from 0.013 (± 0.003) mg/L pre-flow to 0.05 (± 0.003) mg/L on October 12 (Figure 3.3f). FRP concentrations in the estuarine sites decreased gradually from October 12 concentrations to 0.04 mg/L at both sites by November 2 (Figure 3.3f). However, these concentrations rose to 0.10 (± 0.001) mg/L at Site 3 on the November 8 and 0.12 (± 0.02) mg/L on November 14 at Site 4. FRP concentrations then dropped considerably by December 12 to 0.003 (± 0.002) and 0.01 (± 0.002) mg/L at Site 3 and 4 respectively (Figure 3.3f).

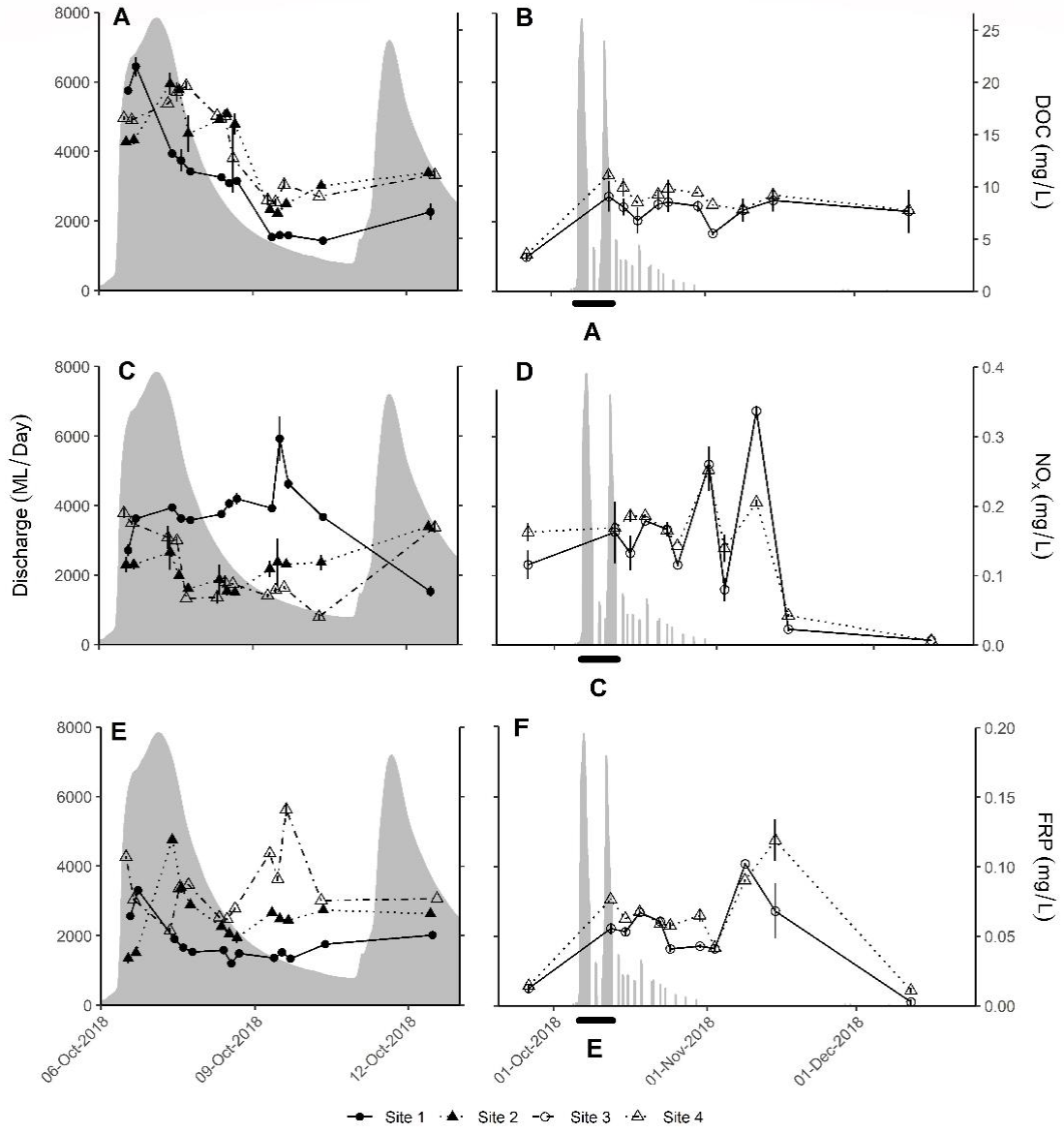

Figure 3.3: Discharge (ML/Day) and concentrations of dissolved organic carbon (DOC), dissolved inorganic nitrogen (NO_x) and filtered reactive phosphorous (FRP) of 4 sites from the Williams River and estuary between the September 26 and December 12, 2018. Figures A, C and E were taken during the flow period and are insets of figures B, D and F. The dark bar on the x-axis of B, D and F indicates the period represented by A, C and E. Fine scale sampling during the inflow event was taken at sites Site 1 and 2, and Site 4, while post- flow samples were only taken at Site 3 and 4. Error bars represent standard error.

3.4.3 Bacterial and Algal Response

Bacterial populations at Site 4 increased rapidly during the initial flow peak (Figure 3.4a), correlating positively with DOC ($R^2 = 0.44$, $p = 0.02$) and turbidity ($R^2 = 0.68$, $p < 0.001$) between October 6 and 10, 2018. A maximum concentration of 2.4 million (± 1.5 e^6) cells/L was recorded at Site 4 during the first inflow at midday on October 7 (Figure 3.4a), before being reduced to 490,000 (± 2.6 e^6) cells/L five days later, on October 12. Bacterial cell

concentrations at Site 3 were 1.0 million (± 2.3 e^6) cells/L on October 12 but decreased to 59,000 (± 1.5 e^5) cells/L by October 15 (Figure 3.4b). Concentrations at both estuarine sites remained below half a million cells/L until October 24 when bacterial concentrations increased to 1.4 million (± 2.0 e^6) and 950,000 (± 2.6 e^6) cells/L for Site 3 and 4, respectively, and then to 2.6 million (± 4.2 e^6) and 1.8 million (± 6.2 e^6) cells/L on October 30 (Figure 3.4b). However, by November 2, these populations had reduced to less than half a million cells/L at both sites. Bacteria concentrations were positively correlated to DOC at Site 4 from the October 12 onward ($R^2 = 51.9$, $p < 0.001$).

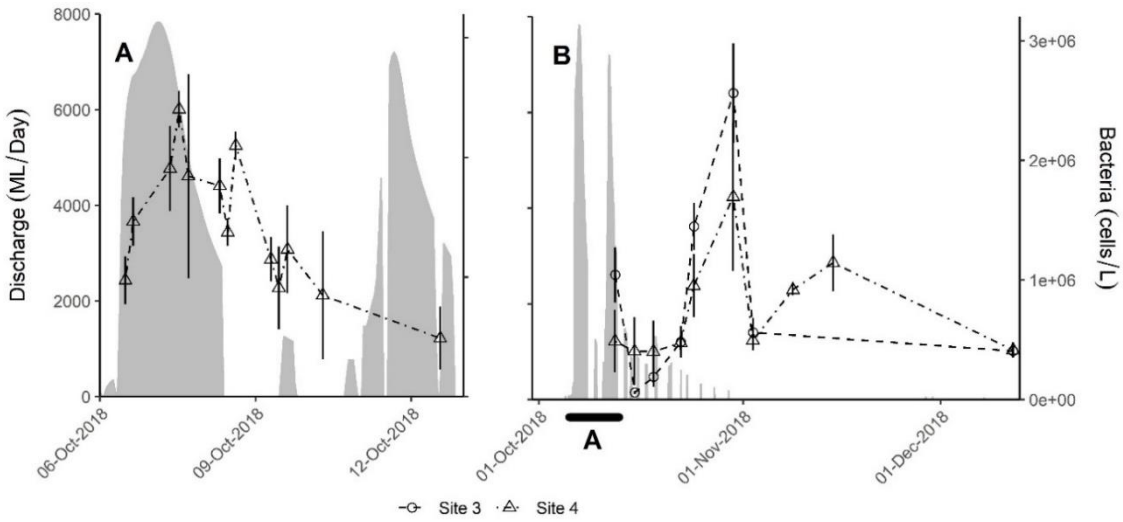

Figure 3.4: Discharge (ML/Day) and concentrations of bacterial cells (cells/L) in the Williams River estuary during (A) and following (B) the inflow event. Site 3 was not sampled intensively through the first flow peak. The dark bar on the x-axis of B indicates the period encompassing A. Samples for Site 3 on November 8 and 14 were compromised during analysis and hence not included. Error bars represent standard error of the mean.

Chl *a* concentration at Site 3 was not altered by the inflow between the September 26 (pre-flow) and October 12, remaining below 5 µg/L (Figure 3.5a). In contrast, Chl *a* concentration decreased at Site 4 from 23.1 µg/L to 3.51 µg/L in the same period (Figure 3.6a). During this time total algal biovolume increased at Site 3 and 4 from 0.29 and 3.63 mm^3/L pre-flow to 7.1 and 9.1 mm^3/L on October 12 (Figure 3.5b). This increase in total biovolume was accompanied by a change from a more diverse algal community at Site 3 to one dominated by cyanobacteria (*Aphanocapsa* sp.) and a change from a cryptomonad dominated community at Site 4 to one comprised mostly of cyanobacteria (*Aphanocapsa* sp.), and euglenoids (*Euglena* sp., Figure 3.5 b, c and d). Biovolumes remained above 5 mm^3/L at both sites between October 12 and 22 (Figure 3.5b), with the dominance of cyanobacteria decreasing and changing to greater concentrations of euglenoids, green (*Cosmarium* sp.,

Scendesmus sp. and *Chlorella* sp.) and cryptomonad (*Cryptomonas* sp.) algae at both sites (Figure 3.5c and d). Total algal biovolumes decreased significantly between October 22 and 24 from 9.2 and 8.0 mm^3/L to 0.31 and 1.3 mm^3/L at Site 3 and 4 (Figure 3.5b), with *Euglena* sp. concentrations decreasing substantially, and an increased representation of *Cryptomonas* sp. at both sites as well as dinoflagellates (*Peridinium* sp.) at Site 2 (Figure 3.5c and d). This decrease in total biovolume coincided with increased Chl *a* concentration at both estuarine sites from 7.5 and 3.7 µg/L on October 18 to 22.0 and 30.0 µg/L by October 24 at Site 3 and 4 respectively (Figure 3.5a).

Chl *a* concentration was decreased 5 days later, on October 30, to 10.6 and 4.9 µg/L at sites 3 and 4 (Figure 3.5a). As Chl *a* was decreasing, total algal biovolume increased to 14.3 mm^3/L at Site 3 on October 30, and to 6.82 mm^3/L at Site 4 on November 2. Total biovolumes at Site 3 on October 30 were comprised of more than 50% *Euglena* sp. (Figure 3.5c), while at Site 4 on November 2 the community was co-dominated by cyanobacteria (*Aphanocapsa* sp.), green algae (*Cosmarium* sp. and *Scendesmus* sp.) and *Euglena* sp. (Figure 3.5d). By November 14 total algal biovolume in the estuary had fallen to 1.9 and 1.3 mm^3/L at sites 3 and 4 respectively (Figure 3.5b). The community here was comprised mostly of dinoflagellates at Site 3 and a combination of dinoflagellates (*Peridinium* sp.), cryptomonads (*Chroomonas* sp.) and *Mallamonas* sp. at Site 4 (Figure 3.5c and d, Appendix 4). At this time, Chl *a* concentration was above 30 µg/L at both estuarine sites (Figure 3.5a). Total algal biovolume then increased to 8.3 and 5.0 mm^3/L on December 12, represented mostly by *Euglena* sp. and green algae (*Cosmarium* sp., *Chlorella* sp. and *Scendesmus* sp.) at both sites (Figure 3.5c and d). By December 12, Chl *a* concentration had been reduced to 10.3 and 7.4 µg/L at Site 3 and 4, respectively. Throughout the period following the inflow, total algal biovolume was positively correlated to euglenoid biovolume ($R^2 = 0.53$, $p < 0.001$) and euglenoids were negatively correlated to DOC ($R^2 = 0.34$, $p < 0.011$) and cyanobacteria were positively correlated to daily discharge ($R^2 = 0.60$, $p < 0.001$). Chl *a* was negatively correlated to total algal biovolume ($R^2 = 0.29$, $p < 0.019$), and the biovolumes of euglenoids ($R^2 = 0.28$, $p < 0.023$) and cyanobacteria ($R^2 = 0.42$, $p < 0.003$), while positively correlated to dinoflagellates ($R^2 = 0.55$, $p < 0.001$).

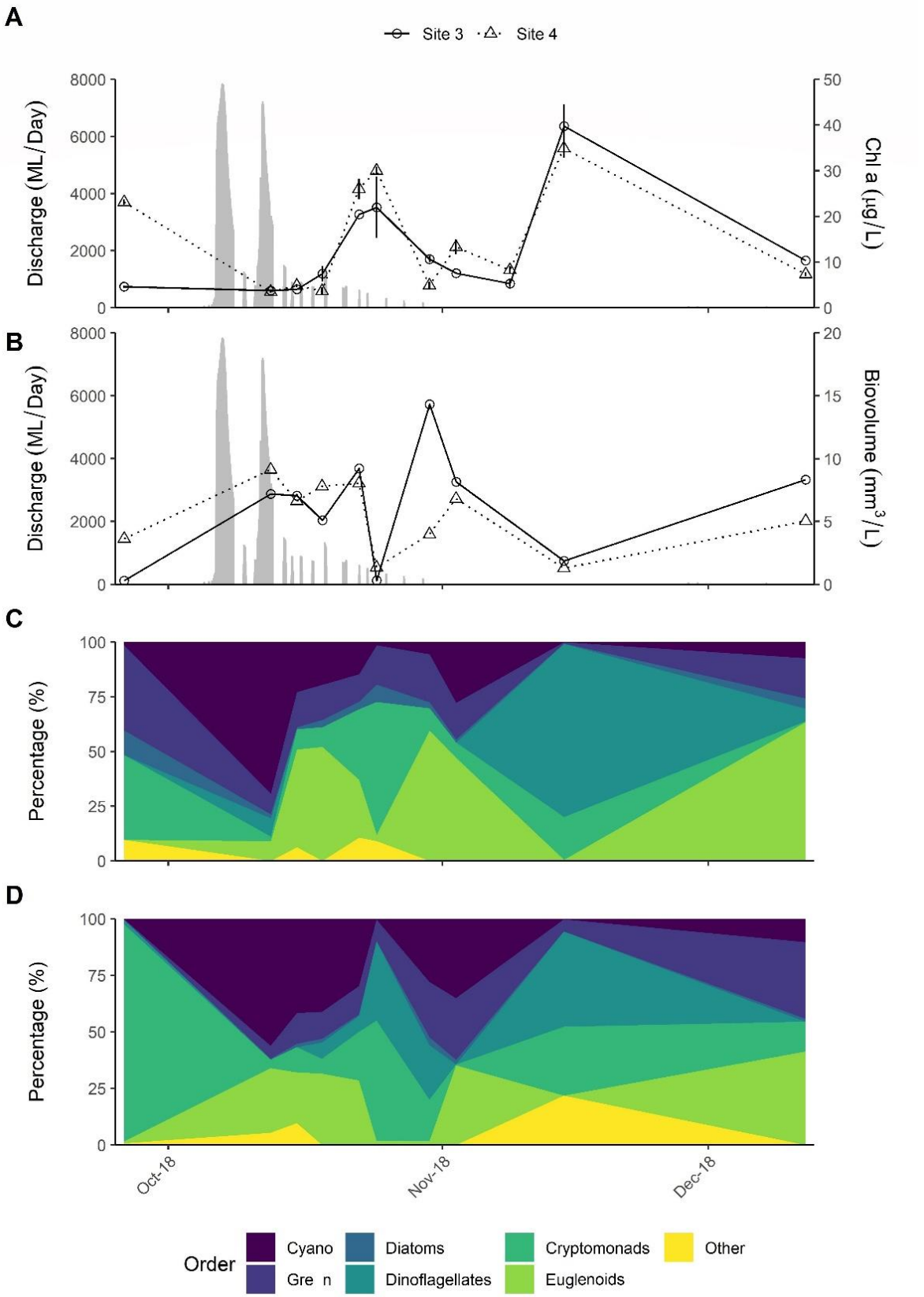

Figure 3.5: Discharge (ML/Day) and, chlorophyll a (A, Chl a, µg/L) total algal biovolume (B, mm^3/L) in the Williams River at Site 3 and 4 prior and following the inflow events. Figures C and D outline the algal community composition as a percentage of biovolume at Site 3 and 4, respectively. Genera comprising these 7 major groups can be found in Appendix 4.

3.4.4 Zooplankton responses to discharge event

Nauplii populations increased from pre-flow concentrations on September 26 of 923 (± 193) and 3,240 (± 401) ind/L (individuals per litre) at Site 3 and 4 to more than 10,000 ind/L at both sites by November 8, within a month of the initial inflow (Figure 3.6a). However, these populations were reduced to less than 1,000 ind/L by December 12 at both sites. Copepodite population responses to the inflow varied between the estuarine sites. At Site 3, densities remained stable throughout the first inflow peak before peaking at 1463 (± 391) ind/L on October 15 and decreasing to 34 (3) ind/L by October 30 (Figure 3.6b). In contrast, copepodite densities at Site 4 reduced from 1,800 (± 283) ind/L prior to the flow to 340 (± 24) ind/L by October 12. Following this they increased to a peak concentration of 2463 (± 314) ind/L on October 22, before decreasing to 93 (± 12) ind/L on October 30, as in Site 3 (Figure 3.6b). While there was a small increase in copepodite concentrations at Site 3 to 777 (± 245) ind/L on October 8, copepodite concentrations were otherwise low through the remainder of the study decreasing to below 50 ind/L at both sites by mid-December (Figure 3.6b).

Adult copepod populations (comprised of both *Gladioferens pectinatus* and *Sulcanus conflictus*) were generally negatively impacted by the inflow events, decreasing from 148 (± 32) and 631 (± 132) ind/L at Site 3 and 4 to less than 50 ind/L at both sites by mid-December (Figure 3.6c). Cladocerans (all *Bosmina meridonalis*) were not present at either site prior to the inflow event (Figure 3.6d). After the inflow they were found at peak densities of 2824 (± 324) ind/L on October 22 at Site 3 and 4328 (± 298) ind/L on October 24 at Site 4. However, cladoceran populations then dropped to under 200 ind/L on November 2 and were no longer present by November 8.

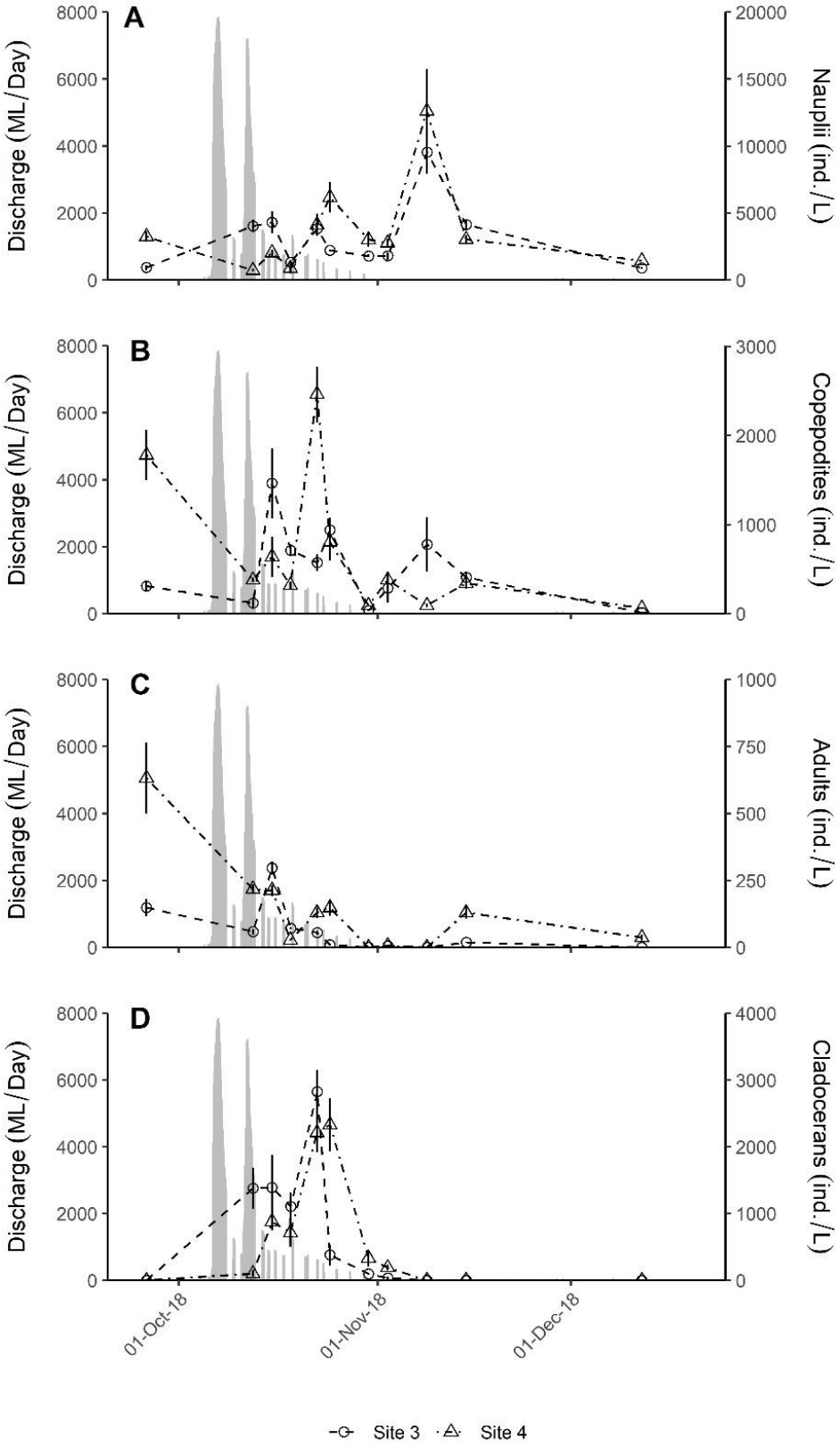

Figure 3.6: Discharge (ML/Day) and abundances of nauplii (A), copepodite (B), adult copepod (C) and cladoceran (D) population concentrations (ind/L = individuals/L) across the October flow peaks. Site 2 was sampled with higher temporal resolution after the first peak to investigate fine scale changes in response to discharge. Error bars represent the standard error of the mean (SEM).

Distance-based redundancy analysis (dbRDA) of the mesozooplankton community showed three distinct communities in the study (Figure 3.7); a “Pre-flow” community (sampled before the flow, September 26, 2018), a “Flow” community (sampled between October 12 and 30, 2018, while the weir gates were still being actively operated) and a “Post-Flow” community (sampled after the final closure of the weir gates on October 30). Monte-Carlo permutational analysis revealed that the constraining environmental variables of salinity, temperature and turbidity all had significant effects on the mesozooplankton community, as did the abundance of cryptomonads, dinoflagellates and euglenoids (Figure 3.7), with constraints explaining 78.7% of variation in the model. The model had two significant axes, with Axis 1, ($F_{1, 13} = 29.7$, $p = 0.001$, 43% of variation) determined by salinity and temperature and Axis 2 ($F_{1, 13} = 14.2$, $p = 0.002$, 23% of variation) determined again by salinity and temperature, as well as the densities of cryptomonads and euglenoids.

Complementary to the dbRDA, multivariate abundance GLM analysis revealed there were significant differences in the three mesozooplankton communities between flow conditions ($LRT_{2, 21} = 52.08$, $p = 0.001$), with “Flow” communities significantly different to “Pre-flow” ($LRT = 37.7$, $p = 0.001$) and “Post-flow” communities ($LRT = 30.8$, $p = 0.002$). Univariate GLM analysis between the flow periods also indicated that concentrations of copepodites ($LRT = 2.26$), *B. meridonalis* ($LRT = 23.5$), *S. conflictus* ($LRT = 13.9$) and *G. pectinatus* ($LRT = 12.28$) were all significantly greater ($p < 0.05$ for all) in the “Flow” period than in the pre- and post-flow periods.

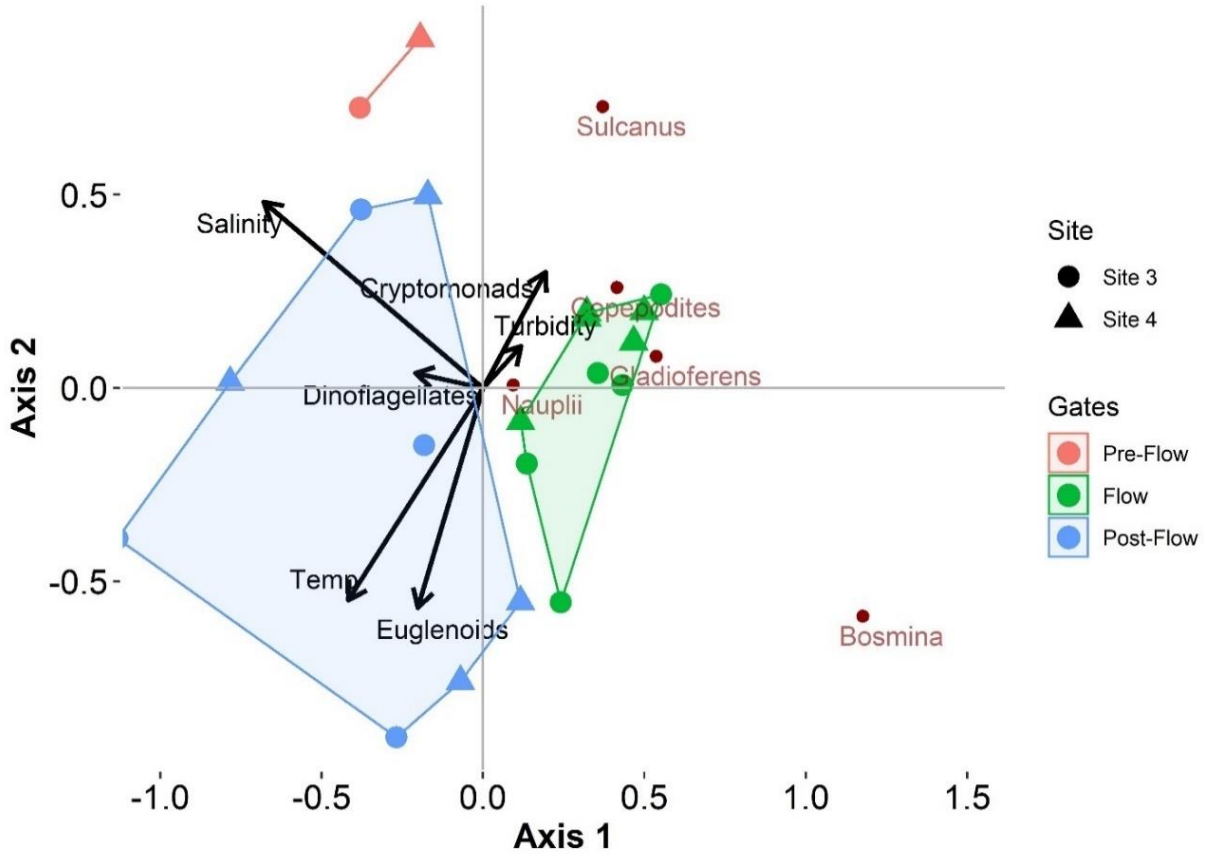

Figure 3.7: Distance-based redundancy analysis of the mesozooplankton community of the Williams River Estuary across an inflow event in 2018. "Pre-Flow" samples (red) are those taken on September 26, 2018, 10 days before the inflow event. "Flow" samples (green) are those from October 12 to 30, 2018, while weir gates were still being actively operated. "Post-Flow" samples (blue) are those from the November 2 to December 12, 2018, during which weir gates were almost exclusively closed. Significant environmental variables ($p < 0.05$) constraining the model are presented.

3.5 Discussion

Routine sampling methods may miss fine scale temporal changes in food webs due to their low temporal resolution (Wilson 2008). Higher temporal resolution sampling is particularly important following inflow events to estuaries as food webs can react to changes quickly and undergo significant changes during these periods (Bruesewitz et al., 2013, Hitchcock et al., 2016). Relying on routine monthly or even weekly sampling may lead to a lack of understanding of the mechanisms by which freshwater inflows shape planktonic and microbial processes and how these feed into the base of estuarine energy flows.

3.5.1 Basal resources increase with flow

Elevated concentrations of DOC and FRP delivered by the inflow event resulted in the stimulation of estuarine microbial and algal communities. This occurred despite the smaller size of the October inflows, approximately 18, 000 ML discharged to the estuary, relative to a previous inflow in late-March, approximately 50,000 ML. While most terrestrial resources

are delivered to estuaries by large storm events (Eyre and Twigg 1997; McKee et al. 2000), moderately sized events such as the one in this study are also capable of stimulating the microbial and algal food web (Hitchcock and Mitrovic 2015a). Increases in bacterial cell concentrations were positively correlated to DOC concentrations during the first inflow peak. Bioavailability of DOC can increase under storm flow conditions, making it more accessible for bacterial populations (Leff and Meyer 1991; Wiegner et al. 2009; Hitchcock and Mitrovic 2015b). Though often positively linked to DOC and discharge, bacterial processes may also be limited by other nutrient availabilities, particularly phosphorous (Hitchcock et al. 2010; Asmala et al. 2014a). The increase of both DOC and FRP in the first inflow (Figure 3.3a and c) may have improved conditions for bacterial growth (Lennon and Pfaff 2005; Hitchcock and Mitrovic 2015a) in the upper estuary during and shortly after the inflow. However, later increases in bacterial concentrations during low flow conditions in late October may be tied to the breakdown of algal cells, with bacteria utilising this autochthonous DOC resource when available (Findlay et al. 1991; White et al. 1991).

Elevated concentrations of FRP following the inflow, relative to pre-flow levels, may have also stimulated the algal community in the estuary as discharge decreased. While increased discharge can reduce estuarine phytoplankton concentrations for a period of time due to advection and decreased light availability (Cloern et al. 1983) the delivery of limiting nutrients can initiate phytoplankton production after storm events (Rudek et al. 1991; Scharler and Baird 2003; Dorado et al. 2015). Due to the already high levels of NO_x in the Williams estuary under low flow periods (Chapter 2, Figure 3.3d) it may be that phosphorous is the limiting nutrient for algal production in the Williams, in contrast to other Australian estuaries where nitrogen is the limiting factor (Thompson and Hosja 1996; Glibert et al. 2006; Woodland et al. 2015). Increases in algal biovolume, co-dominated by euglenoids and cyanobacteria, occurred immediately after the inflow (October 12 to 22), with cyanobacteria decreasing through time before total algal biovolume was substantially reduced on October 24 (Figure 3.5a). This finding is in contrast with previous studies where higher flow periods in estuaries have been associated with increased diatom concentrations as fast-growing diatoms capitalise on flowing conditions and nutrient inputs (Miller et al. 2008; Dorado et al. 2015). The absence of diatoms in this period may be a result of reduced inflows through the weir and reduced mixing of the water column, as diatoms require turbulence to maintain access to the euphotic zone (Borics et al. 2016; Nunes et al. 2018). The co-dominance of euglenoids in this period may also indicate reduced inflows through the weir in the “flow”

period as they are slow growing species and can occur in estuaries following inflows once turbulence subsides partly as they are able to maintain their position in the water column with motility (Roelke et al. 2013; Nunes et al. 2018).

Increases in total algal biovolume following the inflow period correlated positively with euglenoid concentrations ($R^2 = 0.53$, $p < 0.001$) but had weak negative correlations to Chl *a* ($R^2 = 0.29$, $p < 0.019$). Chl *a* is often used as a proxy for algal biomass and primary production but can be suppressed by allochthonous DOC inputs following inflow periods, at concentrations similar to those in my study (5- 10 mg/L), through shading of the euphotic zone (Andersson et al. 2018). The relationships between Chl *a* and algal biovolume are difficult to interpret, as they vary temporally, spatially and with species composition (Felip and Catalan 2000). However, under low light conditions with alternate carbon sources, such as allochthonous DOC, Chl *a* production can be suppressed while biovolumes of mixotrophic algal species increase, including euglenoids, dinoflagellates and cryptomonads (Harris and Kirk 1969; Tittel et al. 2005; Wang et al. 2018). In this study, mixotrophic plankton, e.g., euglenoids, may have outcompeted autotrophs during the "Flow" period by heterotrophically assimilating DOC (Tittel et al. 2003; Nunes et al. 2018) or bacterial cells, which were decreased substantially when euglenoids were present (October 12 to 22 and November 2, 2018, Figure 3.4b and 3.5). Similar processes have been noted in lakes, where coloured dissolved organic matter inputs after inflows result in increased concentrations of mixotrophic protists (Trochine et al. 2015; Hansson et al. 2019) and in regulated estuaries where sudden switches from high flow to low flow periods result in increasingly heterotrophic ecosystems with microbial processes and mixotrophs becoming more important (Bruesewitz et al. 2013; Hemraj et al. 2017b). This may have implications for food web structuring in the upper Williams estuary after inflows, as high concentrations of euglenoids were quickly reduced in this study during periods where cladocerans and copepodites densities were increasing (Figure 3.6b and d). This may indicate possible top-down grazing effects (Buyukates and Roelke 2005; Hoover et al. 2006) and hence increased allochthonous support of zooplankton following the inflow event (van den Meersche et al. 2009).

3.5.2 Mesozooplankton community responses

Two distinct mesozooplankton communities formed after the inflow event which highlighted the influencing roles of discharge and temperature. The "Flow" community, present while the weir gates were actively operated and open, and the "Post-Flow" community, which developed after the closure of the weir gates (Figure 3.7). While I hypothesised that the

zooplankton community would have increased densities of copepods relative to the prior community due to resource inputs, this was not the case. Instead, the highest densities of all mesozooplankton after the event were in the "Flow" community (Figure 3.6 and 3.7), except for nauplii, which increased in the "Post-flow" period (Figure 3.6a). Mesozooplankton taxa in estuaries are generally regulated by temperature and salinity during low flow conditions (Mallin 1991; Wooldridge and Callahan 2000; Marques et al. 2014). However, during high flow periods, discharge does play a role in structuring communities (Hoover et al. 2006; Miller et al. 2008; Kimmerer et al. 2018). Inflows can increase the populations of mesozooplankton in estuaries through advection from upstream areas, with populations capitalising on favourable conditions in their new habitat (Geddes et al. 2016; Kimmerer et al. 2018). This may explain the increasing densities of the freshwater cladoceran *Bosmina meridonalis* following the inflow event between October 15 and 24, 2018 (Figure 3.6b and d) as they capitalised on increased algal resources, namely *Euglena* sp.. Long term data from the Williams estuary demonstrates that *B. meridonalis* only occurs in the study area after inflows (Chapter 2), and this is further supported by a negative relationship of *B. meridonalis* with salinity and a positive relationship with increased biovolumes of euglenoids (Figure 3.7). However, their grazing on phytoplankton, coupled with the grazing of increased concentrations of nauplii and copepodites at this time (Figure 3.6a and b), may have contributed to the reduction of algal biovolumes on October 24 (Figure 3.5a). This reduction in algal biovolume was substantial and the subsequent collapse of the cladocerans and copepodite populations between October 22 and 30 may have been due to a lack of food from prior over-grazing (Figure 3.6b and d). Further, mortality of algae and mesozooplankton may have contributed to the rise in bacterial cells between the October 24 and 30 (Figure 3.3b and 4b). DOC concentrations were low during this period compared to during the peak of the inflow (Figure 3.3a and 3.4a), but DOC from decaying algal cells may have been taken up rapidly by the bacteria (Fouilland and Mostajir 2010; Guillemette et al. 2013; Attermeyer et al. 2014).

In contrast to the "Flow" community, the "Post-Flow" community had significantly reduced concentrations of cladocerans, copepodites and adult copepods (Figure 3.7), with a major increase in nauplii at both estuarine sites on November 8, a feature of this period (Figure 3.6a). The increase in nauplii (both sites) and copepodite concentrations (at Site 3) on November 8 was followed by small increases in adult copepods (*S. conflictus*, Site 4) on November 14, near the conclusion of the study (Figure 3.6). The peaks in nauplii

concentrations quite late after the inflow event may have been a due to a hatching event. Increasing seasonal temperatures (Boyer et al. 2013; Horne et al. 2016; Holm et al. 2018) or the breakdown of saline and oxygen stratification by freshwater inflows (Nielsen et al. 2012; Broman et al. 2015) are known triggers of copepod hatching events and each may have contributed. As basal resource availability was high (Figure 3.5a) and temperatures were increasing over the study period, increased concentrations of nauplii were hypothesised to result in increased adult copepod concentrations after the inflow as they have in previous studies (Mallin 1991; Tan et al. 2004; Hoover et al. 2006; Hitchcock et al. 2016b). However, zooplankton communities followed the long-term pattern in the Williams River estuary of inflows leading to high nauplii concentrations, yet not translating to increased adult concentrations (Chapter 2). As in Chapter 2, it is unknown why nauplii hatching events did not translate into increased adult abundance in the Williams estuary, particularly as temperatures were favourable for development (Hirst and Bunker 2003; Horne et al. 2016). The relatively low abundances of adult copepods compared to other systems in similar seasons (Chapter 2; Furst et al. 2019) suggests predation may be structuring this community (Geddes et al. 2016; Furst et al. 2019). It is also possible that poorer quality heterotrophic food sources were unable to support the development of copepod populations after the inflow (Brett et al. 2009; Isari et al. 2013; Hemraj et al. 2017b). However, without monitoring the birth, growth or mortality rates of copepods it is difficult to interpret the true meaning of adult copepod abundance fluctuations in response to inflows (Kimmerer et al. 2018).

3.5.3 River Regulation and Management

Studies in Australian freshwater and estuarine environments have outlined the importance of the first flush of an inflow in delivering terrestrial resources to rivers and estuaries (Hitchcock et al., 2010; Westhorpe and Mitrovic, 2012). Protection of these inflow periods have been utilised by water resource managers in multiple Australian systems (Baldwin et al., 2016), and are present for the Williams as part of the Hunter River Water Sharing Plan (NOW 2009a). Although this study encompassed only a moderate inflow, my results are a positive affirmation of the effectiveness of first flush rules in delivering important resources to stimulate productivity in the Williams River estuary. However, my study also highlights how the operation of the weir gates may contribute to sudden shifts in the ecology of the upper Williams estuary between high flow and low flow conditions. Mesozooplankton populations were significantly higher under conditions where water was still being delivered to the estuary, compared to when gates were shut. Previous studies have highlighted the negative

impacts of sudden changes in inflow regimes, suggesting that the subsequent rapid shifts in water quality and ecosystem function can be detrimental to estuarine habitats and communities (Bruesewitz et al. 2013; Hemraj et al. 2017b). Rather than flow rules considering the total volume of water delivered, it may be worth considering the timing of operational closing of weir gates to sustain flowing freshwater conditions for longer periods. This may reduce sudden shifts in ecosystem functioning and benefit the ecology and productivity of regulated estuaries (Hemraj et al. 2017b).

This study is limited by its low level of geographical and seasonal scale. However, continued fine scale investigation of estuarine systems with varying catchment sizes, climates, riverine inputs, degrees of regulation, catchment development and morphology would be beneficial in understanding the response of estuarine mesozooplankton food webs to inflows and the influences of river regulation. Future studies including predatory exclusion experiments may also be beneficial in assessing the role of predation in controlling mesozooplankton communities after inflows. Furthermore, investigations into the importance of terrestrial or algal subsidised zooplankton to the food web may provide greater insights into the importance of freshwater inflows and resource delivery in estuaries. In the following chapters I investigate this concept in relation to the role of terrestrial support of zooplankton in estuaries and the energetic benefits they can provide to juvenile fish.

3.5.4 Conclusion

The fine scale sampling approach I used in this chapter highlighted flow-influenced patterns of resource delivery and their influence on bacterial, phytoplankton and zooplankton growth. Initial pulses of DOC and FRP by the inflow resulted in tightly coupled increases in bacterial growth. This was followed by an increase in phytoplankton densities, dominated by *Euglena* sp., and a freshwater zooplankton population which increased in density, utilising available phytoplankton resources. The zooplankton community then shifted to a “Post-Flow” community, during which a large peak in nauplii concentrations occurred. However, increased concentrations of nauplii did not translate into increased adult copepod concentrations, relative to pre-flow conditions, as hypothesised. These fine scale changes in the mesozooplankton food web of the upper Williams estuary may have been missed by traditional monthly sampling regimes. By understanding the short-term impact of freshwater inflows on the plankton community further adaption of river management protocols can be made to improve the delivery of higher quality resources and improve estuarine ecology.

Chapter 4: Inflow events drive resource use changes for mesozooplankton in a regulated estuary

4.1 Abstract:

Research has shown that terrestrial organic matter can contribute to estuarine food webs during and after high flow periods. However, the response of estuarine food webs varies between estuaries and can be altered by river regulation. I examined the changes in mesozooplankton diets using stable isotopes of carbon and nitrogen and mixing model analysis during a period of 8 months with variable hydrology in the regulated Williams River. Mesozooplankton switched from using autochthonous algal resources in periods prior to inflows to allochthonous, terrestrially derived resources in the months following inflows. The $\delta^{13}C$ of suspended particulate organic matter (sPOM) did not follow the same pattern as the mesozooplankton being relatively unaffected by inflows. This suggests that allochthonous resource use potentially relied on selective use of terrestrial sPOM fractions or consumption of terrestrial material through the microbial loop. Furthermore, I found that allochthonous resource use was prolonged in the upper estuary, lasting for up to two months. These findings indicate that terrestrial resource use may be an important part of the estuarine mesozooplankton food web following inflow events.

4.2 Introduction

Freshwater inflows (FWIs) exert a strong influence on the ecological processes and structure within estuaries (Drinkwater and Frank 1994; Gillanders and Kingsford 2002), particularly in the upper reaches of estuaries where river flow is the predominant physical force (Pierson et al. 2002). By increasing longitudinal and lateral connections to catchments and floodplain, FWIs deliver important terrestrial resources to estuaries during inflow events (Junk et al. 1989; Smith and Hollibaugh 1993; Pierson et al. 2002). These events and their inputs of terrestrial organic matter and nutrients can alter basal resource availability and utilisation in estuarine food webs (Buyukates and Roelke 2005; Schlacher et al. 2009; Atwood et al. 2012; Dorado et al. 2015).

There is a general consensus that during low flow periods estuarine food webs are primarily supported by autochthonous phytoplankton driven production (Deegan and Garritt 1997; Jassby et al. 2003; Sobczak et al. 2005; Schlacher et al. 2009). However, rainfall leading to high river flow periods and direct runoff events can suppress autotrophic production due to the

combined effects of inflow turbulence (Hoover et al. 2006; Miller et al. 2008; Anglès et al. 2015) and large inputs of organic matter (OM) and sediment from terrestrial sources, reducing underwater light availability (Andersson et al., 2018). For zooplankton consumers, this suppression of primary production can trigger a switch from autotrophic energy pathways under low flow conditions towards the use of allochthonous energy sources such as terrestrial particulate organic matter (tPOM) or dissolved organic matter (tDOM) following inflow events (Hoffman et al. 2008; Schlacher et al. 2009; Atwood et al. 2012).

Allochthonous OM is sometimes considered a poor-quality food resource (Brett et al. 2009; Taipale et al. 2014), yet there is evidence suggesting it can be assimilated into the food web through microbial processing or direct consumption by zooplankton (Jansson et al. 2007; van den Meersche et al. 2009; Cole et al. 2011). In estuaries, there is a growing body of literature suggesting the assimilation of terrestrial carbon into food webs occurs following high flow periods and can contribute to increased productivity of zooplankton and fish (Hoffman et al. 2007; Abrantes et al. 2013; Lautenschlager et al. 2014; Hitchcock et al. 2016b). While inflows clearly support a range of ecological processes in estuaries, patterns of organic matter delivery and assimilation can be disrupted by river regulation (Pierson et al. 2002; Ru et al. 2020; Liu et al. 2020). Understanding and assessing the relative importance of inflows on estuarine food webs in these types of estuaries is an important step in managing for favourable ecological outcomes (Scheltinga et al. 2006).

Mesozooplankton provide an important link between basal resources and the productivity of higher order consumers such as fish (Newton 1996; Liu and Dagg 2003; Castonguay et al. 2008). Consequently, this group has been widely utilised to assess the use and importance of allochthonous resources in estuaries (Hoffman et al. 2008; Schlacher et al. 2009; Atwood et al. 2012). Many contemporary studies have explored the diet of mesozooplankton using stable isotope techniques, with comparisons of the carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) ratios of suspended particulate organic matter (sPOM) and zooplankton indicating autochthony (use of autochthonous resources) or allochthony (use of allochthonous resources) in mesozooplankton diets (Dias et al. 2016; Hitchcock et al. 2016a; Edje and Chigbu 2021). Variations in sPOM isotopic signatures can occur temporally and spatially following flow events to estuaries, driven by changing salinities, algal densities and inputs of organic matter from terrestrial sources or sediments, allowing parent OM sources of sPOM to be identified (Fry 2002; Chanton and Lewis 2002; Reyna et al. 2017; Bibi et al. 2020). Mesozooplankton consumers reflect these changes in sPOM sources in their diet with trophic enrichment factors (TEF) between

consumers and their diet (e.g., 0.4 ‰ $\delta^{13}C$ and 2.2 ‰ $\delta^{15}N$ for zooplankton, McCutchan et al., 2003) allowing an interpretation of changing diets and trophic positions following inflows (Hoffman et al. 2008; Schlacher et al. 2009; Atwood et al. 2012; Edje and Chigbu 2021).

Considering the results of Chapter 2 in the Williams River estuary where inflows were linked to increased concentrations of dissolved organic carbon (DOC) and nauplii, I sought to investigate if terrestrial resources contributed to estuarine food webs following inflows in the same system. This study was conducted over 8 months between January and August 2018, during a period with high discharge variability. Using stable isotope analysis of carbon and nitrogen for sPOM and dominant mesozooplankton taxa, coupled with ongoing monitoring of water quality, phytoplankton and mesozooplankton communities from Chapter 2, I investigated potential changes in mesozooplankton resource use following inflows. I hypothesised that 1) mesozooplankton would change resource use patterns from autochthony, in low flow periods prior to inflows, to allochthony, in months following inflows, as indicated by changes in $\delta^{13}C$ signatures, and 2) $\delta^{13}C$ signatures of mesozooplankton would be positively related to changes in sPOM $\delta^{13}C$ signatures following inflows.

4.3 Methods

4.3.1 Site Description

The Williams River estuary is located on the south-east Australian Coast (Figure 4.1) and has a catchment area of 974 km^2. It is a sub-catchment of the Hunter River catchment area and joins with the Hunter River at Raymond Terrace. The system is regulated upstream by Chichester Dam and downstream by Seaham Weir, which enforces a tidal limit 42km upstream from the mouth of the Hunter River at Newcastle. This allows extraction to nearby Grahamstown Dam for drinking water for the Newcastle area. The reach between Seaham Weir and the confluence with the Hunter River can range in salinities between 0 ppt during inflows to 20 ppt during prolonged low flow periods. Much of the Williams catchment is developed with 80% of the catchment as agricultural land, 14% national park and 6% held by local water management authorities. As a tributary to the Hunter River, the majority of saltmarsh and mangroves associated with the system are downstream of Raymond Terrace, although there are small populations of mangroves throughout the Williams, up to the tidal limit at Seaham. Two sites were used along the estuary (Figure 4.1) to account for any longitudinal effects of the estuarine salinity gradient which can impact biogeochemical processes regulating the $\delta^{13}C$ signatures of sPOM and zooplankton (Fry 2002; Asmala et al.

2014b). Site 1 was 500 m downstream of Seaham Weir at the imposed tidal limit of the Williams estuary, Site 2 was 10 km downstream of the weir (Figure 4.1).

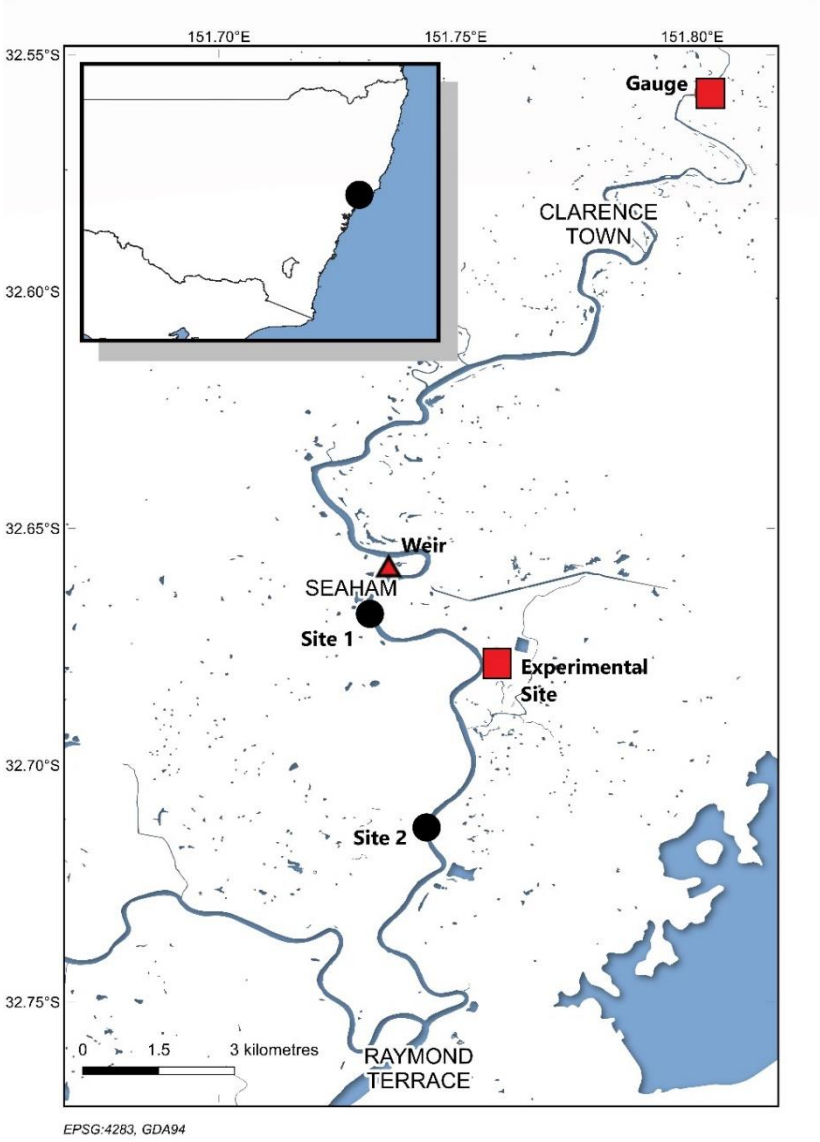

Figure 4.1: Site, weir and discharge gauge positions for the Williams River. Estuarine sites are denoted by black circles on the river channel. Site 1 is the most upstream black circle (near Seaham) with Site 2 being the downstream black circle nearer to Raymond Terrace. Seaham Weir is denoted by the red triangle upstream of Seaham. WaterNSW gauge 210010 (Williams at Glen Martin) is indicated by the red square upstream of Clarence Town. The red square between Site 1 and 2 is where the nutrient amendment experiment site.

4.3.2 Physicochemical, DOC and chlorophyll *a* data

Sampling was conducted approximately monthly on 7 trips between January 2018 and late-August 2018 as part of the ongoing monitoring in Chapter 2. Samples were taken within 2 hours of high tide at Raymond Terrace (Figure 4.1) moving upstream from Site 2 to Site 1. Salinity was measured at the surface with a Hydrolab Surveyor 4 and MS5 sonde probe. Turbidity surface samples were measured using a Hach 2100 turbidimeter. Water samples for

dissolved organic carbon (DOC) were taken in duplicate from surface water subsamples. DOC samples were filtered through 0.45 µm syringe tip filters (Minisart, 16533Q) and collected in sample rinsed 250 mL PET bottles. Samples were then frozen as soon as possible (within 4 hours). DOC samples were analysed by the high temperature combustion method (Sugimura and Suzuki, 1988). Chlorophyll *a* (Chl *a*) was determined with duplicate samples by filtering 500 mL of surface water onto GF/C filters. Filters were frozen until Chl *a* was determined using the grinding technique and an ethanol solute with corrections for phaeophytin (APHA 2005). Chl *a* concentrations were analysed with a detection limit of 1 µg/L, anything below this level is displayed as < 1 µg/L. Duplicate samples for DOC and Chl *a* were used due to the high cost of external analysis. All flow data was obtained from the WaterNSW gauge 210010 at Glen Martin (Figure 4.1).

4.3.3 Stable Isotope Analysis

Zooplankton samples for stable isotope analysis were collected by duplicate horizontal tows of a 60 cm diameter and 150 µm zooplankton tow net for 5 to 10 minutes at approximately 6 km/h. Due to the mesh size of this tow net, smaller zooplankton including copepod nauplii were excluded from these samples. Samples were rinsed and stored with GF/C (Whatman, 47 mm) filtered river water from the sampled site to prevent anoxic conditions developing and to remove food sources and allow gut purging (Feuchtmayr and Grey 2003; Hitchcock et al. 2016a). Water from the sampled site was used to provide organisms with a similar salinity to conditions at the time of sampling. After a minimum 4 hour purging period, samples were filtered, concentrating the sample to between 20 and 40 mL with a small 20 µm net. This concentrated sample was then preserved with ethanol, being made up to approx. 200 mL (1:9 ratio) with pure ethanol (Feuchtmayr and Grey 2003; Fanelli et al. 2010).

Zooplankton were identified and enumerated through microscopy as part of data collection for Chapter 2 and therefore followed the same methods. In the laboratory, samples for zooplankton stable isotopes were rinsed and size fractionated with reagent grade water, removing ethanol from the sample (Feuchtmayr and Grey 2003). Fractions above 200 µm were transferred to petri dishes for cleaning underneath a dissecting microscope. Though present in zooplankton enumeration samples from Chapter 2, fractionation and rinsing further removed copepod nauplii from stable isotope samples due to their small size. This resulted in samples being comprised of >90% adult zooplankton taxa, with the dominant species being the same as that enumerated in Chapter 2. Species were not isolated from each other due to their low abundances

and the need to meet minimum weight requirements (>1mg) for staple isotope analysis. Samples for isotopic analysis were cleaned of larger detritus with tweezers and were transferred to pre-weighed tin (Sn) or silver (Ag) cups for nitrogen and carbon stable isotope analysis, respectively. Technical duplicates for both carbon and nitrogen analysis were taken from each sample of zooplankton totalling in 4 pseudo replicates for each sample point.

Approximately 4 drops of Hydrochloric acid (HCL) solution (10%) were added to carbon samples to demineralize dissolved inorganic carbon (DIC) (Carabel et al. 2006). Once DIC had been removed (indicated by the halt in effervescence) samples were dried for 48 hours at 60ºC (Feuchtmayr and Grey 2003). Carbon samples were not rinsed of HCL solution prior to drying (Jacob et al. 2005; Carabel et al. 2006). Nitrogen samples were not rinsed with HCL solution. Samples were weighed after drying for a target weight of above 1 mg.

Samples of sPOM were taken in conjunction with zooplankton samples to provide an estimate of the sources of sPOM in the water column. To attain these samples, approximately 10 L of surface water was filtered through a 40 µm tow net. The retained sample was then filtered through pre-combusted GF/C filters (Whatman, 47 mm). Samples were taken in duplicate and frozen. Prior to stable isotope analysis samples were oven dried for 24 hr at 60°C. Samples were analysed by a continuous flow-isotope mass spectrometer (GV IsoprimeEurovector EA 3000, Manchester, UK). Results were determined against laboratory standard reference material IAEA-CH-6 using Equation 1. Stable isotope results are expressed as permil (‰) deviations away from the standard used in accordance with said equation.

Equation 1: Example equation for calculating ^{13}C, the ratio of ^{13}C: ^{12}C in obtained samples to international standards expressed in units of permil (Fry, 2007). ^{15}N is calculated similarly.

$$\delta^{13}C = \left(\frac{\left(\frac{^{13}C}{^{12}C}\right)_{sample}}{\left(\frac{^{13}C}{^{12}C}\right)_{standard}} - 1 \right) * 1000\ ‰$$

4.3.4 Stable Isotope Endmembers

As an added component to this Chapter, I performed a novel experimental method to obtain localised and more accurate $\delta^{13}C$ signatures of autochthonous algal carbon from the estuary. Algal $\delta^{13}C$ signatures from aquatic systems are often derived from samples of sPOM, however, in turbid or coloured systems terrestrial matter may represent a substantial component of sPOM (del Giorgio and France 1996; Pace et al. 2004; Carpenter et al. 2005), such as in estuaries after inflows. It is therefore important to obtain localised signatures to represent autochthonous

production. Furthermore, algal signatures in upper estuarine areas may vary with reported signatures from the Mattaponi River being between -31 and -36 ‰ (Hoffman et al. 2008), while other estuaries have reported signatures between -21 to -28‰ depending on salinities (Fry and Sherr 1989; Fry 2002). Methods to isolate $\delta^{13}C$ of algae have been trialled previously in freshwater systems and have been recommended as the best way to estimate algal signatures within aquatic systems (Marty and Planas 2008). Rather than cleaning samples and fractionating them through centrifuging as performed by Marty and Planas (2008, Method 5) I aimed to overwhelm sPOM with algal matter through nutrient amendments to stationary mesocosms of estuarine water. It was hypothesised that dominant algae would increase in concentration and detrital matter would fall out of suspension, resulting in a sPOM sample more indicative of estuarine algal $\delta^{13}C$. I hypothesised that this method would result in a more accurate algal sPOM signature relative to the sPOM of the estuary as the influence of organic detritus and terrestrial material, which are usually depleted in $\delta^{13}C$ relative to estuarine phytoplankton (del Giorgio and France 1996; Fry 2002), would be removed from the sample.

The nutrient amendment experiment was conducted between the 20/9/18 and the 24/9/18. Six 70 L bins were filled with approximately 60 L of estuarine water (salinity = 7 ppt) from the Williams River estuary between Sites 1 and 2 (Figure 4.1). The bins were stationed on level grass on a private residence in full sunlight. Inorganic nitrogen and phosphorous were added to 3 bins as $NaNO_3$ and $NaPO_4$ at 1 mg/L and 0.4 mg/L respectively, with 3 bins left as controls. The bins were left for 4 days to allow estuarine phytoplankton to increase in biovolume and suspended sediments to settle before sPOM samples were collected (a single replicate from each bin, n=3 for control and treatment groups) and stored following the methods above before undergoing analysis. Phytoplankton samples were also taken before and after the experiment for identification and biovolume measurements (methods as in Chapter 3). At the end of the experiment the control groups were dominated by *Mallomanas sp. and Stombidium sp.* cells with a total algal biovolume of 1.76 mm^3/L (Appendix 5) and a $\delta^{13}C$ of -27.52 ‰ ± 0.17. In contrast the amended treatments were dominated by *Chroomonas sp.* with 2.27 mm^3/L of the total algal biovolume 2.81 mm^3/L (Appendix 5) and a more enriched $\delta^{13}C$ of -25.73 ‰ ± 1.6. The increased cell density in the nutrient amended treatment provided a cleaner sample and a more enriched autochthonous signature for $\delta^{13}C$ relative to the controls (Appendix 6) which was used in analysis (Table 4.1). The $\delta^{15}N$ signatures from the amended treatment was not useable due to the effect of the added nutrients (Appendix 6) so the $\delta^{15}N$ of the unamended mesocosm samples were used instead (Table 4.1), though these were slightly lower than

autochthonous signatures used in other studies (~5 to 10 ‰, Hoffman et al., 2008; Schlacher et al., 2009).

To establish the range of terrestrial $\delta^{13}C$ values for the study samples of terrestrial plants *Lomandra longifolia*, a perennial herb, and the coastal tree *Casuarina glauca,* both very common along rivers and estuaries in Eastern Australia, were collected. Leaves of *L. longifolia* and *C. glauca* were collected from the Williams River upstream of the Seaham Weir (-32.581324, 151.782468E) and nearby Seaham weir (-32.656703S, 151.744654E). Samples of stream bank sediment organic matter (OM) were also taken from nearby Seaham Weir to provide an endmember for suspended sediment OM. Vegetation and sediment samples (n=3 from each site) were frozen and returned to the laboratory before being sent for analysis. Endmember averages for $\delta^{13}C$ and $\delta^{15}N$ and standard errors are displayed in Table 4.1.

Table 4.1: Stable isotope ratios (‰) of organic matter sources for the Williams River estuary for carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) with standard deviation (±SD), and relevant C:N elemental ratios. The estuarine algal signature of $\delta^{13}C$ is derived from the treatment samples of the amendment experiment while $\delta^{15}N$ or estuarine algae is from the control samples of the amendment experiment, due to the effects of additional nutrients on the $\delta^{15}N$ of treatment samples.

Source	$\delta^{13}C$ (± SD)	$\delta^{15}N$ (± SD)	C:N Ratios
Estuarine Algae	-25.73 (± 1.6)	3.38 (±1.4)	5.6
Sediment OM	-26.49 (±1.2)	2.63 (±1.2)	15.2
Lomandra longifolia	-29.84 (±1.0)	4.70 (±1.9)	20.2
Casuarina glauca	-30.92 (±1.1)	-1.17 (±0.6)	22.8

4.3.5 Statistical Analyses

Statistical analysis was conducted using R (4.1.1) (R Development Core Team 2020). Correlation analysis was performed using Pearson's R (presented as R^2) through the "Hmisc" package (Harrel 2015). Two-way ANOVAs (site*month) were used to test for significant differences ($p < 0.05$) in water quality variables (Chl *a*, DOC and turbidity) and sPOM $\delta^{13}C$ between sites and time points using base R, with assumptions of ANOVA checked using

visual inspection of boxplots and Levene's Test. One-way ANOVAs were conducted to test for significant differences between sites for mesozooplankton $\delta^{13}C$ and $\delta^{15}N$, with the two-way ANOVAs (site*month) unable to be transformed to satisfy the assumptions of ANOVA. Further single factor investigation of mesozooplankton $\delta^{13}C$ and $\delta^{15}N$ were undertaken using nonparametric Kruskal-Wallis testing, followed by a Dunn's *post-hoc* test, to reveal potential temporal patterns relating to inflows. These tests, and visual inspections of biplots, indicated that there was a group of months following inflows where $\delta^{13}C_Z$ was significantly depleted (April, July and August). As these months followed inflow events, I continued to contrast them with the remaining months where $\delta^{13}C_Z$ was relatively enriched (January, March, May and June).

To calculate the relative contribution of OM sources to mesozooplankton diets in the study I used a 2-isotope ($\delta^{13}C$ and $\delta^{15}N$), 4 source (Table 4.1) Bayesian mixing model through the "simmr" package in R (Parnell 2021). Trophic enrichment factors (TEFs) used in the model were 2.2‰ (± 0.3) for nitrogen and -0.21 (± 0.21) for carbon per trophic level (McCutchan et al. 2003) and concentration dependence means (proportion of C or N in samples) were also applied. Trophic levels for mesozooplankton were determined from literature based on feeding ecology of the dominant species (Ough and Bayly 1989; Zamora-Terol et al. 2014) with 1 being assigned to primary consumers and 2 being assigned to predators, with omnivores being assigned 1.5. Probability distributions of dietary contributions to mesozooplankton were provided at a 50% confidence interval level for each sampling point as in Atwood et al., (2012). The mean dietary contributions of estuarine algae and *L. longifolia* (the major terrestrial energy source in the study) produced by mixing model analysis were further analysed by a two-way ANOVA comparing them between time periods where $\delta^{13}C_Z$ was relatively enriched (January, March, May and June) or depleted (April, July, August). Calculations of the difference between $\delta^{13}C$ and $\delta^{15}N$ of sPOM and that of the dominant zooplankton (e.g., $\delta^{13}C_Z - \delta^{13}C_{POM}$) were used to investigate potential grazing behaviours on sPOM and trophic levels of zooplankton relative to sPOM across the same groupings of months. Small differences in $\delta^{13}C_Z - \delta^{13}C_{POM}$ (e.g., ± 1‰) can imply non-selective grazing while larger differences (e.g., ± 5‰) indicate selective or preferential feeding behaviours (Deegan and Garritt 1997; Hoffman et al. 2008). Meanwhile, smaller differences between $\delta^{15}N$ of sPOM and zooplankton are indicative of shorter food chain lengths, compared to larger differences, which indicate additional trophic levels may be involved (Deniro and Epstein 1981; McCutchan et al. 2003). This data was compared

between the enriched and depleted $\delta^{13}C_Z$ groups for $\delta^{13}C$ using a Kruskal-Wallis test with a Dunn's *post-hoc* test, as data did not satisfy the assumption of homoscedasticity and normality for $\delta^{13}C_Z$ - $\delta^{13}C_{POM}$ and could not be transformed to do so. Figures were constructed using "ggplot2" (Wickham 2016) and "ggpubr" (Kassambara 2020).

4.4 Results

The study began during a low flow period in January 2018 and no major inflows had occurred since July 2017. There was limited connectivity between the weir pool and the estuary through Seaham Weir in January and February with the weir gates only being open for 1 hour on January 10, 2018. The weir gates were next opened on the March 1, 2018, with minor releases occurring over the next 2 weeks in response to a small inflow event (1,649 ML/Day). This was followed by a large inflow event peaking at 40,444 ML/Day on March 23, 2018 (Figure 4.2). After this event, operation of the weir gates maintained connectivity between upstream of the weir and the estuary with weir releases on a daily to weekly basis until the next inflow event on June 20, 2018, which had a peak discharge of 8,070 ML/Day (Figure 4.2). Following this inflow, the weir released on a daily to fortnightly basis. Each major inflow event reduced salinity (Figure 4.2a) being most pronounced at Site 1. Salinities decreased from 14.6 (± 0.01) and 16.4 ppt (± 0.01) in January at Site 1 and 2, to 0.22 (± 0.01) and 1.28 ppt (± 0.001) by April (Figure 4.2a). Salinities remained below 5 ppt at both sites between April and July, increasing to 9.9 (± 0.04) and 10.7 ppt (± 0.06) by August (Figure 4.2a).

Chl *a* concentration was variable throughout the study, with increases and decreases observed through time in response to inflows. There was no significant difference in Chl *a* throughout the study as determined by 2-way ANOVA (site*month, Appendix 7). The highest concentrations at Site 1 occurred after the releases of freshwater from the weir in early March, increasing the Chl *a* from 13 µg/L (± 0.01) in January to 36.5 µg/L (± 0.36) in March (Figure 4.2b) during a *Chrysosporum* sp. bloom (Appendix 8 and 9). This was then reduced to 5.75 µg/L (± 1.5) by April, following the larger March inflow (Figure 4.2b). At Site 2, Chl *a* concentration increased slightly through the first half of the study, from 14.5 µg/L (± 1.06) in January to 17.5 µg/L (± 4.5) in April (Figure 4.2b). This was followed by the highest Chl *a* concentration at Site 2 of 36.15 µg/L (± 1.66) in May (Figure 4.2b) during increased densities of *Chlamydomonas* and *Cryptomonas* cells in a low flow period (Appendix 8 and 9). However, this was soon reduced to 3.95 µg/L (± 0.25) by June 26, shortly after the June 20

inflow. Chl a was low at both sites after the June inflow but did increase to 9.63 (± 5.68) and 19.57 µg/L (± 1.14) in August at sites 1 and 2 respectively (Figure 4.2b).

Dissolved organic carbon (DOC) concentrations showed no significant differences between sites or months across the study (Appendix 7). DOC concentrations were below 5 mg/L at both sites through January and March but increased following the larger March inflow to 7.95 (± 0.04) and 8.05 mg/L (± 0.04) at sites 1 and 2, respectively (Figure 4.2c). DOC concentrations continued to increase to their highest concentrations of the study to 29.13 mg/L (± 5.36) at Site 1 and 22.49 mg/L (± 1.21) at Site 2 on the June 26 (Figure 4.2c), shortly after the inflow event on June 20, 2018. Turbidity has no significant differences between sites or months (Appendix 7); however, it was also noticeably increased by the larger March and June inflow events. Turbidity increased from 13.6 (± 0.39) and 5.23 NTU (± 0.66) at Site 1 and 2 in March to 19.2 (± 1.98) and 34.2 NTU (± 0.14) in April following the larger March inflows, reducing to 6.9 (± 0.40) and 9.96 NTU (± 0.24) by May (Figure 4.2d). Turbidity then increased to 47.1 (± 0.25) and 37.8 NTU (± 0.07) in June following the June inflow, before falling to 8.75 (± 0.03) and 10.0 NTU (± 0.07) in August (Figure 4.2d).

There were no significant differences in sPOM $\delta^{13}C$ ($\delta^{13}C_{POM}$) between sites ($F_{1, 10} = 0.12$, $p = 0.737$) or months ($F_{1, 10} = 0.75$, $p = 0.4.8$) with minimal fluctuation outside of the estuarine algae range established by the nutrient amendment experiment (-25.73 ± 1.6). $\delta^{13}C_{POM}$ signatures only became depleted relative to the estuarine algae signature range (Table 4.1) during periods where high Chl *a* concentrations and low salinities occurred, e.g., Site 1 in March where sPOM $\delta^{13}C$ was -31.1 ‰ ± 0.27 and at Site 2 in May -29.6 ‰ ± 0.42 (Figure 4.2a, b and e) and were negatively correlated to Chl *a* ($R^2 = 0.74$, $p < 0.001$). Inflows had a minimal influence on $\delta^{13}C_{POM}$ throughout the study with no significant correlations present for any antecedent discharge metrics, associated changes in DOC or turbidity or significant differences between months.

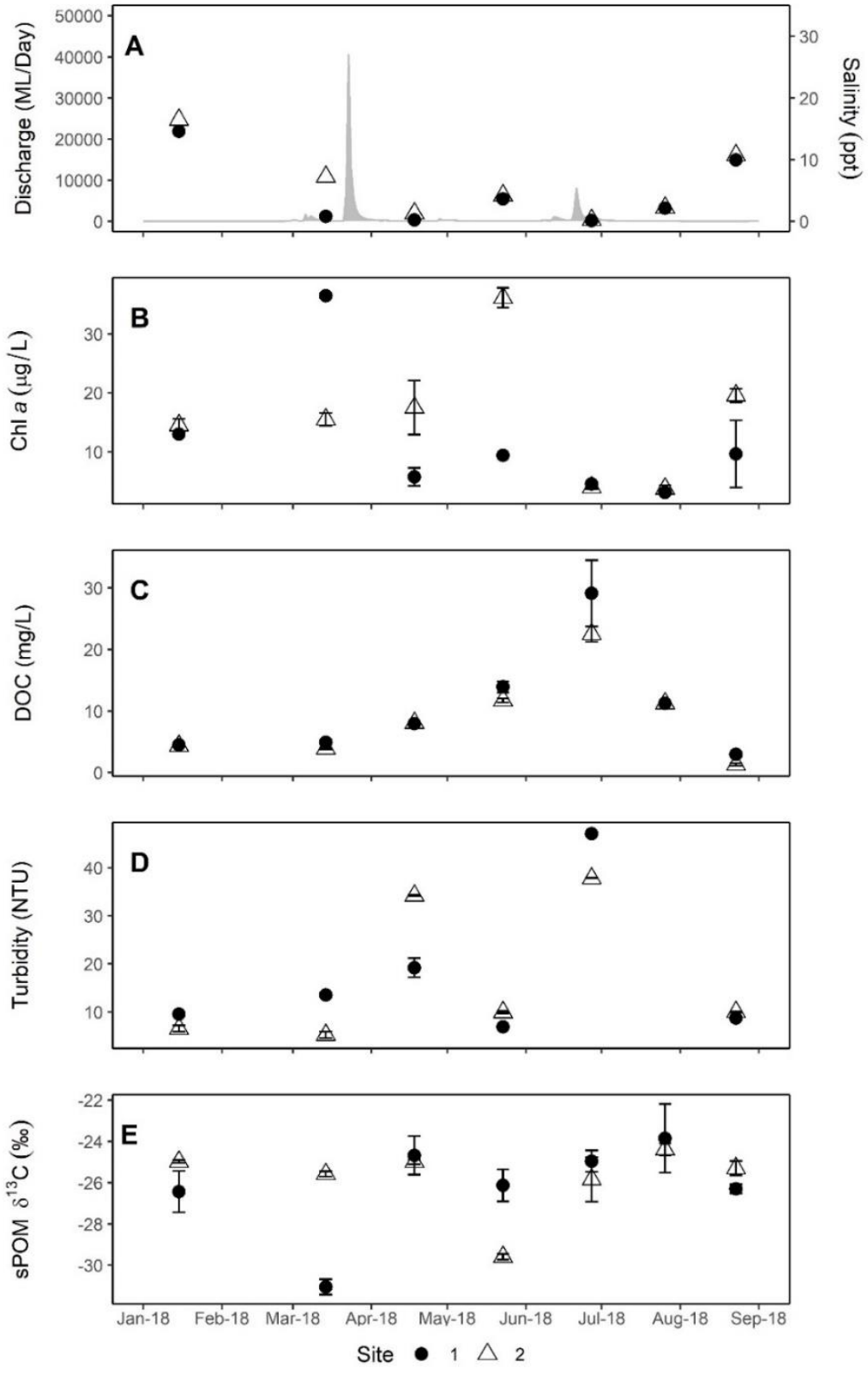

Figure 4.2: A) Discharge (shaded peaks) and salinity, B) chlorophyl a (Chl a), C) dissolved organic carbon (DOC) D) turbidity and F) $\delta^{13}C$ of suspended particulate organic matter (sPOM) through the study period, January 2018 to August 2018, at Sites 1 and 2 in the Williams River estuary. Error bars represent the standard error of the mean.

ANOVAs indicated there were no significant differences between sites for mesozooplankton $\delta^{13}C$ ($\delta^{13}C_Z$, $F_{1,10} = 0.54$ $p = 0.47$) or $\delta^{15}N$ ($\delta^{15}N_Z$, $F_{1,10} = 0.48$ $p = 0.50$), though differences could be seen visually between sites in March for $\delta^{13}C$ (Figure 4.3, Table 4.2) and April for both isotopes (Figure 4.3, Table 4.2). Kruskal Wallis analysis of $\delta^{13}C_Z$ signatures showed significant differences between months (KW, $H_2 = 43.2$, $p < 0.001$) highlighting 2 distinct groups in the study; with samples from April, July and August significantly depleted relative to those from January, March, May and June (Figure 4.3, Dunn's, $p < 0.05$). Zooplankton $\delta^{13}C$ from the months of April, July and August averaged -29.4 ‰ (± 0.16) while samples from other months (January, March, May and June) averaged -26.7 ‰ (± 0.29). Kruskal-Wallis testing also revealed significant differences in $\delta^{15}N_Z$ between months (KW, $H_2 = 43.7$, $p < 0.001$) with Dunn's *post-hoc* test indicating that samples from July and August were significantly more enriched in $\delta^{15}N$ compared to other months. However, there was overlap between months and no distinct groupings as there was for $\delta^{13}C_Z$. Correlation analysis indicated that there were no relationships between $\delta^{13}C_{POM}$ and $\delta^{13}C_Z$ across the study but $\delta^{15}N_{POM}$ and $\delta^{15}N_Z$ did correlate throughout the study ($R^2 = 0.37$; $p = 0.024$).

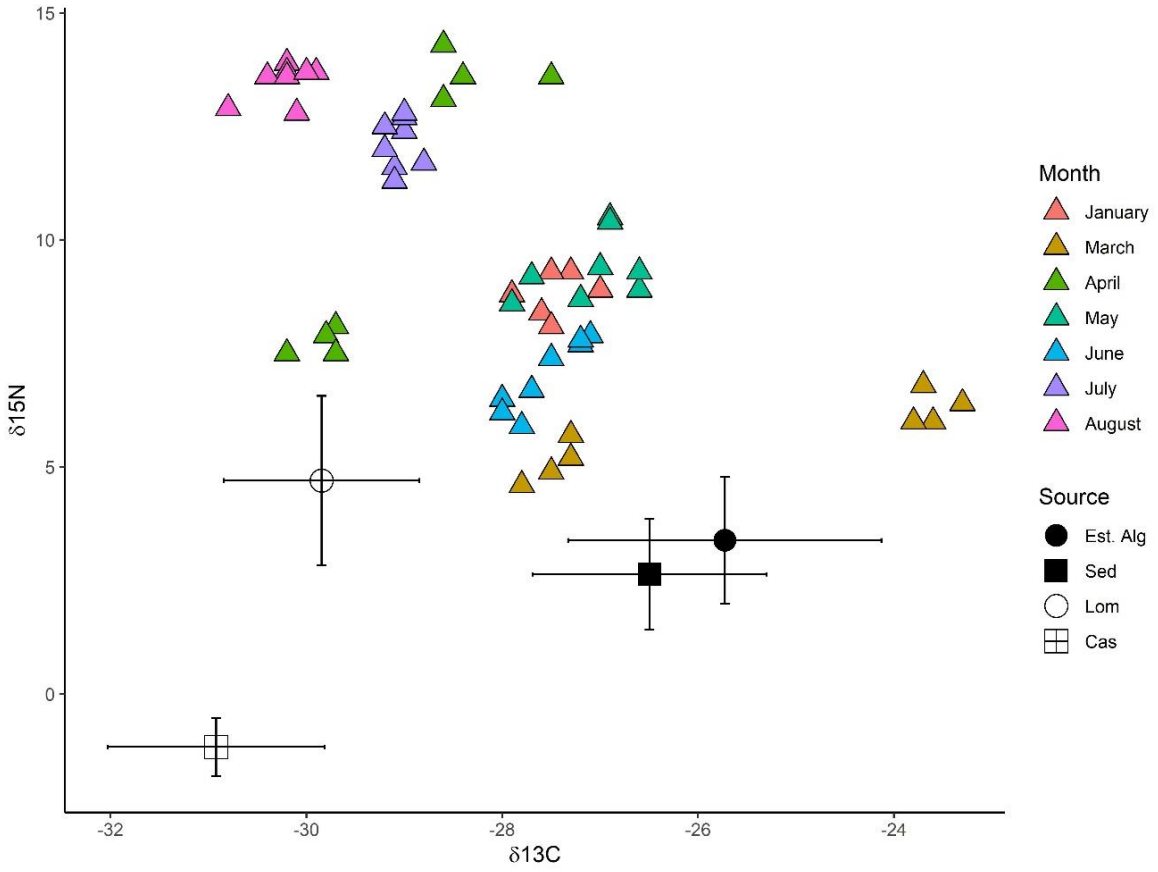

Figure 4.3: Stable isotopic signatures of carbon ($\delta^{13}C$, ‰) and nitrogen ($\delta^{15}N$, ‰) for mesozooplankton consumers from the Williams River estuary tidal freshwater zone during pre- and post-flow periods between January and August 2018. June samples were assigned their own group "flowing" due to their temporal proximity to an inflow in June. Mean endmember source signatures (± SE) are also shown; Est. Alg = estuarine algae, Sed = sediment organic matter, Lom = Lomandra longifolia, Cas = Casuarina glauca.

Mixing model analysis suggested that in periods where $\delta^{13}C_Z$ was more enriched (January, March, May and June) dominant mesozooplankton utilised a combination of estuarine algae and sediment OM as primary food web resources regardless of species or site (Table 4.2). These food web resources each accounted for up to half of the mesozooplankton diet in these periods, being as high as 71.4 % for estuarine algae (May – Site 1, Table 4.2) and up to 61.8% for sediment OM (March – Site 1, Table 4.2). Mesozooplankton in January (*G. pectinatus* and *Oithona* sp.) and May (*S. conflictus*) were more likely to have utilised estuarine algae over sediment OM, while in March combinations of *Oithona* sp. and *B. meridonalis* at Site 1 and *Oithona* sp. and *S. conflictus* at Site 2 were more likely to have used sediment OM (Table 4.2). Dominant mesozooplankton in June showed similar resource use patterns with combinations of estuarine algae and sediment OM again making up most of their diets (Table 4.2).

Mesozooplankton from April, July and August, when $\delta^{13}C_Z$ was significantly depleted, exhibited contrasting resource use patterns with increased contributions of terrestrial material and reduced contributions of estuarine algae and sediment OM (Table 4.2). In April, following the larger March inflow, *B. meridonalis* showed an increased likelihood of *L. longifolia* resource use, between 37.8 and 67.6 % of its diet, the highest in the study, and the lowest likelihood of estuarine algae utilisation in the study (7.4 – 22.3 %, Table 4.2). The dominant mesozooplankton in July and August, *S. conflictus*, also showed increased contributions of *L. longifolia* to its diet (up to 56.9 %) compared to pre-flow conditions (19.1 %, Table 4.2). Contributions of *L. longifolia* to the diet of *S. conflictus* were greatest in August (up to 56.9 %), when *C. glauca* also contributed significantly to their diet (37.5 %), compared to July where *L. longifolia*, sediment OM and estuarine algae contributed similarly (Table 4.2). Only *Oithona* sp. had a minimal contribution of terrestrial material to their diet in post-flow conditions (Table 4.2).

By continuing to contrast the periods of relative $\delta^{13}C_Z$ enrichment and depletion (Figure 4.3, Table 4.2) I found that values of $\delta^{13}C_Z - \delta^{13}C_{POM}$ (mesozooplankton signature minus sPOM signature) were also significantly different between these periods (KW, $H_2 = 42.4$, $p < 0.001$). Results from April, July and August were significantly more negative in $\delta^{13}C_Z - \delta^{13}C_{POM}$, ranging from -5.2 to -3.3 ‰ (Table 4.2), compared to results from January, March, May and June (Dunn's, $p < 0.001$), which ranged between -2.5 and 3.6 ‰ (Table 4.2).

Variations in the $\delta^{15}N$ of mesozooplankton minus the $\delta^{15}N$ of sPOM ($\delta^{15}N_Z - \delta^{15}N_{POM}$) outside of the expected TEF range occurred consistently within the study. $\delta^{15}N_Z - \delta^{15}N_{POM}$ was highest for predatory species in higher trophic levels, being as high as 10.3‰ for *S. conflictus* at Site 1 in July. However, interpreting food chain lengths relative to expected TEFs was invalid as the sources selectively consumed by zooplankton had different $\delta^{13}C$ ratios to sPOM and may have also had different $\delta^{15}N$ ratios. On the two occasions when $\delta^{13}C_Z - \delta^{13}C_{POM}$ was close to ± 1 ‰, $\delta^{15}N_Z - \delta^{15}N_{POM}$ values were similar to the expected TEF for G. *pectinatus* and *Oithona* sp. in January at Site 1, but much higher than expected for *S. conflictus* at Site 1 in May.

Table 4.2: Dominant mesozooplankton taxa in the Williams River estuary sorted by relative enrichment or depletion of $\delta^{13}C_Z$ through January to August of 2018, including where and when they were sampled, average $\delta^{13}C_Z$, $\delta^{13}C$ minus sPOM ($\delta^{13}C_Z$ - $\delta^{13}C_{POM}$), average $\delta^{15}N$, $\delta^{15}N$ minus sPOM ($\delta^{15}N_Z$ - $\delta^{15}N_{POM}$), expected TEF (trophic enrichment factor) range based on their trophic level (TL), and the primary, secondary and tertiary sources utilised by each group as identified by Bayesian mixing model (50% confidence interval). ± symbols indicate standard error, GP = Gladioferens pectinatus, OS = Oithona spp., BM = Bosmina meridonalis, SC = Sulcanus conflictus, Est. Alg = estuarine algae, Sed = sediment organic matter, Lom = Lomandra longifolia and Cas = Casuarina glauca.

Relative $\delta^{13}C_Z$	Dominant Zooplankton	Month-Site	TL	$\delta^{13}C$ ‰	$\delta^{13}C_Z$ - $\delta^{13}C_{POM}$ ‰	$\delta^{15}N$ ‰	$\delta^{15}N_Z$ - $\delta^{15}N_{POM}$ ‰	Expected TEF range	Primary Source (%)	Secondary Source (%)	Tertiary Source (%)
Enriched	GP/OS	Jan – 1	1	-27.4 (± 0.45)	-1.1	8.8 (± 0.24)	1.8	2.2 (± 0.3)	Est. Alg (20.2 – 61.1)	Sed (15.9 – 55.7)	
	GP/OS	Jan – 2	1	-27.5 (± 0.16)	-2.5	9.0 (± 0.59)	3.6	2.2 (± 0.3)	Est. Alg (20.3 – 58.3)	Sed (17.6 – 55.5)	
	OS/BM	Mar – 1	1	-27.5 (± 0.23)	3.6	5.1 (± 0.47)	6.0	2.2 (± 0.3)	Sed (27.5- 60.8)	Est. Alg (17.7-48.9)	
	OS/SC	Mar – 1	1.5	-23.6 (± 0.21)	2.0	6.3 (± 0.38)	7.3	3.3 (± 0.45)	Sed (16.6 – 51.1)	Est. Alg (15.7 – 44.6)	
	SC	May – 1	2	-26.8 (± 0.17)	-0.6	9.8 (± 0.80)	7.3	4.4 (± 0.6)	Est. Alg (22.4 – 71.4)	Sed (11.0 – 48.7)	
	SC	May – 2	2	-27.5 (± 0.42)	2.1	9.0 (± 0.39)	2.7	4.4 (± 0.6)	Est. Alg (24.9 – 65.2)	Sed (13.0 – 48.6)	
	OS/BM	June – 1	1	-27.2 (± 0.17)	-2.3	7.7 (± 0.22)	4.1	2.2 (± 0.3)	Est. Alg (21.8-63.3)	Sed (14.8- 54.6)	
	BM/SC	June – 2	1.5	-27.9 (± 0.15)	-2.0	6.3 (± 0.35)	4.0	3.3 (± 0.45)	Sed (27.8-47.4)	Est. Alg (17.4 – 46.2)	
Depleted	OS	April – 1	1	-29.9 (± 0.24)	-5.2	7.8 (± 0.30)	2.0	2.2 (± 0.3)	Sed (17.2 -48.9)	Est. Alg (16.4 – 48.7%)	
	BM	April – 2	1	-28.3 (± 0.53)	-3.3	13.7 (± 0.49)	5.3	2.2 (± 0.3)	Lom (37.8 – 67.6)	Est. Alg (7.4 – 22.3)	
	SC	July – 1	2	-29.0 (± 0.14)	-5.1	11.8 (± 0.47)	10.4	4.4 (± 0.6)	Lom (16.7 – 38.7)	Sed (14.8 – 40.2)	Est. Alg (14.8 – 39.6)
	SC	July – 2	2	-29.1 (± 0.12)	-4.7	12.5 (± 0.36)	7.8	4.4 (± 0.6)	Lom (16.8 – 38.9)	Sed (14.6 – 38.2)	Est. Alg (14.1 – 38.9)
	SC	Aug – 1	2	-30.3 (± 0.39)	-4.0	13.3 (± 0.56)	6.9	4.4 (± 0.6)	Lom (23.7 – 56.9)	Cas (13.0 – 37.5)	
	SC	Aug – 2	2	-30.2 (± 0.16)	-4.9	13.7 (± 0.06)	7.1	4.4 (± 0.6)	Lom (23.5 – 55.4)	Cas (13.9 – 37.4)	

Median contributions of the two dominant dietary resources, estuarine algae and *L. longifolia* (representing terrestrial sources), to mesozooplankton diets (as determined by mixing model analysis) were significantly different between the enriched and depleted $\delta^{13}C_Z$ groups (ANOVA, $F_{2,24} = 53.9$, $p < 0.001$). Combining the results from the months of January, March May and June, where $\delta^{13}C_Z$ was enriched, estuarine algae accounted for 38.9 % of dietary resources of the mesozooplankton community, a significantly larger contribution than terrestrial resources, 11.8 %, in the same months (Figure 4.4, Tukey's HSD, $p < 0.001$). In contrast, during the months of April, July and August, where $\delta^{13}C_Z$ was depleted, the average contribution of estuarine algae was significantly less at 18.8 % (Figure 4.4, Tukey's HSD, $p = 0.001$), while contributions of terrestrial sources were significantly higher at 35.3% (Figure 4.4, Tukey's HSD, $p < 0.001$).

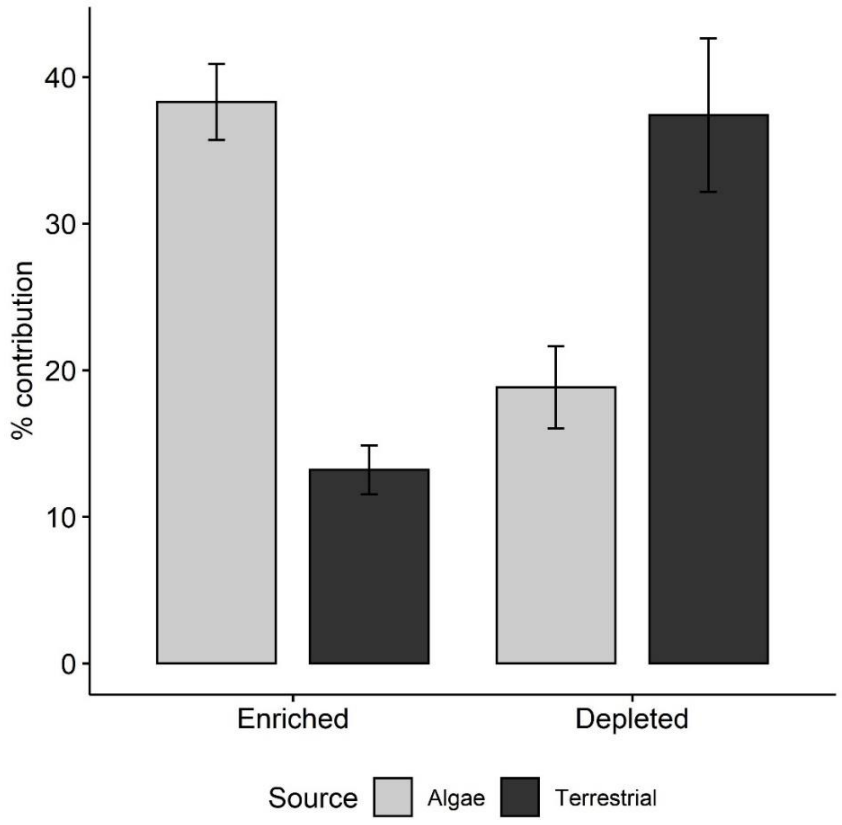

Figure 4.4: Mean percentage contributions of algae (estuarine algae) and terrestrial (Lomandra longifolia) food web resources to mesozooplankton diets between months where zooplankton δ13C was enriched (January, March, May and June) and depleted (April, July and August) between January 2018 to August 2018 in the Williams River estuary as determined by Bayesian mixing model. Error bars represent standard error of the mean.

4.5 Discussion

Freshwater inflows to estuaries influence the spatial and temporal distributions of organic matter resources and their assimilation (Atwood et al. 2012; Liu et al. 2020). Most investigations of these dynamics have been conducted in larger bays, lower estuaries and nearshore plumes while upper estuarine areas are relatively ignored (Xu et al. 2021). River regulation is increasing in Australia (Arthington et al. 2018b) and may negatively impact the magnitude and timing of inflows and the trophic dynamics of estuaries (Ru et al. 2020; Liu et al. 2020), particularly in areas where freshwater inputs are key to ecological processes (Pierson et al. 2002). It is therefore important that we understand how regulated estuaries and their food webs respond to inflows and the utilisation of terrestrial resource inputs, to better inform resource management decisions (Scheltinga et al. 2006).

4.5.1 Allochthony following inflows

The data presented in this chapter suggests that mesozooplankton in the upper William River estuary switched from relying predominantly on autochthonous food sources during lower flow periods prior to inflows (January, March and May) to allochthonous sources in the months following inflows (April, July and August; Figure 4.3 and 4.4, Table 4.2). This supported my first hypothesis – that inflows deliver terrestrial carbon resources that, in turn, are used in mesozooplankton diets. This was consistent across species tested and longitudinal position in the estuary. Interestingly, the mesozooplankton sampled in June, six days after an inflow event, were more similar in $\delta^{13}C$ signatures to the months of January, March, and May. This may be due to a temporal lag in zooplankton isotopes, which can take several days to weeks to occur following changes in diet (Tiselius and Fransson 2016), suggesting that the June signatures may be reflective of resources utilised prior to the inflow.. While many studies have shown that autochthonous carbon is a major resource for estuarine food webs (Cloern et al. 2002; Martineau et al. 2004; Delong and Thorp 2006), these results support the growing body of literature demonstrating allochthony in estuarine mesozooplankton (van den Meersche et al. 2009), and particularly after inflow periods (Hoffman et al. 2008; Atwood et al. 2012; Liu et al. 2020). Studies from large bays and in near shore plumes have reported dietary shifts in estuarine mesozooplankton with similar mesozooplankton $\delta^{13}C$ ratios, between -28 and -32‰ $\delta^{13}C$, and mixing model analysis results indicating increased allochthony following inflow events and monsoon seasons (Schlacher et al. 2009; Atwood et

al. 2012; Liu et al. 2020). Moreover, the few studies conducted in more upstream areas of estuaries have reported increased terrestrial support of mesozooplankton following inflow events and terrestrial inputs in years of increased freshwater discharge (Hoffman et al. 2008; Hitchcock et al. 2016a). In particular, Hoffman et al.'s (2008) study on the Mattaponi river showed a negative relationship between discharge and autochthony and in periods of increased discharge there was increased mesozooplankton allochthony, which is consistent with my results (Table 4.2, Figure 4.4). Given the similarity of my results to previous studies, the increased allochthony following inflows suggests that terrestrial resources are an important part of the mesozooplankton diet when delivered to the Williams estuary, reinforcing the relationships outlined in Chapter 2 and highlighting the importance of freshwater inflows supporting estuarine food webs and ecology.

Employing stable isotope mixing model methods to the interpretation of terrestrial resource use is reliant on the endmember signatures used (Hoffman et al. 2008; Bartels et al. 2018). While there are difficulties in applying mixing model analysis due to overlapping signatures (Deegan and Garritt 1997) and temporal lags in isotopic signatures for both sPOM and zooplankton (Middelburg and Herman 2007; Tiselius and Fransson 2016), this approach is commonly used (Hoffman et al. 2008; Schlacher et al. 2009; Berggren et al. 2015). The endmember signatures in this study were determined through several approaches. The estuarine algal $\delta^{13}C$ range was derived from a novel and localised experiment during low flow conditions, which ensured sPOM was dominated by algal cells at the time of sampling (Appendix 5), providing a more accurate autochthonous algal signature (Appendix 6), although this could have been influenced by the algal genera that dominated these samples (Vuorio et al. 2006). Regardless, this signature was isotopically distinct from terrestrial endmembers and stoichiometrically (C:N ratios) distinct from sediment OM endmembers (Table 4.1), with both terrestrial endmembers locally obtained and similar to sediment and terrestrial organic material signatures used in other studies (Deegan and Garritt 1997; Cloern et al. 2002; Hoffman et al. 2008; Atwood et al. 2012). Moreover, I utilised a 4-source model inclusive of a dominant nitrogen fixing terrestrial species (*C. glauca*, Zhong et al., 2013) and sediment OM that may have also contributed to the organic matter pool in the Williams estuary, increasing the diversity of sources in my model. Other potential sources of organic matter in Australian estuaries such as salt marsh or mangroves (Connolly et al. 2009; Schlacher et al. 2009) were excluded from analysis due to their low presence at the study

sites. My model was further strengthened by using elemental concentrations of carbon and nitrogen for endmembers and consumers as well as the use of trophic enrichment factors for specific groups of dominant zooplankton (Parnell 2021). Considering these approaches, and the similarity of my results to previous studies (Hoffman et al. 2008; Schlacher et al. 2009; Atwood et al. 2012), I am confident in the outputs, suggesting that inflow events drove a switch to allochthonous resource use in periods following inflows in the Williams estuary.

4.5.2 Allochthony and relationships to sPOM $\delta^{13}C$

While $\delta^{13}C_Z$ data suggested that terrestrial resource use by mesozooplankton was evident for several months after inflows, zooplankton signatures did not positively correlate with $\delta^{13}C_{POM}$ following inflows as hypothesised. Increased differences in $\delta^{13}C_Z$ - $\delta^{13}C_{POM}$ away from ±1 ‰ have previously suggested selective feeding behaviour by zooplankton (Deegan and Garritt 1997; Hoffman et al. 2008). In this case of my study, $\delta^{13}C_Z$ - $\delta^{13}C_{POM}$ was rarely within the ±1 ‰ range (Table 4.2) indicating that feeding on sources other than sPOM may be occurring. Positive $\delta^{13}C_Z$ - $\delta^{13}C_{POM}$ values occurred in March at both sites and in May at Site 2 indicating assimilation of isotopically enriched sources relative to sPOM (Table 4.2). These occurred during periods of increased algal biovolume at those sites (Appendix 8), with the $\delta^{13}C_{POM}$ changing significantly from the reference autochthonous signature with the presence of different dominant algal groups (*Chrysosporum, Chlamydomonas* and *Cryptomonas)*. Different algal taxa can produce influence $\delta^{13}C_{POM}$ (Vuorio et al. 2006), however, the only notable variation in $\delta^{13}C_{POM}$ was during periods of increased algal density rather than changes in taxa. Meanwhile, negative $\delta^{13}C_Z$ - $\delta^{13}C_{POM}$ results demonstrated the assimilation of isotopically depleted food sources relative to sPOM, notably in the months of April, July and August where $\delta^{13}C_Z$ was more depleted, as well as in June (Table 4.2). Only in January and May at Site 1 was the $\delta^{13}C_Z$ - $\delta^{13}C_{POM}$ close to ±1 ‰.

Selective feeding is common in mesozooplankton as they choose resources that are suitable for their dietary requirements and reject others (Mitra et al., 2014b). Selective grazing can also aid consumers avoid harmful foods such as cyanobacteria (Demott and Moxter 1991), a phenomenon that may have led to the positive differences in $\delta^{13}C_Z$ - $\delta^{13}C_{POM}$ in March during the *Crysosporum* sp. bloom at both sites (Table 4.2, Appendix 8 and 9). However, selective feeding behaviour is usually associated with a selection for autochthonous resources (Tackx et al. 2003; Sobczak et al. 2005), even when allochthonous material is dominant (Cloern et al. 2002; Martineau et al. 2004). In this study, $\delta^{13}C_{POM}$ did not reflect terrestrial signatures

during periods of high turbidity and low Chl *a* as expected, indicating it may not have been the dominant fraction of sPOM available in the estuary following inflows (Figure 4.2). This suggests that for zooplankton to utilise terrestrial resources they may have had to select for terrestrial fractions of sPOM. These results are distinct from other studies which indicate post-flow allochthony in estuaries is facilitated by passive assimilation of terrestrially material when it dominates sPOM following inflows (Hoffman et al. 2008; van den Meersche et al. 2009). While it is impossible to be certain I offer two reasons which may account for my contrasting results.

Selective use of tPOM by mesozooplankton may have been due a process known as co-metabolism which can occur to various extents in copepods and cladocerans (Brett et al. 2009; Harfmann et al. 2019). This process can allow organisms to increase their growth and survival rates by selectively consuming larger amounts of lower quality, allochthonous particulates simultaneously with less abundant but high quality, autochthonous material (Harfmann et al. 2019). In periods where allochthonous resources become plentiful, e.g., after inflows, co-metabolism and selective use of tPOM may be a beneficial feeding strategy for mesozooplankton (Harfmann et al. 2019). This process would concur with the findings of several studies which purport that direct consumption of terrestrial particulates are the primary way terrestrial material enters zooplankton food webs (Cole et al. 2006; Hoffman et al. 2008; van den Meersche et al. 2009). An alternative explanation for the larger differences in $\delta^{13}C_Z$ - $\delta^{13}C_{POM}$ following inflows may be the use of tDOM and microbial energy pathways, which can occur in turbid estuaries (Mitra et al., 2014; van den Meersche et al., 2009). A similar process has also been recognised in lake systems with mixotrophic protists facilitating the transfer of tDOM to mesozooplankton and changing their $\delta^{13}C$ signatures after terrestrial resource inputs (Jansson et al. 2007; Trochine et al. 2015). Manipulative experiments have shown tDOM can contribute to increased adult zooplankton densities through microbial processes at dissolved organic carbon (DOC) concentrations > 10 mg/L and for nauplii at concentrations > 3 mg/L (Hitchcock et al. 2010, 2016b). In my own study, DOC concentrations were elevated above 10 mg/L following inflows (Figure 4.2), the dominant algal species in post-flow conditions were mixotrophic flagellates (*Cryptomonas* and *Chlamydomonas*, Appendix 8 and 9) and nauplii were the dominant zooplankton (Appendix 10). Hence, this pathway could be potentially transferring tDOM to *S. conflictus*. Unfortunately, nauplii were not retained in this experiment due to the mesh size of the nets

but must be considered in future studies. The role of mixotrophic protists in zooplankton diets such as omnivorous nauplii, particularly in transferring terrestrial carbon after inflows, is one that requires further exploration (Stoecker et al., 2019).

4.5.3 Prolonged allochthony

Previous studies have reported that allochthony is only exhibited for a limited time in mesozooplankton consumers following inflows, e.g., for only a few weeks after inflows (Schlacher et al. 2009; Bartels et al. 2018). This considers the temporal lags in zooplankton isotopes which may take several days to a week to shift following changes in resource use, exacerbating the periods of allochthony (Tiselius and Fransson 2016). For example, in this study, six days after the June flow mesozooplankton $\delta^{13}C$ was similar to pre-flow conditions (Figure 4.2) and had not begun to show changes in diet that became apparent in July. Beyond this short-term period, my results indicate that terrestrial support of mesozooplankton following inflows may continue for over a month in the upper estuary, particularly following the June inflow (Table 4.2). Spatial gradients in estuaries can determine resource use, with increased terrestrial resource use closer to riverine sources under low and high flow conditions (Chanton and Lewis 2002; Lautenschlager et al. 2014; Bartels et al. 2018). For example, allochthonous support of mesozooplankton during an inflow was strongest in zooplankton closest to the mouth of the Ore River estuary following an inflow compared to near shore areas (Bartels et al., 2018). The proximity of the upper estuarine area to riverine sources may therefore increase the relative importance and availability of allochthonous carbon sources to planktonic food webs and, by extension, prolong allochthony relative to more marine influenced areas of estuaries (Lautenschlager et al. 2014). This may be particularly relevant in estuaries where tidal resuspension of deposited sediments may occur (van den Meersche et al. 2009), although the similarity in salinity between the two upstream sites and the low salinity throughout the study indicated that tidal action was not strong in the Williams, though the tidal limit is at Seaham Weir. Additionally, seasonal patterns are known to govern allochthonous resource use in zooplankton, with colder temperatures suppressing autochthonous algal production (Dorado et al. 2015), which potentially increasese the reliance of zooplankton on allochthonous resources (Rautio et al. 2011; Berggren et al. 2015; Dias et al. 2016). This may explain the extended periods of mesozooplankton allochthony following the inflow in June (up to two months) compared to the larger inflow in April (1 month) as well as other studies (up to two weeks, Schlacher et al. 2009; Bartels et al. 2018).

A growing body of work documents the impacts of river regulation on aquatic systems and their trophic structures (Sabo et al. 2018; Ru et al. 2020; Liu et al. 2020). These studies suggest that disruptions to riverine continuums can influence the transport of organic matter and subsequently alter the food webs of rivers and estuaries, (Ru et al. 2020), with food web complexity and trophic length increasing with terrestrial inputs (Sabo et al., 2018). While limited studies exist documenting the impact of river regulation on estuarine food webs, a study from the Coorong River estuary has shown that inflows to regulated systems increase the importance of heterotrophic energy resources to food webs, resulting in longer food chains and a greater importance of bacteria and flagellates to estuarine food webs (Hemraj et al. 2017b). These findings were attributed to the rapid shifts between high and low flow conditions in regulated estuaries and the increased organic loading this can cause in the upper estuary following inflows (Connell et al. 1981; Hemraj et al. 2017b). Similar processes may also be contributing to the prolonged periods of allochthony in the Williams estuary after inflows. In Chapter 2 it was shown that Seaham Weir may increase the retention of terrestrial resources in the upper estuary and that copepod nauplii were positively related to inflows, DOC and turbidity. Meanwhile in Chapter 3, bacteria and flagellates were important elements of the post-flow food web in the upper estuary, suggesting more complex food webs and longer trophic chains. While the true effect of river regulation on resource use and food web structure in the Williams estuary is outside of the scope of this study, Seaham Weir may be increasing the importance of allochthonous resources to productivity following inflows. However, the response of estuaries and their food webs to inflows is highly individual (Abrantes et al. 2013; Roelke et al. 2013) and requires more detailed study to fully understand the influence of river regulation on post-inflow food web dynamics. Considering river regulation can impact on the transfer and quality of terrestrial organic matter entering estuaries (Drinkwater and Frank 1994; Palinkas et al. 2019) and the food web structure of aquatic systems (Sabo et al. 2018; Ru et al. 2020) further research may allow more informed decisions to be made regarding inflow management, such as the timing and size of inflows.

4.5.4 Limitations

There are multiple limitations in this experiment which must be mentioned, particularly in regard to the nutrient amendment experiment portion of the study and the additional endmembers used. The estuarine algal signature derived from the nutrient amendment experiment came from a single algal taxon, *Chlamydamonas sp.*. It is well known that $\delta^{13}C$

can vary between algal taxa (Vuorio et al. 2006) and is also dependent on salinity in which photosynthesis is occurring due to the fractionation of DIC (Fry 2002). It is possible that if the nutrient amendment experiment was conducted at a different salinity and had a different dominant algal taxon that the endmember $\delta^{13}C$ value for estuarine algae may have been different and changed the interpretation of the results. However, considering the approaches used and the caveats noted in section 4.5.1 we still feel that our interpretation, and use of this method is valid, though these limitations must be taken into account. Likewise, a more detailed collection of terrestrial endmember sources may have provided a more extensive understanding of terrestrial resources potentially contributing to mesozooplankton diets post flow and this limitation must also be acknowledged.

4.5.5 Conclusions

The results of this study indicate the importance of terrestrial resources and inflows to productivity in the Williams estuary, building on the findings of Chapter 2. Critically, the results show a shift from use of basal autochthonous sources during lower flow periods to use of allochthonous sources following flow events. Interestingly, my results contrast with other studies where sPOM and zooplankton $\delta^{13}C$ signatures positively correlate following inflows highlighting the complexity of terrestrial resource assimilation by mesozooplankton food webs. Prolonged periods of allochthony, of up to two months, were also evident in the Williams estuary following inflows. Where Chapter 2 highlighted a positive association between inflows, DOC, turbidity and copepod nauplii concentrations in the Williams River estuary, this chapter demonstrates the importance of terrestrial resources and inflows to the mesozooplankton food web of the Williams River estuary.

Chapter 5: Can zooplankton fed allochthonous carbon diets support and sustain juvenile fish?

5.1 Abstract

Freshwater inflows have long been linked to fish abundance and fishery catch rates in estuaries. While inputs of allochthonous nutrients are purported to increase algal production subsequently supporting increased fish growth and abundance in estuaries, the role of allochthonous carbon in this process is still debated. I performed a study where the effects of varying levels of terrestrial dissolved organic matter (tDOM) additions to a food limited diet of juvenile Australian bass (*Macquaria novemculeata*) were investigated. Nauplii of the crustacean zooplankton *Artemia franciscana* (*Artemia*) were reared for two days under control conditions (no addition) or with additions of tDOM leachate made up from terrestrially derived native *Casuarina glauca* and *Eucalyptus* sp. leaves at dissolved organic carbon (DOC) concentrations of 5 mg/L or 10 mg/L. *Artemia* were fed to juvenile bass in their treatment tanks over 42 days at feeding rates reduced by 65-75% of *ad libitum*. Juvenile fish fed the 5 mg/L reared *Artemia* exhibited no statistical difference for weight, standard length, fork length or total length compared to the control treatment. However, fish fed *Artemia* grown in the 10 mg/L tDOM treatment had significant increases ($p < 0.05$) in all length parameters after 28 days compared to other treatments and were significantly greater in weight and dorso-ventral depth by 42 days. Stable isotope analysis of fish tissue did not show significant changes towards signatures of the terrestrial carbon. Decreased highly unsaturated fatty acid (DHA, EPA and ARA) concentrations of fish tissue and weights of juveniles across all treatments suggested utilisation of endogenous lipids and proteins to facilitate growth under food limitation. I suggest that increased terrestrial resource availability in juvenile fish diets subsidised energetic needs, allowing greater proportions of endogenous resources to contribute to growth and development, resulting in greater lengths of fish where tDOM was available. In this manner, organic material of terrestrial origin brought to estuaries by inflows may ecologically link the improved recruitment of juvenile Australian bass and other species to increased freshwater discharge.

This study was approved by the University of Technology Sydney Animal Care and Ethics Committee under permit ETH19-3895.

5.2 Introduction

Freshwater inflows have been linked to estuarine and nearshore fisheries abundance and catch rates for several decades (Loneragan and Bunn 1999; Gillanders and Kingsford 2002; Connolly et al. 2009). The links between inflows and estuarine fish abundances are thought to be driven by a combination of facilitation movement for diadromous fish, terrestrial nutrient delivery stimulating algal growth, increased habitat access and favourable changes in environmental conditions (ter Morshuizen et al. 1996; Robins et al. 2005; Gillson 2011). In recent decades researchers have also suggested that terrestrial organic carbon delivered during freshwater inflows may provide an energetic subsidy to fish productivity (Salen-Picard et al. 2002; Darnaude et al. 2004; Hoffman et al. 2007; Abrantes and Sheaves 2010).

Terrestrial organic carbon in aquatic food webs has been considered a poor energy source for production of zooplankton and presumably fish (Brett et al. 2009; Kelly et al. 2014; Taipale et al. 2014). Firstly, it has poor nutritional quality compared to algae, with low concentrations of essential highly unsaturated fatty acids (HUFA) necessary for growth and development of zooplankton and fish (Sargent et al. 1999a; b; Brett et al. 2009). Secondly, most terrestrial organic carbon is available in dissolved form (Aitkenhead-Peterson et al. 2003) requiring a higher number of trophic levels before energy is transferred to fish. This results in the majority of its energy and nutritional value being respired by microbes and other microplankton (Ducklow et al. 1986). However, when the flux of terrestrial carbon substantially outweighs the bulk of algal carbon, such as after inflows when terrestrial inputs are large and comparatively more bioavailable (Hitchcock and Mitrovic 2015b), terrestrial resources can become a dominant energy source supporting growth of bacterial, microzooplankton, mesozooplankton and benthic invertebrate communities in estuaries (Asmala et al. 2014a; Hitchcock et al. 2016a; Bartels et al. 2018; Berggren et al. 2018).

Increased prey densities following freshwater inflows, supported by terrestrial carbon, have been suggested as a reason for increased recruitment success of juvenile fish following higher inflow periods (Darnaude et al. 2004; Hoffman et al. 2007; Kostecki et al. 2010). Increases in prey density can result in increases in larval and juvenile fish growth and body size, which in turn increases the success rate of juvenile recruitment (Houde 1997; Sogard 1997). Research has suggested that terrestrial subsidies may be more important for early life-history stages of fish and sub-adults as they feed on lower trophic levels within the food web (von Biela et al.

2013). However, studies exploring terrestrial support of zooplankton suggest that terrestrial carbon is unlikely to contribute to growth and development (metabolism) and is instead selectively used for energetic demands (catabolism) due to the lack of physiologically important HUFA (Brett et al. 2009; Taipale et al. 2014). Developing a better understanding of the role terrestrial carbon plays in supporting the growth and survival of fish during early life-stages is important in understanding recruitment success following inflows.

Manipulative studies on the impact and role of terrestrial resources on the somatic growth of juvenile fish are limited. Degerman et al. (2018) found that additions of dissolved organic carbon (DOC) as glucose to an existing algal food web decreased the growth of planktivorous juvenile fish **(***Gasterosteus aculeatus***)** due to the channelling of energy through inefficient bacterial pathways. Meanwhile, Growns et al. (2020) found additions of terrestrial dissolved organic matter (tDOM) as a leachate had no positive influence on the growth of carp gudgeons (*Hypseleotris* spp.), although they acknowledged an abundance of algal resources may have overshadowed any potential influence of tDOM on growth. They therefore proposed future investigations use a single source diet where terrestrial resources were abundant and algal resources excluded (Growns et al., 2020). This single source diet may be more representative of food web conditions that can occur in estuaries after larger inflows where terrestrially derived resources and heterotrophic processes dominate the food web and algal resources are limited, if only for a short period (Bruesewitz et al. 2013; Hemraj et al. 2017b).

Australian bass (*Macquaria novemculeata*) is an endemic catadromous species that has been widely impacted by river regulation and reductions in riverine-estuary connectivity (Growns and James 2005; Harding et al. 2017). Multiple links between Australian bass and inflows have been established, with the importance of inflows during spawning seasons well documented (Growns and James 2005; Morrongiello et al. 2014; Harding et al. 2017). However, the mechanisms underlying these relationships are not fully understood (Harding et al. 2017; Stoessel et al. 2018). This study aimed to improve this understanding by undertaking an experiment investigating if terrestrial resource availability could positively influence somatic growth of juvenile Australian bass. My experiment used varying levels of tDOM additions to a planktonic food web, energetically enriching zooplankton prey with terrestrial material and limiting algal resource availability. I hypothesised that increased carbon availability from the leachate would result in increased microbial production,

transferring additional terrestrial energy to *Artemia* and enriching them as a prey resource for juvenile Australian Bass (tDOM → microbes → *Artemia* → fish). *Artemia* exposed to the higher concentrations of carbon leachate would theoretically become more energy dense, transferring more terrestrial energy to juvenile Australian Bass resulting in increased somatic growth of juvenile fish over the experimental period. I also monitored the stable isotope ratios of fish tissue, to see if tDOM contributed to new tissue growth, and concentrations of key HUFA (DHA, EPA and ARA) to see if tDOM additions contributed HUFA to juvenile fish and to assess their nutritional health.

5.3 Methods

5.3.1 Experimental Setup and Acclimation

Prior to the experiment, leachates were prepared from a mix of senesced *Eucalyptus* sp. and *Casuarina glauca* leaves (approximately 2 kg wet weight) added to 40 L bins of reverse osmosis (RO) water and left in a refrigerator overnight (3.8 ºC). After 24 hours, the leachate was filtered through pre-combusted glass fibre filter papers (GF/F grade, 47 mm, Whatman) and refrigerated, stored in batches. Batches were independently tested for dissolved organic carbon and nutrient levels to allow treatment additions to be regulated across batches. The average ratio of dissolved C:N:P in the leachate used was 156.3 (±41.2): 6 (± 0.0): 1 (± 0.0) highlighting how the added leachate would not be a significant source of nitrate and phosphorous to the system. This leachate was stoichiometrically representative of terrestrial organic matter in aquatic systems after freshwater inflows (Lennon and Pfaff 2005).

Australian bass juveniles were acquired 1 hr post feeding from Port Stephens Fisheries Institute (PSFI) hatchery at 40 days after hatching (DAH). Prior to collection, fish were reared by PSFI Hatchery following methods of Fielder and Heasman (2011). Fish were housed in 27 separate 20 L plastic tanks (length = 45 cm; width = 30 cm; depth = 30 cm) in a filtered flow through system (5 L/h water exchange) with aeration systems in a temperature and light insulated room located at Port Stephens Fisheries Institute, NSW, Australia. A total of 200 fish was stocked into each tank to provide 10 fish/L; a density previously used in experimental studies on the species (Fielder and Heasman 2011). Tanks were labelled for treatment (control, 5 mg/L and 10 mg/L, n=3) and sampling time point (n=3) and randomly allocated to 4 large water baths and set up as part of a flow through system maintained at 20 ºC (Table 5.1). Replicate tanks for different time points were used to minimise the effects of

manual handling, repeated measures and growth depensation. Juvenile Australian bass (mean ± S.E. standard length = 12.8 m ± 0.2 mm) of unknown sex were randomly allocated to each tank (n=200 fish). Fish were acclimated to a salinity of 20 ppt (Table 5.1) over the next 24 hours using gradual additions of pre-mixed 20 PSU water until it had filled the system. This salinity level was selected to reduce mortality rates of juvenile Australian bass (Fielder and Heasman 2011). Full spectrum artificial light (Ledzeal, Malibu S series) was provided on a 12-hr light:12 hr dark cycle and water was filtered (10 µm) prior to re-entering the system to reduce particulates entering the tanks. Tanks and filters were cleaned daily prior to feeding. Water temperature, salinity, pH and oxygen saturation were monitored daily with a multiprobe (WTW, Multi3430) to ensure conditions were stable (Table 5.1) and ammonia was measured with a test kit (Aqua One® Quick Drop Ammonia Test Kit). Fish behaviour was observed in the half hour prior to and post feeding with any deceased fish removed prior to feeding. Mortality rates throughout the experiment were low with an average loss of 17% of fish from each replicate across the study (Appendix 11), similar to the mortality rates found in the PSFI hatchery under optimal feeding conditions (van der Wal and Nell 1986; Fielder and Heasman 2011).

Table 5.1: Average water quality conditions in system over the 42-day experimental period.

	Temperature(ºC)	DO mg/L	pH	Salinity (ppt)
Average	19.6	9.5	8.0	20.1
SEM	0.083	0.046	0.018	0.11

5.3.2 *Artemia* and Feeding

Artemia were used to transfer the leachate across trophic levels, a process similar to the enrichment of *Artemia* by the stocking program by PSFI hatchery (Fielder and Heasman 2011). *Artemia* nauplii were chosen for two reasons; firstly, they are a commonly used food source for rearing juvenile fish (Sorgeloos et al. 2001) and secondly, they maintain a similar food web position to the planktonic crustacea which are the dominant food source of juvenile Australian Bass in estuaries until they reach a size > 25 mm (Harris 1985). *Artemia* were hatched in 100 L of seawater at 30ºC over 20 hrs using 30 g of *Artemia* cysts (Sep-Art® GSL Magnetic *Artemia* Cysts) yielding approximately 10 L of *Artemia* at a concentration of 500-600 *Artemia*/mL at the end of the hatching cycle. *Artemia* were then transferred to 20 L tanks

of filtered (10 µm) brackish water (20 ppt, 20ºC) at a density of 40 *Artemia*/L and exposed to the DOC amendment treatments (0 mg/L, 5 mg/L and 10 mg/L) for 40 hours (the standard timeframe for *Artemia* growth for use as live feed – Fielder and Heasman, 2011). DOC concentrations and the length of the experiment were analahgous to the range and duration of DOC concentrations (between 5 and 12 mg/L) found in a post-inflow monitoring study of the nearby Williams River estuary (Chapter 3, Figure 3.3b); with elevated DOC concentrations present for up to a month and a half following the inflow, roughly the length of this experiment. Carbon additions were administered to the on-growing tanks as they were filled with brackish water to homogenise the addition. Treatment batches of *Artemia* were prepared daily to ensure 40-hour enriched *Artemia* were available for daily feeding with on-growing tanks cleaned with freshwater before being restocked. Prior to feeding, *Artemia* were enumerated daily to determine their concentration after on-growing. Triplicate 5 mL samples of *Artemia* were enumerated for each tank by light microscopy (Leica, MZ6). *Artemia* were fed to their corresponding treatment fish at a concentration of 2/mL/day which equated to approximately 200 *Artemia*/fish/day. Feeding concentrations were selected with the advice of PSFC hatchery staff and post-flow prey densities of copepod nauplii from Chapter 4, being between 2 and 5 individuals/ml. Fish were fed daily between 8 and 10am. During feeding, tank flow through was turned off for half an hour to prevent loss of *Artemia* from the tank and to maximise access to prey for fish. To optimise growth in the PSFI production process juvenile bass are generally fed *ad libitum* 600-800 *Artemia*/fish/day (Fielder and Heasman 2011). By reducing this feeding rate, I was able to underfeed fish, to better reveal the effects on growth of terrestrial carbon which have been previously masked by feeding *ad libitum* (Growns et al. 2020), while still ensuring high survival rates (Appendix 11).

Technical replicates of bacterial samples were taken in triplicate directly from the *Artemia* on-growing tanks immediately after they were prepared (0 hrs) on the three fish sampling days to assess differences in microbial activity due to the addition of leachate. Bacterial samples were also taken from the on-growing tanks used for feeding on the day of fish sampling, after the homogenising of the tank for *Artemia* enumeration, to characterise microbial activity across the 2 day on-growing period. The 3 analytical replicates for each treatment (control; 5 mg/L; 10 mg/L) from the 3 fish sampling days resulted in 9 replicates for each bioencapsulation time level (0 hours; 40 hours). Samples were taken with a 1 mL pipette and were stored in 1.6 mL cryovials (Sarstedt, CryoPure) with 0.1 mL of

glutaraldehyde for preservation. Samples were refrigerated (3ºC) before they were snap frozen in liquid nitrogen within 2 days of sampling. Samples were then stored at -80 ºC until they were processed.

5.3.3 Fish Sampling

Fish were sampled at 2, 4 and 6 weeks after the commencement of their new diet (referred to in figures as T1 = 2 weeks, T2 = 4 weeks and T3 =6 weeks). Fish were not fed on the day of sampling, functionally "purging" their digestive systems prior to homogenised tissue analysis. Replicate tanks for different time points were used to eliminate the impacts of density dependant growth, manual handling and repeated measures. Survival rates (as percentage) of each treatment and timepoint are detailed in Appendix 11 and were all above 75%. All fish were removed from tanks using dipnets and then euthanised in a 150 mg/L benzocaine bath. After the cessation of opercular movement fish were removed and rinsed with RO water. Thirty (30) fish from each tank were randomly selected and placed on glass microscope slides, weighed, measured with callipers for standard, fork and total length, and photographed under a microscope (MC120 HD, Leica, Germany) for morphometric assessment. Fish were photographed in a lateral position with head-to-top-left orientation. Images were analysed using the software Fiji (Schindelin et al. 2012) to determine morphometry (Strauss and Bookstein 1982) using body landmarks as described in Cavalcanti et al. (1999) and Appendix 12 and 13. All remaining fish from each replicate tank were placed into plastic bags and frozen (-14ºC) for tissue analysis.

5.3.4 POM and Tissue analyses

Triplicate samples of on-grown *Artemia* from each on-growing treatment were taken on fish sampling days by filtering 500 mL of water from the on-growing tanks through a small 40 µm tow net, separating the *Artemia* from POM. Water that passed through the tow net was then filtered through GF/F filter papers to allow POM to be isotopically analysed. *Artemia* retained in the tow net were also placed on pre-combusted GF/F filter papers for stable isotope analysis. All filter papers were placed into plastic bags and then frozen (-14ºC). Frozen fish were dried at 50ºC for 96 hours and crushed to homogenise tissue and stored at -20ºC. POM, *Artemia* and homogenised fish material were analysed for stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) using a continuous flow-isotope mass spectrometer (GV IsoprimeEurovector EA 3000, Manchester, UK) and a Sercon Hydra 20-22 stable isotope

ratio mass spectrometer, respectively. Results were determined against laboratory standard reference material IAEA-CH-6 and atmospheric nitrogen.

Fatty-acid methyl ester analyses

Homogenised fish tissue was also reserved for Fatty-acid methyl ester (FAME) analysis. Lyophilised samples were extracted with 3 mL of chloroform/methanol (2/1, v/v) by vortexing and centrifugation. Supernatants were collected and residues re-extracted thrice with 2 mL chloroform/methanol (1/1, v/v). Pooled supernatants were washed with 2 mL of saturated sodium chloride solution. Lower organic phases were collected and evaporated to dryness under nitrogen and total lipid content were determined gravimetrically.

Extracted lipids were trans methylated to FAME as described. Dried lipid mass was treated with 1 mL of 1% NaOH in MeOH, followed by heating for 15 min at 55 °C, adding 2 mL of 5% methanolic HCl and again heated for 15 min at 55 °C and then adding 1 mL Milli-Q water. Nonadecanoic acid (C19:0) was used as the internal standard. FAMEs were extracted by hexane (3 × 1 mL) and evaporated to dryness under nitrogen. They were redissolved in 200 µl of hexane and stored at −20 °C in glass vials until analysed by GC–MS.

FAME analysis was carried out using a GC-MS-QP2020 (Shimadzu Corporation, Kyoto Japan) equipped with an AOC-20is autosampler (Shimadzu Corporation). The column used was an SH-Rxi-5Sil MS fused silica capillary column (30.0 mm x 0.25 mm x 0.25 µm) operating in electron impact mode at 70 eV. Helium was used as the carrier gas at a constant flow of 1.0 mL/min and an injection volume of 1 µL, with an injector temperature of 280°C and an ion source temperature of 230°C. Injection volume was 2 µL. The oven temperature was programmed for 50^{o}C (2 min) to 220^{o}C (4 ^{o}C/min); 220^{o}C to 300^{o}C (60 ^{o}C/min) and held for 3 min. FAME peaks were identified by comparison of their retention times with authentic standards and quantified by area normalisation and response factor measurements.

5.3.5 Statistical analyses

We conducted a two-way ANOVA to determine if mean bacterial cell counts (n = 9) differed among treatments (control; 5 mg/L; 10 mg/L) and bioencapsulation times (0 hours; 40 hours). Similarly, we conducted a two-way ANOVA to test for statistical differences between tDOM treatments ((control; 5 mg/L; 10 mg/L) and experimental timepoints (Initial, T1, T2 and T3) for weight and length measurements as well as HUFA concentrations. HUFA analysis focussed on docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3)

and arachidonic acid (ARA, 20:4n-6) due to their importance in fish health and nutrition (Sargent et al. 1999b; Glencross 2009). Parametric assumptions were checked for all ANOVAs and where not met; transformations were applied to satisfy assumptions. This was only necessary for the weight (g) of juvenile fish, where a square root transformation was applied. Significant differences between treatment and bioencapsulation time/timepoints were determined using Tukey's post hoc test. All statistical analyses were undertaken in R (R Development Core Team 2020). Figures were also constructed in R using "ggplot2" and "ggpubr (Wickham 2016; Kassambara 2020). Principal components analysis (PCA) was used to determine differences in truss-based morphometric size of fish between treatments at the end of the experiment. Measures contributing to the majority (>80%) of variation in fish morphology were identified and used to describe the PCA axes (Michie et al. 2020). This analysis was conducted in R using the "vegan" package (Oksanen et al. 2013) .

5.4 Results

There were significant differences in bacterial concentrations with the interaction between treatments and bioencapsulation time in the *Artemia* on-growing tanks (ANOVA, $F_{2,6}$ = 5.891, p = 0.0384, Table 5.1). Post hoc testing indicated that bacteria concentrations were significantly higher in the 10 mg/L treatment at 0 hours after the DOC additions compared to the other two treatments (Figure 5.1, Tukey's HSD, $p < 0.05$). After 40 hours of bioencapsulation bacterial cell concentrations in the control treatment (0 mg/L) on-growing tanks were less than in the 5 and 10 mg/L treatment tanks (Figure 5.1) though non-significantly (Tukey's HSD, $p > 0.05$).

Table 5.1:Two-way ANOVA results for bacterial concentrations in the bio-encapsulation tanks for treatments (0 mg/L, 5 mg/L, 10 mg/L) and bioencapsulation time (Bio-Time – 0hours, 40 hours). Df = degrees of freedom, Sum Sq = sum of squares, Mean sq = mean of squares

	df	Sum Sq	Mean Sq	F-value	p-value
Treatment	2	5.75×10^{11}	2.88×10^{11}	8.66	0.017
Bio-Time	1	4.50×10^{11}	4.50×10^{11}	13.53	0.010
Treatment*Bio-Time	2	3.92×10^{11}	1.96×10^{11}	5.891	0.038
Residuals	6	1.99×10^{11}	3.23×10^{10}		

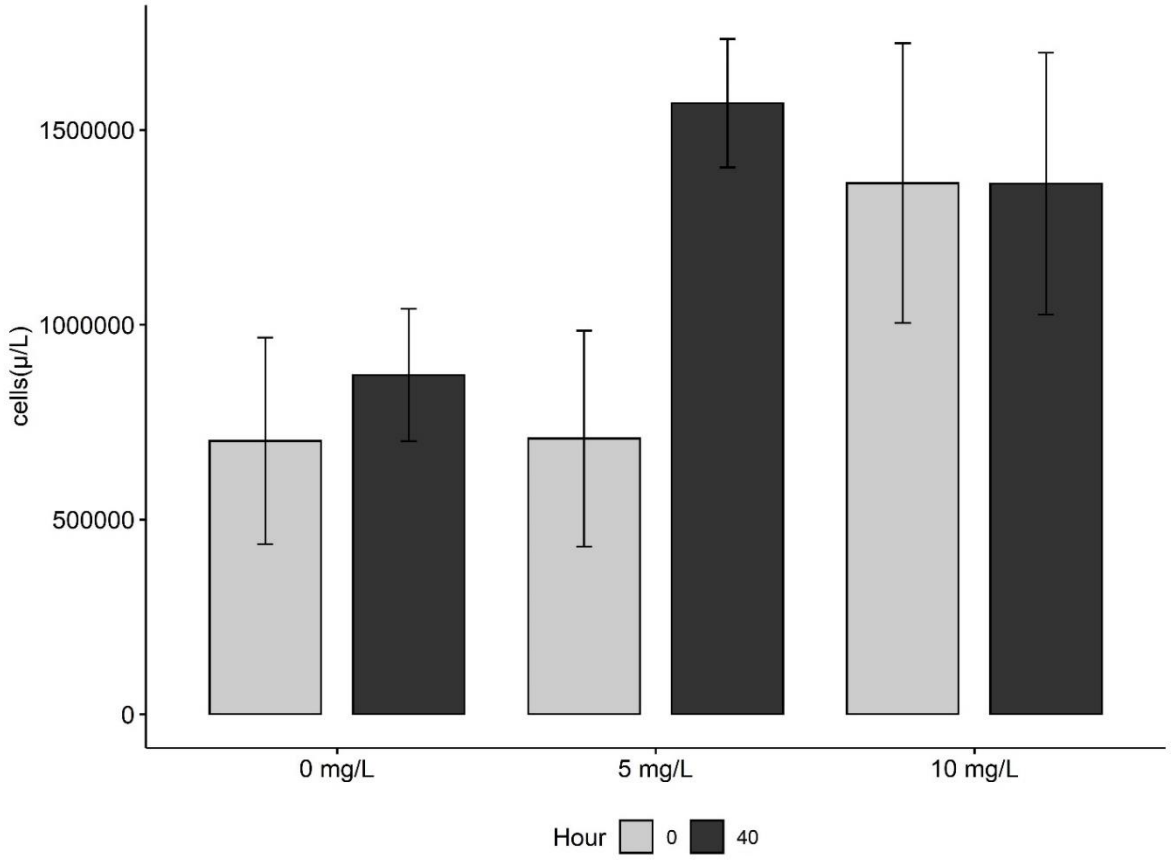

Figure 5.1: Bacterial cell concentrations (cells/µL, n=9)) for the 3 DOC addition treatments 0 mg/L, 5 mg/L and 10 mg/L in the Artemia on-growing chambers during the bio-encapsulation period. 0 hr measurements were taken minutes after the addition of leachate and Artemia to freshly prepared on-growing tanks. 40 hr measurements were taken from pre-prepared on-growing tanks to be used for feeding that day, approximately 40 hours after the addition of leachate and Artemia. Error bars represent standard error of the mean (n =9).

The leachate used in the experiment had an average $\delta^{13}C$ and $\delta^{15}N$ ratios of -29.4 ‰ (±0.529) and 3.2 ‰ (±0.834). Particulate organic matter in the on-growing tanks was more $\delta^{13}C$ and $\delta^{15}N$ depleted in the 10 mg/L treatment than in the control and 5 mg/L treatments (Figure 5.2). Despite the difference in isotopic signatures of POM, *Artemia* had similar $\delta^{13}C$ ratios between treatments, although $\delta^{15}N$ ratios for *Artemia* in the 5 and 10 mg/L treatments were higher than the control, being highest in the 10 mg/L treatment. Initial fish tissue measurements of $\delta^{13}C$ and $\delta^{15}N$ were -18.2 ‰ (±0.018) and 13 ‰ (±0.119), respectively (Figure 5.2). At the conclusion of the experiment fish in all treatments and control largely resembled their initial signatures and did not become closer to the leachate or *Artemia*. Fish from the 10 mg/L treatment were slightly more depleted in $\delta^{13}C$ and more enriched in $\delta^{15}N$ than the other treatments which had similar $\delta^{13}C$ and $\delta^{15}N$ ratios (-17.7 ‰ and 12.5 ‰, respectively).

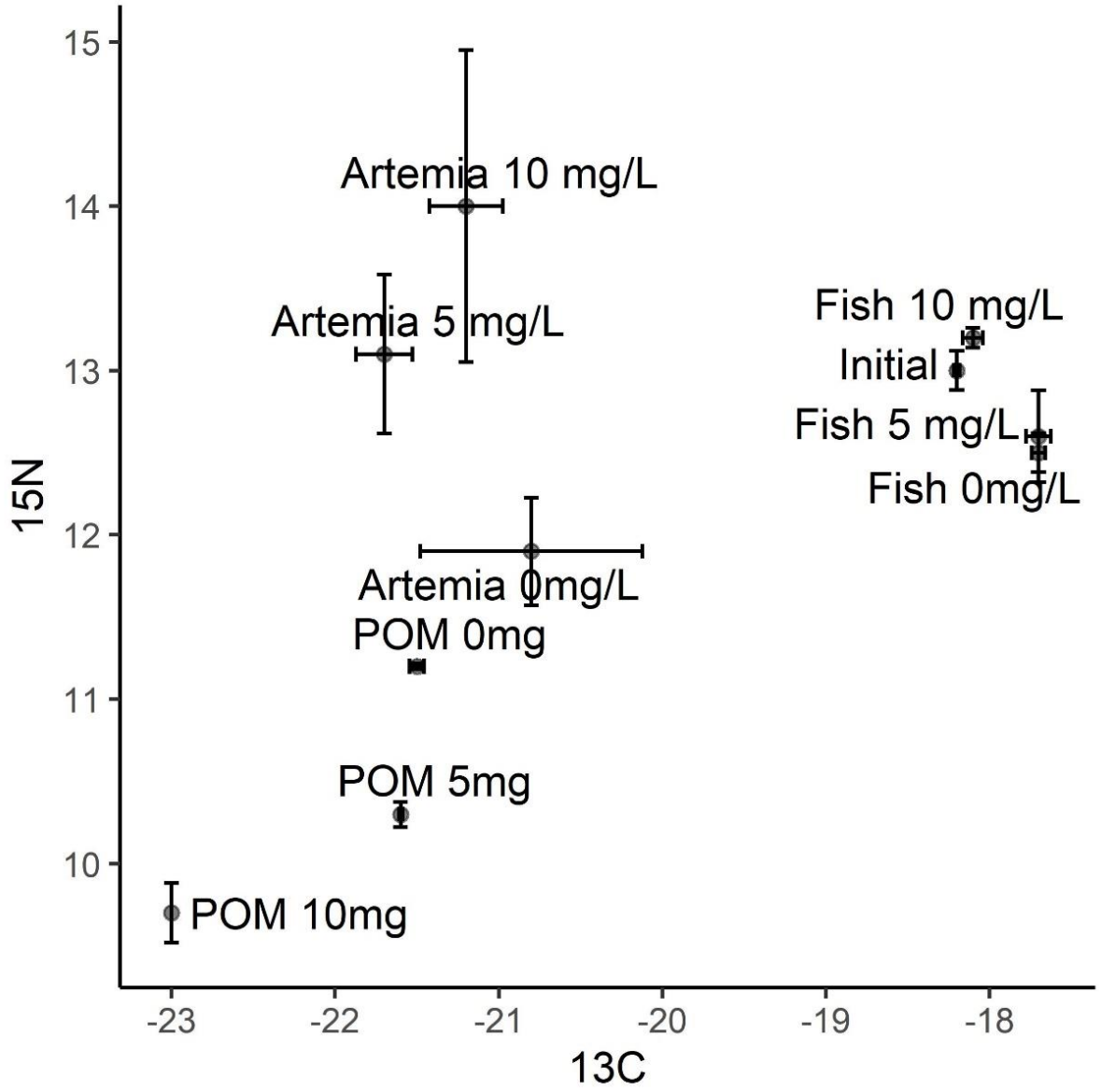

Figure 5.2: $\delta^{13}C$ (13C) and $\delta^{15}N$ (15N) stable isotope ratios for fish tissues at the beginning (Initial) and conclusion of the experiment for all 3 treatments (Fish 0 mg/L, Fish 5 mg/L and Fish 10 mg/L). Isotopic ratios are also displayed for particulate organic matter samples from the Artemia on-growing tanks (POM 0 mg/L, 5 mg/L and 10 mg/L) and Artemia (Artemia 0 mg/L, 5 mg/L and 10 mg/L) from the 3 treatments. Error bars represent standard error of the mean for each sample.

Analysis of average wet weight of fish through ANOVA showed there was a significant difference in weight between treatments (0 mg/L, 5mg/L and 10mg/L) and timepoints (T0, T1, T2, and T3) (ANOVA, $F_{4,839} = 5.99$, $p < 0.001$, Appendix 14). There were notable decreases in weight for all treatments during the first fortnight of the experiment (Tukey's HSD, $p < 0.001$) with decreases from 0.064 g (± 0.002) at T0 to 0.044 g (± 0.001) for the control treatment, 0.042 g (± 0.001) for the 5 mg/L treatment and 0.044 g (± 0.001) for the

10mg/L treatment by timepoint T1, 2 weeks into the study (Figure 5.3a) . In the control treatment, wet weight continued to fall across the experimental period to 0.037 g (± 0.001) T3, the end of the experiment (Figure 5.3a). Similarly, wet weights in the 5 mg/L treatment also decreased between T1 and T2 to 0.035 (± 0.001), but then increased to 0.040 g (± 0.001) by T3, the end of the experiment (Figure 5.3a). Like the 5mg/L treatment, fish from the 10 mg/L treatment decreased in average wet weight between T1 and T2 to 0.042 g (± 0.001), and increased to 0.045 g (± 0.001) by T3 at the conclusion of the study (Figure 5.3a). By the end of the experiment fish from the 10 mg/L treatment were significantly heavier than those from the control (Tukey's HSD, p <0.001) and the 5mg/L (Tukey's HSD, p = 0.024) treatments.

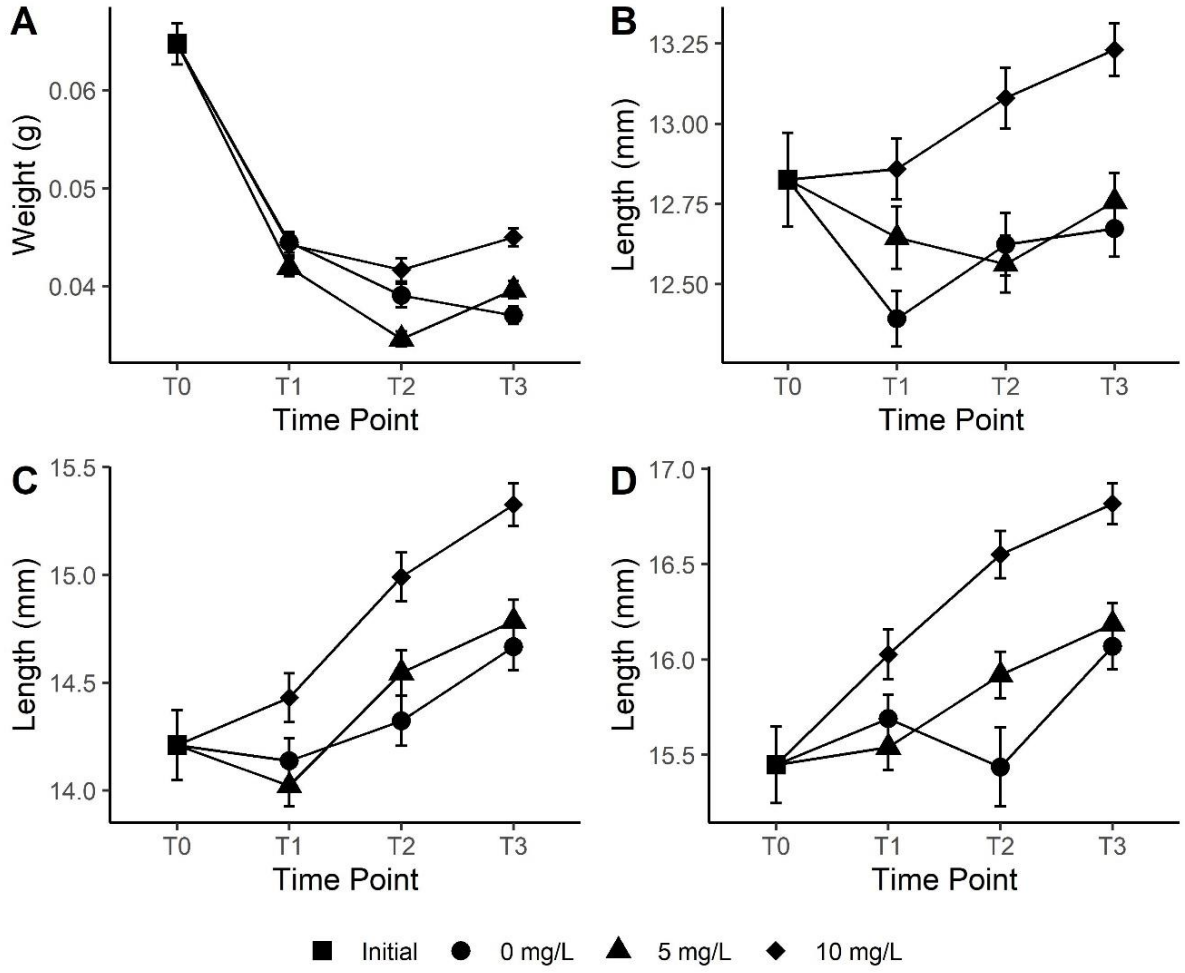

Figure 5.3: Weight (A), standard length (B), fork length (C) and total length (D) of juvenile Australian bass for experimental carbon treatments of 0 mg/L, 5 mg/L and 10 mg/L across the study period. Measurements of fish when acquired are presented as "Initial" measurements. Error bars represent standard error of the mean (n = 30). Time points refer to T0 = start of experiment, T1 = 2 weeks, T2 = 4 weeks and T3 =6 weeks into the experiment. T3 was the final point of the experiment

There was no interaction factor between the treatment and time factors for any fish length measurements (standard, fork and total lengths), though there were significant differences between time points and treatments for all length measurements (all $p < 0.001$, Appendix 14). Fish length was significantly greater in the 10 mg/L treatment for standard and total length compared to other treatments by the conclusion of the study (Figure 5.3B and D, Tukey's HSD, $p < 0.001$). While fork length was greatest in the 10 mg/L treatment by T3 and significantly different to the control group, there was no significant difference in fork length between the 5 mg/L and 10 mg/L treatments by the end of the study (Figure 5.3C, Tukey's HSD, $p = 0.0501$). Over the course of the experiment all length metrics increased continually regardless of treatment, except for the 5 mg/L treatment for standard length and the control treatment for total length, which both decreased between T1 and T2 before increasing again by T3, at the end of the experiment.

Principal components analysis (PCA) of the morphology of juvenile Australian bass measured at the end of the experiment (T3) produced 2 significant axes, explaining a cumulative 69.5 % of accumulated variance (Figure 5.4). The recognised gradients (Figure 5.4) indicate that fish from the 10 mg/L treatment had greater dorso-ventral depth in the anterior section of their body (PC1) and greater length between the origin of the pectoral and anal fins (PC2) than fish from the 0 mg/L and 5 mg/L. Principle component (PC) 1 explained 58.5 % of variance, describing a gradient negatively determined by 1) the length between the anterior tip of the upper snout and the origin of spinous dorsal fin; 2) the length between the origin of spinous dorsal fin and the origin of pelvic fin; and 3) the length between the origin of spinous dorsal fin and the origin of anal fin (Appendix 12 and 13). PC2 explained 11.0 % of variance describing a gradient negatively determined by the length between the origin of the pectoral fin and the origin of the anal fin and positively determined by 1) the length between the origin and insertion of the anal fin; and 2) the length between the origin of the anal fin and the insertion of the 2nd dorsal fin (Supplementary Figure 1).

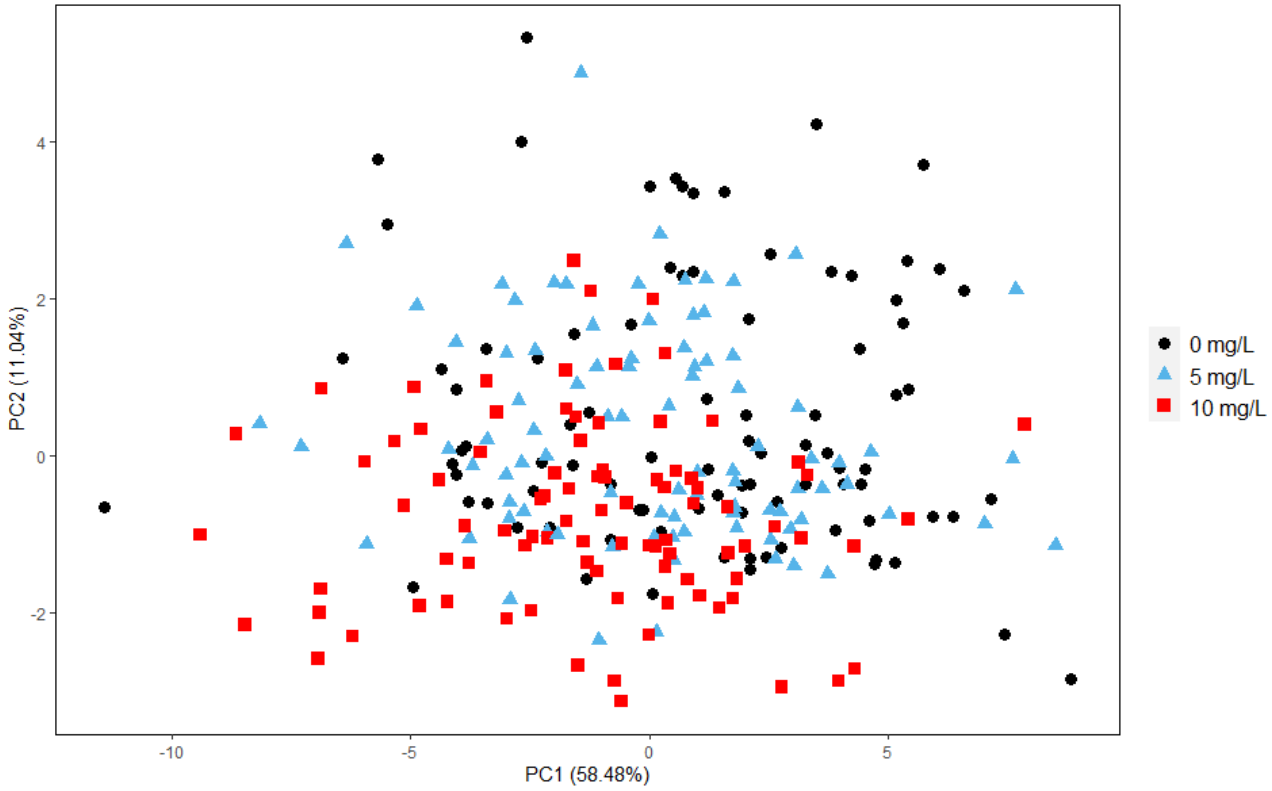

Figure 5.4: Principal component analysis of truss-based morphology for juvenile Australian bass from treatment A (control – black circles), B (5 mg/L DOC addition – blue triangles) and C (10 mg/L DOC addition – red squares). PC1 was characterised by lengths of X2, X6 and X10 while PC2 represented X13, X8 and X12 (Appendix 13). X2 = length between the anterior tip of the upper snout and the origin of spinous dorsal fin, X6 = the length between the origin of spinous dorsal fin and the origin of pelvic fin, X10 = the length between the origin of spinous dorsal fin and the origin of anal fin, X13 = the length between the origin and insertion of the anal fin and X12 = the length between the origin of the anal fin and the insertion of the 2nd dorsal fin

Concentrations of HUFA in fish tissue decreased significantly in all treatments during the experiment (Figure 5.5, ANOVA, $F = 122$ (DHA), 31 (EPA), 164 (ARA), $p < 0.001$ for all). There were significant decreases in concentration of all key HUFA between T0 and T1 (Tukey's HSD, $p < 0.001$). Concentrations of DHA (22:6n-3) and ARA (20:4n-6) in fish of the 5 mg/L treatment were significantly lower than in other treatments at T1 (Figure 5.5a and c, Tukey's HSD, $p < 0.05$). There was otherwise no significant difference between treatments for any HUFA at any other timepoint.

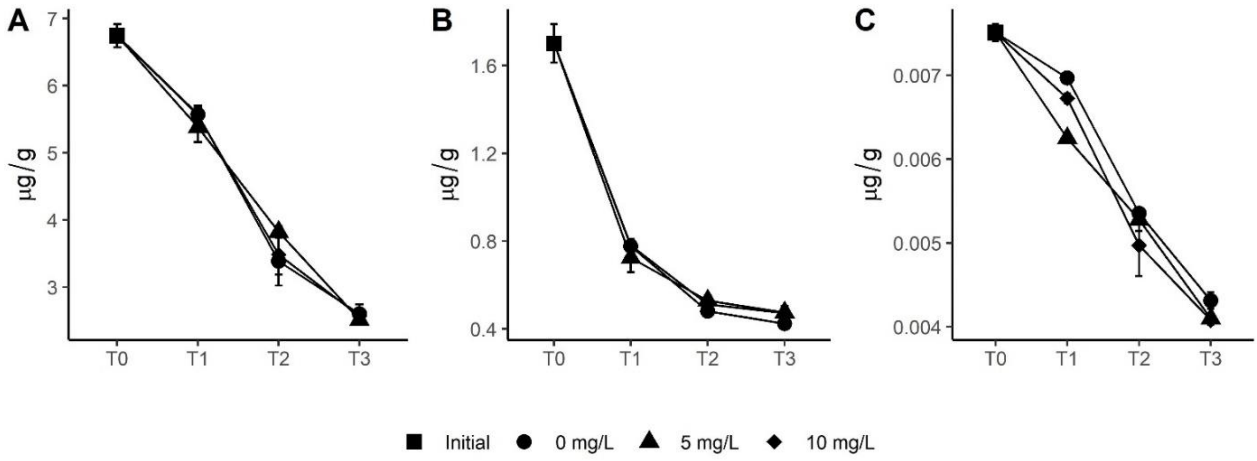

Figure 5.5: Highly unsaturated fatty acid concentration of homogenised fish tissues for initial measurements and the control (0 mg/L), 5 mg/L and 10 mg/L treatments over the 6-week experimental period. A = docosahexaenoic acid (DHA, 22:6n-3), B = eicosapentaenoic acid (EPA, 20:5n-3) and C = arachidonic acid (ARA, 20:4n-6). Error bars represent standard error of the mean (n= 3). Time points refer to T0 = start of experiment, T1 = 2 weeks, T2 = 4 weeks and T3 =6 weeks into the experiment. T3 was the final point of the experiment

5.5 Discussion

Somatic growth and body size of larval and juvenile fish are key determinants in the recruitment and success of yearly fish cohorts. As such, resources that can aid the growth of larval and juvenile fish are important to fish populations. In this study I investigated the influence of terrestrial dissolved organic matter (tDOM) on the somatic growth of juvenile Australian bass. My results indicate that terrestrial carbon could be transferred across several trophic levels (Figure 5.1 and 5.2) and positively influence growth of juvenile Australian bass (Figure 5.3 and 5.4), though only when additions of DOC were at higher concentrations (10 mg/L). Despite increases in length, stable isotope and fatty acid analysis of fish tissue suggested that tDOM did not contribute to new growth or provide HUFA to juvenile fish.

5.5.1 tDOM influence on growth

Juvenile fish in the 10 mg/L treatment were significantly larger and heavier than those of other treatments (Figure 5.3 and 5.4), indicating that increased tDOM availability had a positive influence on somatic growth. The small increases in size may be due to the slow growing nature of Australian Bass (Harris 1987). By the end of the study juveniles were roughly the same size (15-18 mm) as similarly aged individuals (12 weeks) found in the Sydney Basin by Harris (1987), though smaller than optimally fed PSFI hatchery juveniles of the same age (20-25 mm, *pers comms.* Cheviot, 2021). Increased dorso-ventral size of fish from the 10mg/L treatment as indicated by PCA (Figure 5.4) also suggests that these fish are

in a healthier condition than those of other treatments and are able to contribute growth to areas other than length, which is the priority growth area of juvenile fish (Stearns 1992; Garvey and Marschall 2003). Although increases in total length of fish in all treatments continued through the experiment, there were significant reductions in weight (Figure 5.3a). The most significant reductions in weight occurred between T0 and T1 in all treatments. While osmotic water loss due to a movement between fresh and brackish water may have contributed to wet weight loss, these effects are generally short lived, approximately 96 hours (Maceina and Shireman 1979; Altinok et al. 1998). It is more likely that the significantly reduced wet weights in all treatments are an indication of energetic limitation, with fish potentially utilising endogenous lipids and proteins to support themselves (Fielder and Heasman 2011; Güroy et al. 2011; Falahatkar et al. 2013). As the experimental design purposefully limited food availability by between 66 and 75% relative to PSFI hatchery diets (Fielder and Heasman 2011), this process likely explains the weight loss of fish in all treatments.

The small enrichment of $\delta^{13}C$ signatures of juvenile fish relative to initial signatures, across all treatments, indicated that additional terrestrial carbon was not assimilated into new tissue by juveniles, though tissue turnover was occurring (Weidel et al. 2011). It may mean therefore, that tDOM did not contribute directly to increased somatic growth in the 10 mg/L treatment. However, enrichment of $\delta^{15}N$ and small depletion of mean $\delta^{13}C$ for *Artemia* in the 5 mg/L and 10 mg/L treatments relative to control group may suggest the transfer of terrestrial energy to *Artemia* was occurring and that additional trophic levels were involved, rather than direct consumption of tDOM, as indicated by the enrichment of $\delta^{15}N$ of fish tissue in the 10 mg/L treatment (Deniro and Epstein 1981; McCutchan et al. 2003). The additional trophic levels involved in these results were likely due to the increased bacterial concentrations in the *Artemia* on-growing tanks (Figure 5.1). Bacterial concentrations were higher after 40 hours in the on-growing tanks with leachate additions compared to the controls (Figure 5.1), except for the 10 mg/L treatment which had an equivalent bacterial concentration between the 0 and 40-hour bioencapsulation timepoint. It is not likely this increase at the 0-hour point is a result of increased bacterial productivity in the on-growing chambers as bacterial samples were taken minutes after the addition of the leachate and it is unknown what may have caused this as the additions of leachate, which may have contained bacteria, to the 5mg/L treatment did not affect bacterial concentrations there relative to the

control. Nonetheless, it is likely that increased bacterial concentrations in the treatment groups played a role in transferring tDOM to *Artemia*, extending the length of the food chain and increasing the $\delta^{15}N$ of *Artemia* by between 1 and 2 ‰ (McCutchan et al. 2003; Karlsson et al. 2004). But if additional trophic levels were involved in the transfer of energy to fish of the 10 mg/L treatment, and these fish were longer, dorso-ventrally deeper and heavier than in other treatments, but did not assimilate terrestrial carbon, how is tDOM contributing to their improved somatic growth?

It is possible that tDOM may have contributed to somatic growth by subsidising the daily energetic demands of juvenile fish. This phenomenon has been seen previously in zooplankton where terrestrial resources low in HUFA, were selectively catabolised to meet energetic demands while HUFA from algal resources were retained for growth and reproductive development (Brett et al., 2009). However, as HUFA rich algal sources were not provisioned in this experiment, fish may have utilised endogenous reserves of these lipids (e.g., DHA, EPA and ARA) to facilitate continual growth (Figure 5.5). During periods of energetic and nutritional limitation, juvenile fish can utilise endogenous reserves of lipids and proteins to meet energetic and metabolic demands, which can reduce their weight (Kooijman 2010; McCue 2010; Nepal et al. 2021). For juvenile fish, lengthwise growth is commonly prioritised over dorso-ventral growth, gonad development and energy storage (increased weight) as a larger eventual body size, predicated by fish length, can lead to better future reproductive output once resources become abundant (Stearns 1992; Garvey and Marschall 2003). Differences in the length and dorso-ventral depth of fish between treatments may therefore be due to terrestrial resources subsidising the energetic demands of juvenile fish, allowing more endogenous tissue to be metabolised for growth and development in the 10 mg/L treatment, resulting in larger fish compared to the other treatments, where fish also had to catabolise endogenous tissue to meet energetic demands. This process may also account for the consumption of terrestrial carbon yielding no response in terms of assimilation into fish tissue, instead being allocated to energetic demands rather than contributing to growth (Brett et al. 2009). My results contrast to findings by field studies which have shown the assimilation of terrestrial carbon into tissue by juvenile fish (Hoffman et al. 2007; Abrantes and Sheaves 2010). However, this may be due to food limitation conditions masking changes in $\delta^{13}C$, as metabolic processes in fish tissues under energetic limitation can limit the equilibration of $\delta^{13}C$ signatures between food source and tissue, particularly in fish muscle

tissue (Colborne and Robinson 2013).

The 5 mg/L treatment presented no significant effect on juvenile fish growth, relative to the control, possibly indicating the concentration of tDOM (as DOC) needed to overcome food web respiration losses and influence somatic growth of juvenile fish (Ducklow et al. 1986). Limitations of energetic transfer due to tDOM quantity were also found by Hitchcock et al., (2016), where a 3 mg/L addition of DOC had a reduced influence on copepod productivity compared to an addition of 16 mg/L, and Karlsson (2007), where an increasing gradient of glucose additions between 0.11 and 3.4 mg/L produced increasingly positive responses in zooplankton growth rates. However, the transfer of terrestrial energy through the food web may have been enhanced due to the relatively short food web used to transfer energy to juvenile fish (tDOM → microbes → *Artemia* → fish) and use of tDOM with a high bioavailability (Lennon and Pfaff 2005). Further experimentation would be required to produce more accurate estimate for terrestrial carbon requirements in estuarine ecosystems to support juvenile fish growth.

As concentrations of HUFA did not differ significantly between treatments (Figure 5.5) we can also assume that terrestrial carbon resources did not provide additional HUFA to juvenile fish during the experiment. Terrestrial carbon resources have low concentrations of long-chain HUFA compared to algal resources (Brett and Müller-Navarra 1997; Sayanova and Napier 2004). Although they do possess shorter chain HUFA, e.g., linoleic (LIN), alpha linoleic (ALA) and stearidonic acid (SDA), that can be elongated by animals in metabolic processes this is not an efficient pathway for obtaining long-chain HUFA (Arts et al. 2009). The decreasing concentrations of key HUFA (DHA, EPA and ARA) are also indicators of poor nutritional health of fish (Sargent et al. 1999b; Glencross 2009). It is likely that the continuation of a diet based solely on terrestrial resources and lacking in sufficient dietary sources of HUFA in this experiment, such as algae, would have resulted in increased mortality of juveniles (Takeuchi 2014). The continued decreases in HUFA during this experiment further highlight the need for phytoplankton and rich HUFA sources in the diets of aquatic organisms to facilitate physiological development (Glencross 2009; Brett et al. 2009).

5.5.2 tDOM, inflows and recruitment

Similar experiments with terrestrial carbon additions to juvenile fish diets have found

contrasting responses in juvenile fish growth. Experiments on freshwater gudgeons (*Hypseleotris* spp.) found no significant increase in fish size with increased terrestrial DOC availability as part of a zooplankton diet based on mixed algal/tDOM sources (Growns et al. 2020). Food web efficiency experiments with juvenile three-spined stickleback (*Gasterosteus aculeatus*) as an apex predator and increased DOC availability (in the form of glucose) in the presence of an existing phytoplankton energy pathway resulted in reduced growth of juvenile fish (Degerman et al. 2018). My contrasting results to these experiments may be due to 1) the use of a single source diet in my experiment reducing the influence of algal resources and 2) a sufficient concentration of tDOC to overcome energy respiration losses by microbial and zooplankton trophic levels (Karlsson 2007). My experiment, and use of a single source diet, was designed to mimic the potential concentrations and duration of elevated DOC levels after inflows in Australian estuaries (Chapter 3, Figure 3.3b) as well as heterotrophic conditions which can occur following inflow periods; where algal production is suppressed, and terrestrial carbon becomes the dominant food web resource (Hemraj et al. 2017b; Meunier et al. 2017; Andersson et al. 2018). Numerous studies have demonstrated links between inflows, terrestrial carbon assimilation and growth of juvenile fish in estuaries, attributing this relationship to increased prey resources (Darnaude et al. 2004; Hoffman et al. 2007; Wai et al. 2012; von Biela et al. 2013). My results provide a more detailed analysis of this relationship, suggesting that terrestrial carbon and associated increases in juvenile fish prey resources after inflows (Asmala et al. 2014a; Hitchcock et al. 2016a; Berggren et al. 2018) can positively influence the somatic growth of juvenile fish indirectly, providing increased energetic resources and allowing what limited algal resources may be available after inflows (Andersson et al. 2018) to be used for growth and development (sensu Brett et al. 2009). Previous studies on zooplankton have suggested that terrestrial carbon plays a similar role in lower trophic levels, becoming a more effective energetic supplement when co-metabolised with HUFA rich algal resources in a ratio between 1:1 and 1:3 (Brett et al. 2009; Harfmann et al. 2019).

Multiple studies have documented relationships between the success of Australian bass populations and inflows, attributing this relationship to increased prey resource availability and increased habitat connectivity (Harris 1986; Growns and James 2005; Stoessel et al. 2018). Of particular importance is the connectivity of riverine and estuarine environments, allowing migration of spawning adults (Harding et al. 2017), and the timing of inflow events

with spawning seasons (Stoessel et al. 2018). My results show that tDOM delivery by inflows could also play some role in this relationship by subsidising energetic needs of juvenile fish. However, further research is required to understand the value of terrestrial resource subsidies to other species of juveniles. The majority of similar research so far has been conducted on benthic feeding fish and has demonstrated a benefit of terrestrial subsidies to their growth and development, though this may be due to their closer link to benthic food webs (Darnaude et al. 2004; Kostecki et al. 2010; Wai et al. 2012). Further research on planktivorous juvenile fish is required given the ability of terrestrial subsidies to influence the growth of zooplankton as well (Hoffman et al. 2008).

River regulation can significantly reduce the input of terrestrial carbon and nutrient resources to lowland rivers, wetlands and estuaries (Robertson et al. 1999; Kingsford 2000; Gillanders and Kingsford 2002). However, appropriately managed environmental flows can be utilised to improve the delivery of terrestrial organic matter to end of system food webs such as estuaries (Baldwin et al. 2016). Considering my results, and the importance of terrestrial resources to estuarine fish juveniles in other studies (Hoffman et al. 2007; Kostecki et al. 2010; Abrantes et al. 2013), the timing and delivery of tDOM to estuaries may be an important element in integrating inflow management with fisheries stocking or monitoring programs. With the importance of riverine connectivity for catadromous species such as Australian bass for spawning (Harding et al. 2017), pairing inflows designed for connectivity during spawning seasons with higher tDOM loads could have a positive impact on Australian bass populations by providing important energetic subsidies to their diet alongside algal based food resources.

5.5.3 Limitations

There are several inherent limitations in the study that must be acknowledged in the interpretation of the results and in future studies. Firstly, the choice to underfeed fish may have resulted in the drastic reductions in weight seen across all fish in the first 2 weeks of the experiment. These concentrations were based on a combination of advice from PSFC staff and the results of Chapter 4, where post-flow concentrations of copepod nauplii were between 2 and 5 individuals/ml. While *ad libitum* feeding is not representative of real-world scenarios, its use in this experiment may have eliminated the weight loss of fish in the first 2 weeks and could have changed the experiment, rather than feeding at the lower end of expected natural prey densities, with a reduced quality basal resource. Secondly, while the

experiment was designed as a single-source experiment (one dietary source), this is not representative of natural conditions or diets of fish. As such the results of this experiment only have limited applicability and must be considered in this context. Future work could investigate the impacts of different dietary ratios on juvenile fish as Harfmann et al. (2019) did for zooplankton. Lastly, the changes in fish size and weight are minimal across the experimental period. Australian bass are a slow growing species and running the experiment for an extended period of time may have yielded clearer results. Further investigations using alternate, faster-growing species and longer experimental periods may provide clearer results.

5.5.4 Conclusion

My results indicate that terrestrial carbon can positively influence somatic growth of juvenile Australian bass (*Macquaria novemculeata*) after additions of tDOM to the food web. Additions of tDOM in concentrations of 10 mg/L of DOC to *Artemia* nauplii on-growing tanks, led to increased growth of food limited juvenile Australian bass fed these zooplankton. However, tDOM was not assimilated into new tissue by juvenile fish as revealed by SIA and did not contribute essential HUFA to juvenile Australian bass, though these results may have been influenced by food limitation. My results suggest tDOM can be catabolised for energetic demands and contribute indirectly to somatic growth by allowing more high-quality dietary resources to be used for growth when resources are scarce. With the importance of body size for survival and fitness of juvenile fish, the provisioning of terrestrial carbon through freshwater inflows during spawning periods may aid recruitment and adult populations in estuaries by providing additional energetic resources.

Chapter 6: General Discussion and Conclusions

6.1 General Discussion

The aim of this thesis was to understand the influence of FWI events on basal resources, estuarine processes and planktonic food webs. It addresses two major questions in estuarine ecology and management; 1) how do estuarine biota respond to inflows in regulated systems and 2) how important are terrestrial carbon resources to estuarine food webs? These questions were addressed through the lens of the planktonic food web using a combination of field-based monitoring and laboratory-based manipulative studies. The evidence from these studies highlights the importance of both freshwater inflows and terrestrial resource inputs, including carbon, in the productivity and ecology of estuarine systems, particularly in upper estuary reaches which are more strongly influenced by freshwater inflows.

6.1.1 Planktonic responses in regulated systems

Zooplankton are a key component of estuarine food webs (Newton 1996; Ferdous and Muktadir 2009) and understanding how they respond to freshwater inflows in regulated systems is an important step in appropriately managing estuaries (Scheltinga et al. 2006). In Chapter 2, I found contrasting zooplankton communities and inflow responses between a regulated and unregulated estuary. Despite the lack of geographical replication, this research did indicate some potential effects of river regulation on zooplankton communities in upstream estuarine areas. In the unregulated Manning River estuary, zooplankton communities responded positively to seasonal increases in temperature, being dominated by herbivorous copepods during these periods, and negatively to inflows in the short-term; following patterns commonly reported in estuaries (Huntley and Lopez 1992; Kobayashi et al. 1998; Hitchcock et al. 2016a). In contrast, the zooplankton community of the regulated Williams River estuary was regularly dominated by copepod nauplii, which responded positively to FWI events and did not respond to temperature increases, suggesting that other factors may have been influencing zooplankton populations in this estuary. These factors may have included changes to the estuarine salinity gradient (Devreker et al. 2004; Hemraj et al. 2017a), reduced DO following inflows influencing zooplankton development (Broman et al. 2015) and/or eutrophication of the estuary and subsequent changes in algal resources (Marcus 2004) due reduced flushing trapping nutrients in the upper estuary (Connell et al., 1981). While comparative studies between regulated and unregulated estuaries are lacking in the

literature, studies have noted the impacts of river regulation on zooplankton communities, noting changes in the composition of zooplankton communities toward smaller individuals and short-term positive responses to inflows (Wooldridge and Callahan 2000; Chícharo et al. 2006; Furst et al. 2019). Along with these studies, my thesis suggests that river regulation may impact on the key functions FWIs perform in upper estuaries, as they change both water quality conditions and the zooplankton communities that live there (Chapter 3). Importantly, this suggests that current flow rules outlined in the WSP are not suitably alleviating the full impacts of river regulation and the presence and operation of Seaham Weir. Continued impacts of river regulations may have ecological consequences within the Williams estuary, including potential harmful algal blooms due to eutrophication and reductions in secondary productivity.

Using environmental flows to influence the ecological function of rivers and estuaries is a key water management technique for achieving specific ecological outcomes (Baldwin et al. 2016; Arthington et al. 2018b). This is particularly relevant in the context of climate change in Australia where reduced inflows are forecast to have a raft of negative ecological effects, including reduced estuarine productivity (Gillanders et al. 2011). As part of the development and implementation of environmental flows it is important to understand how food webs may respond to releases of freshwater so that better ecological outcomes can be achieved (Davies et al. 2014). In Chapter 3, fine-scale monitoring of the planktonic food web following an inflow showed that increases in basal resources do not necessarily translate into increased secondary productivity of adult zooplankton. Initially, increases in DOC and nutrients resulted in increased bacterial and algal production in the estuary, with a freshwater zooplankton community present in the estuary after the inflows able to capitalise on these resources. Juvenile copepods, initially decreased in concentration by the inflow, began to increase in concentration as did freshwater cladocerans, which may have also been advected into the estuary by the inflow. However, upon the closure of the weir gates, the biomass of the zooplankton community declined to a level below that observed in pre-flow conditions. This reduced concentration of zooplankters occurred following decreases in the abundance of algal resources present after the inflow event. Decreased zooplankton concentrations following the inflow may be related to several processes including the overgrazing of algal resources stimulated by the inflow (boom and bust dynamics), predation by planktivores (Geddes et al., 2016) or negative impacts on food web productivity brought about by rapid

shifts from high to low flow conditions in regulated systems (Hemraj et al., 2017b). Further investigation is required to understand why inflows did not lead to sustained adult zooplankton productivity in the Williams and the current patterns outlined in Chapter 2 and 3 should be considered in future flow management proposals.

6.1.2 Role of terrestrial carbon

The role of terrestrial carbon in food webs has been long debated in aquatic ecology (Ducklow et al. 1986; Brett et al. 2009; Cole et al. 2011). Within estuaries, shifts from algal resource use to terrestrial resources have been recorded in lower estuarine and near-coastal areas, especially after inflows (Schlacher et al. 2009; Atwood et al. 2012). However, studies have suggested that terrestrial support of zooplankton was limited, compared to zoobenthos, and short lived, between 1-2 weeks (Schlacher et al. 2009; Bartels et al. 2018). Results from research presented in Chapter 4, conducted in upper estuarine areas, indicated that inflows increased the degree to which terrestrial sources of carbon support zooplankton and that these subsidies were utilised for between 1-2 months. While differences in estuaries and the magnitude of flow events makes direct comparisons across studies difficult, these findings may highlight an elevated role for terrestrial material supporting zooplankton communities in upper estuarine areas relative to near-shore and coastal areas. Links have been made between the spatial proximity of estuarine communities to sources of terrestrial organic matter (rivers/river mouths) and their assimilation of terrestrial material (Chanton and Lewis 2002; Lautenschlager et al. 2014; Bartels et al. 2018). This is particularly important in the context of this thesis and the focus on regulated river systems, as the impact of river regulation is known to alter the spatial and temporal dynamics of organic matter and energetic resources in rivers and estuaries (Sabo et al. 2018; Ru et al. 2020; Liu et al. 2020).

My studies suggests that river regulation may have aided the retention of terrestrial matter in the upper estuary, through reduced discharge and export to the ocean (Connell et al. 1981), increasing the amounts available to consumers and potentially its importance as a food web resource following inflows (Hemraj et al. 2017b). The evidence presented in this thesis also suggests that terrestrial material may support zooplankton for considerable time periods, of up to two months, especially during cooler months in south-east Australian estuaries. Similar results have been found in both freshwater and estuarine environments with terrestrial material becoming more important to consumer diets during cooler months (Rautio et al. 2011; Berggren et al. 2015; Dias et al. 2016). This may be related to the balance of

autotrophic and heterotrophic pathways, whereby decreased algal production during periods of reduced light and lower temperatures (Dorado et al. 2015) may increase the energetic reliance on terrestrial material and the microbial processes in these periods (Karlsson et al. 2003; Berggren et al. 2015). This is an important finding in a south-east Australian context where the majority of rainfall is between late summer and early winter (Callaghan et al. 2014) and may transport a significant load of organic material to estuaries (Ford et al. 2005; Hitchcock and Mitrovic 2015a) during these cooler periods.

An interesting finding from this thesis was the relationship of some zooplankton to inflows and potentially to terrestrial organic carbon. In Chapter 2, copepod nauplii responded positively to increases in discharge, DOC and turbidity, while in Chapter 4, the predatory *S. conflictus*, which prefers nauplii as its prey (Ough and Bayly 1989), showed an increased reliance on terrestrial resources following inflows, as confirmed by stable isotope signature analysis, indicating nauplii may be utilising terrestrial carbon after inflows. The appearance of *Euglena* sp. in high densities after the inflow period in Chapter 3, concurrent with the comparatively low Chl *a* concentration, indicated that heterotrophic behaviour may have been occurring for this population during this period. Collectively, these results suggests that terrestrial sources of carbon are important resources fuelling the lower planktonic food web in the regulated Williams estuary. These results are similar to other studies which highlight the increased importance of terrestrial resource subsidies to the bacterial and protist food web following freshwater inflow periods (Hoffman et al. 2008; Bruesewitz et al. 2013; Meunier et al. 2017), including in a regulated system (Hemraj et al. 2017b). With increased sediment load through changing land practices and periods of higher magnitude flows due to climate change (Chilton et al. 2021), terrestrial organic matter may have a greater importance in estuarine food webs in the near future and should be investigated further.

While inflows can support microbial production in estuaries, the results of Chapters 2 and 4 highlight the potential importance of smaller mesozooplankton in transporting terrestrial energetic resources to higher trophic levels. For example, copepod nauplii, which can access bacterial and protist communities as food resources, can provide a clear link between terrestrial carbon, microbial production and higher-level consumers in the metazoan food web (Roff et al. 1995; Vogt et al. 2013). This potential role of smaller mesozooplankton, like copepod nauplii, in supporting estuarine productivity following inflows in regulated estuaries requires further research attention. Investigation of this role was unfortunately missed in

Chapter 4 due to experimental errors but may have yielded interesting results indicating diet switching of nauplii following inflow events. Further analysis of the role of nauplii as a food source is necessary especially as they are often underrepresented in current sampling methods (see Chapter 4) and analysis but can be an important part of consumer diets (Williams et al. 2013).

The results from Chapters 2 to 4 indicated that terrestrial carbon may be an important energetic resource for estuarine food webs, especially after inflow events. However, the ability of terrestrial resources to contribute to trophic levels higher than zooplankton remains equivocal (Ducklow et al. 1986; Brett et al. 2009; Wilkinson et al. 2013), despite several studies showing terrestrial resources contributing to increased estuarine fish populations (Salen-Picard et al. 2002; Hoffman et al. 2007). I therefore explored this by investigating the link between terrestrial carbon, zooplankton and higher food web consumers in Chapter 5, by feeding juvenile fish zooplankton that had been grown with a leachate of terrestrial organic material. The results indicated that terrestrial carbon could be passed to higher trophic levels under experimental conditions, contributing to the growth of juvenile Australian bass, providing another potential link between inflows and population success for the species (Growns and James 2005). In contrast to other experiments investigating terrestrial support of juvenile fish (Degerman et al. 2018; Growns et al. 2020) my experiment utilised a single source food web under food limited conditions, with few trophic levels and high concentrations of terrestrial carbon (Lennon and Pfaff 2005).

Importantly, inflows fundamentally alter estuarine conditions, especially from those of low flow periods where algal resources are the dominant food web resource (Cloern et al. 2014; Andersson et al. 2018). Bioavailable terrestrial material transported to estuaries following inflows supplies a terrestrial resource while simultaneously reducing the availability of algal resources and their productivity (Hitchcock and Mitrovic 2013; Andersson et al. 2018; Paczkowska et al. 2019). These conditions are similar to those used in my experiment. Considering that fish recruitment is predicated on growth (Houde 1997; Castonguay et al. 2008), my results suggest terrestrial carbon resources may contribute to the growth of food limited juvenile fish, e.g., smaller, out competed individuals. My results do not necessarily contend with assertions by nutritional and mass balance studies which suggest terrestrial carbon is unlikely to contribute to somatic growth of organisms (Brett et al. 2009, 2012; Galloway et al. 2014) but do highlight the role that terrestrial carbon can play as an energetic

subsidy in aquatic systems after inflows (Kelly et al. 2014). This role may be similar to that of recent studies of co-metabolism which show that consumption of both terrestrial and algal material increases growth and survival rates in zooplankton (Harfmann et al. 2019).

6.2 Further Research Directions

6.2.1 Temporal and spatial replication

While certain elements of my studies align with previous research and can be applied to other estuaries, this is difficult due to the idiosyncratic nature of estuaries (Roelke et al. 2013). Temporally, Chapter 2 encompassed a weak to moderate El Nino period in Australia (2015 – 2016), characterised by slightly reduced rainfall on the south-eastern seaboard (BOM 2020). While seasonal rainfall events still occurred in this study during these years (Chapter 2) the impact of longer periods of drought or significantly wetter La Nina periods on estuarine food webs requires further investigation. Drought or periods of decreased rainfall may drive food web patterns, by increasing algal production due to reduction in discharge and longer water residence times, especially in upper estuaries. As terrestrial organic matter is transported to estuaries during significantly wetter periods, this may potentially result in the opposite pattern, increasing the use of terrestrial resources (Whitworth et al. 2012; Bruesewitz et al. 2013; Cira et al. 2021). These changes in rainfall, discharge and resource availability would likely impact on the zooplankton communities present in the estuaries, and by extension, the food web functioning (Marques et al. 2014). Further research could also investigate the influence of lateral linkages to the floodplain by larger inflows on terrestrial resource availability in estuaries in relation to the 'flood pulse concept' (Junk et al. 1989). While inflow events in this study did bring terrestrial resources to the estuary, all were considered 'in-stream' flows and had little to no connectivity with their floodplains. It is expected that increased connectivity with floodplains due to larger inflows may lead to greater terrestrial resource availability in estuaries (Junk et al., 1989, Baldwin et al., 2016).

This thesis was also spatially limited in 3 ways, *i*) contrasting only two estuaries in Chapter 2, *ii*) focussing on a single regulated system in Chapters 3 and 4 and *iii*) having a specific focus on upper estuarine areas. As estuaries can behave differently in response to inflows between systems and within different areas of a single system (Roelke et al. 2013) it is important to study individual estuaries in detail to inform our scientific understanding and better inform management decisions (Adams 2014). However, this does not necessarily limit the

applicability of findings to wider contexts. Further research contrasting patterns throughout a range of regulated and unregulated systems in Australia, and globally, may uphold or contrast some of the patterns seen in this thesis. For example, a potentially closer relationship to freshwater inflows and terrestrial carbon inputs by food webs of regulated systems. Some of the patterns presented in this thesis may only be present in upper estuarine areas, however, it would be useful to assess their strength throughout estuarine gradients. Despite these limitations, the negative impact of river regulation on water quality and fish production has been found globally (Drinkwater and Frank 1994; Gillanders and Kingsford 2002). In the context of the work presented in this thesis, it is likely that there will be some consistent impacts of river regulation on the planktonic food web of estuaries worldwide.

6.2.2 Terrestrial carbon resources and co-metabolism

Terrestrial carbon can support the metazoan food web in estuaries. The evidence presented in Chapter 4 demonstrated the use of terrestrial carbon by the zooplankton food web and in Chapter 5 showed its potential to be transferred to higher trophic levels such as juvenile fish. However, my experiments are limited by the chemical resolution of their investigation and the specific mechanisms by which carbon contributes to these trophic levels requires further investigation. Previous research has indicated that terrestrial carbon, due its lack of HUFA compounds, is more likely to subsidise energetic needs rather than contribute to growth compared to algal carbon (Brett et al. 2009; Taipale et al. 2014). However, recent studies have suggested that zooplankton may actively select for terrestrial material in the presence of algal material and can improve their survival and growth rates when consuming both energetic sources together (Harfmann et al. 2019). The use of additional stable isotopic compounds, like deuterium (Brett et al. 2018), or specific chemical biomarkers and DNA metagenomics (Harfmann et al. 2019) as food web tracing techniques may be effective in advancing investigations into these specific uses of basal resources.

6.2.3 The role of mixotrophs

Further investigations into terrestrial resource use by aquatic consumers could also focus on the role of mixotrophic protists in energetic transfer. While Chapter 3 does include mixotrophic protists in its analysis, the scope and aim of the experiment limited the exploration of the protist role and response to freshwater inflows in estuaries. Since 1990 the classification of protists into autotrophic and heterotrophic categories has become more

difficult, with many protists now recognised as being capable of mixotrophy (Mitra et al. 2014b; Stoecker and Pierson 2019). The role of mixotrophs remains unclear, but recent studies have shown the ability of terrestrial carbon to be transferred to zooplankton through mixotrophic protists (Trochine et al. 2015) while their ability to "trophically upgrade" terrestrial material, improving its nutritional quality, has also been documented (Arts et al. 2009). Further investigation into the importance of protists to zooplankton diets and in terrestrial energy transfer is required (Stoecker and Pierson 2019).

6.2.4 Responses of higher trophic levels to terrestrial inputs and heterotrophy

There is widespread documentation of the responses of planktonic food webs to terrestrial carbon inputs (Hitchcock et al. 2016b; Degerman et al. 2018; Berggren et al. 2018) and emerging work investigating the effects of changing patterns of terrestrial resource delivery to estuaries on lower food web components (Paczkowska et al. 2017; Andersson et al. 2018; Bartels et al. 2018). However, it is not known how increases in terrestrial carbon inputs to estuaries, through climatic changes to rainfall and runoff (Andersson et al. 2018), or through river regulation (Hemraj et al. 2017b), will affect higher trophic levels such as fish and birds. Heterotrophy in aquatic systems requires longer food chains to transport energy to higher trophic levels and as a result more energy is respired by the food web (Ducklow et al. 1986; Brett et al. 2009). How this will impact higher trophic levels is currently unknown but could result in changes to species composition, for example, detritivorous fish communities maybe favoured as their feeding ecology could reduce the lengths of their food chain relative to planktivores (Darnaude 2005)

6.3 Management Recommendations

This study contributed to the assessment and evaluation of the *WSP for the Hunter Alluvial Water Sources 2009*, specifically addressing the effectiveness of existing environmental flow protocols for the Williams River estuary. The findings of this thesis suggest that the effects of river regulation were still present in the Williams River estuary under the current rules established in the WSP. Therefore, several key recommendations stemming from the work presented in this thesis were included in a report to NSW DPIE Water (O'Brien et al. 2020) to enhance ecological outcomes in the Williams River estuary. These recommendations may, however, be applicable to other regulated estuarine systems within Australia and globally.

My first recommendation centres on the importance of mimicking natural flow patterns and

establishing riverine connectivity (Poff et al. 1997). While this is a common environmental flow practice, there are specific elements of natural flows that may be beneficial to the Williams River estuary, including low-flow riverine connectivity and post-peak releases. While it must be recognised that there are some managed flow releases from Seaham Weir the results of this thesis indicate the frequency, timing and duration of these releases does not adequately mimic natural flow patterns, particularly in dry periods where water in the weir for extraction and may be contributing to some water quality and ecological issues in the upper estuary. Increased riverine connectivity during low flow periods, where water is available through daily releases, may aid in maintaining an estuarine gradient in the upper Williams estuary as well as providing riverine connectivity, allowing the migration of fauna through this important transitional habitat. Furthermore, it may promote the replenishing of freshwater algal and zooplankton communities in the estuary which may be important resources for estuarine consumers. Low flow releases may also mediate water quality and productivity patterns in the system, reducing the likelihood of algal blooms by reducing water residence times in the weir pool and the upper estuary. Currently there are no daily release provisions through Seaham weir in the WSP for the Williams River (NOW 2009a), though some managed releases are made by the Hunter Water Corporation who manage the weir. In part, this may be related to the inability of the current Seaham Weir gates to maintain low flow releases to the estuary. However, Seaham Weir is scheduled for an upgrade in the near future with a focus of this upgrade being the ability of the new structure to facilitate daily releases at lower flow conditions. Implementation of this recommendation, coupled with the weir upgrade, will likely improve conditions in the estuary downstream of the weir.

Secondly, it is important that first flush protections are enacted on the Williams River estuary. Chapter 3 demonstrated the importance of the resources, namely organic carbon and phosphorous, being transported from the catchment during the early stages of inflows or the "first flush". While these resources are currently being transported under exiting flow rules, further protection is needed to ensure their continued delivery to the estuary. First flush protections are a common part of other WSPs, preventing the extraction of water during the first 24 hours of inflows. Although it is generally understood that first flush water is "dirtier" and is not usually extracted by local water users, first flush protections are not included in the current WSP (NOW 2009a) and could therefore be easily applied in the Williams system. This could help to alleviate the impacts of stratification that can occur in the estuary and

remove concentrations of nutrients and organic matter that may be trapped in the upper estuary. Legislated protection of these first flush periods may also aid in ensuring future flushing of the upper estuarine area, enshrining protection against extraction if there are changes in water demand, leading to the use of poorer quality water in the future due to climate change and decreased water security.

6.4 Conclusions

Freshwater inflows play a significant role in the ecology of the estuaries investigated in this thesis. Inflows influence resource availability, zooplankton communities, patterns of resource use and the productivity of estuarine environments. Reduced inflows (through river regulation) negatively impacted on the water quality of the regulated Williams estuary compared to an unregulated estuary, the Manning, and influenced the composition of zooplankton communities and the environmental factors controlling them. River regulation and the alteration of natural flow patterns may have also played a role in the short-term food web dynamics of the same regulated estuary, where increased food web resources following an inflow did not translate to sustained zooplankton productivity. Freshwater inflows were also found to deliver terrestrial carbon resources to the estuary which were an important energy source for the mesozooplankton food web, especially following inflows. Furthermore, terrestrial carbon inputs yielded responses in copepod nauplii and associated predator species in the regulated Williams River estuary. Further investigations into the importance of terrestrial carbon resources to juvenile fish energetics through an experiment suggest the potential of terrestrial resources to be transferred through the planktonic food web to higher trophic levels. This research may have important implications for the link between freshwater inflows and juvenile fish recruitment in estuarine ecosystems, indicating terrestrial carbon may have a role to play in supporting juvenile fish residing in estuarine environments, especially in upper estuaries. While each of the chapters within this thesis contributes specific knowledge to subdisciplines within estuarine flow-ecology, together they suggest the importance of freshwater inflows, riverine connectivity and natural flow patterns in delivering important food web resources and maintaining estuarine function and productivity. Acting on these findings and the wider body of literature supporting the protection of freshwater inflows to estuaries is the crucial next step in maintaining these complex ecosystems into the future.

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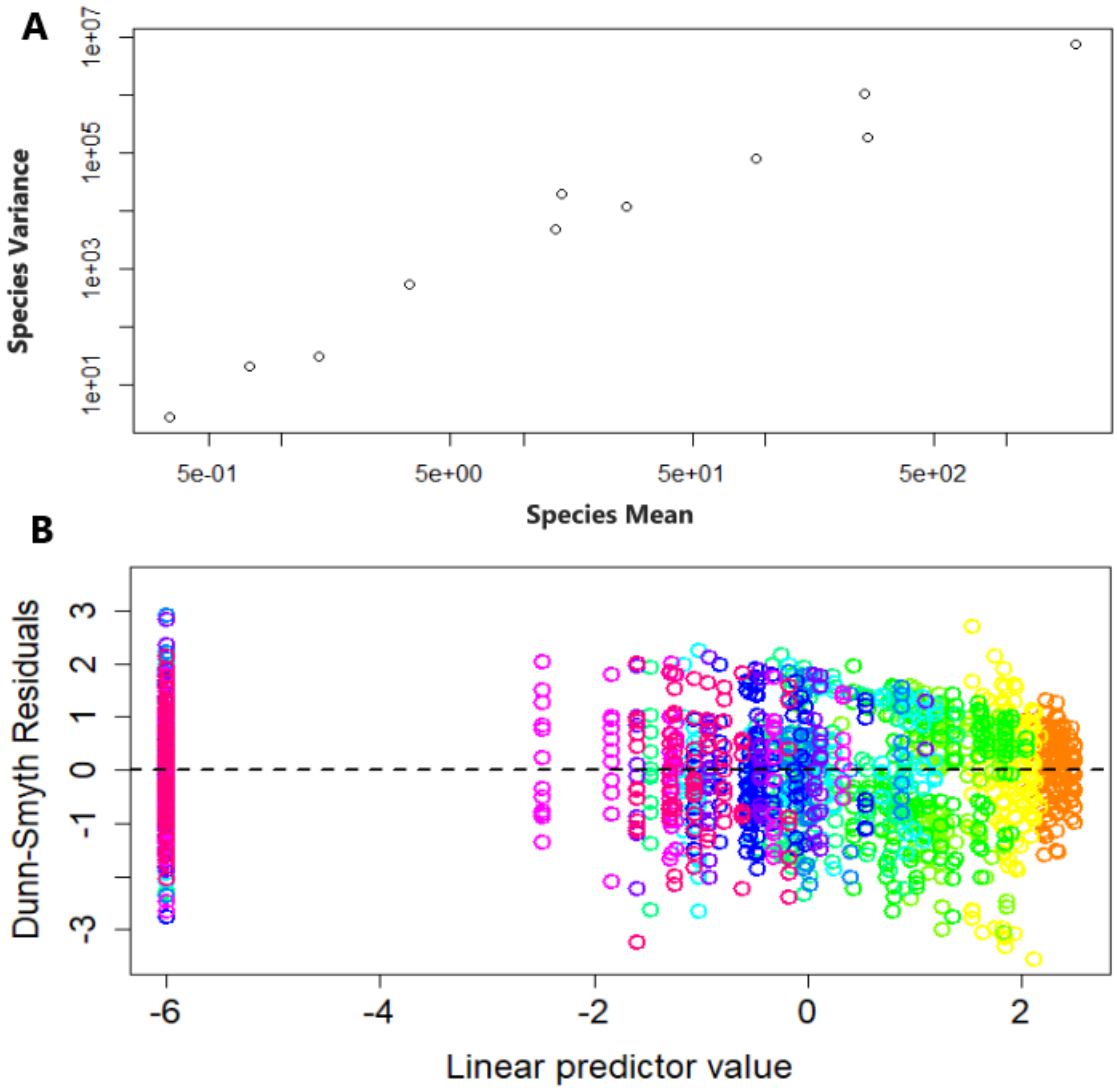
8. Appendices



Appendix 1: Mean variance relationships for the zooplankton multivariate abundance data (A) and fitted residuals with a negative binomial distribution (B) indicating an appropriate model fit.

*Appendix 2: Correlation matrix for each site in the Manning and Williams Rivers between 10, 30 and 60-Day antecedent discharge metrics (ML/Day) and physicochemical and nutrient variables,. Antecedent inflows were transformed by log(x+1). Numbers represent the R2 of Pearson's R while asterisks represent the corresponding p-value, * <0.05, **<0.01, ***<0.001. A negative sign (-) indicates the correlation is negative.*

Estuary	Site	Flow	DOC	Turbidity	NO_x	Chl a	DO	pH	Salinity
Manning	1	Q10			0.39***	-0.13*			-0.49***
		Q30			0.23**	-0.12*			-0.47***
		Q60			0.12*				-0.35***
	2	Q10			0.36***				-0.75***
		Q30		0.17*	0.19**				-0.75***
		Q60							-0.57***
	3	Q10		0.14*	0.35***	-0.13*			-0.78***
		Q30			0.18*	-0.14*			-0.65***
		Q60				-0.15*			-0.54***
Williams	1	Q10		0.26**					-0.54***
		Q30		0.30***			-0.13*	-0.14*	-0.68***
		Q60		0.21**			-0.24**	-0.28**	-0.65***
	2	Q10	0.14*	0.27**			-0.12*	-0.12*	-0.44***
		Q30		0.40***			-0.21**	-0.16*	-0.58***
		Q60		0.24**			-0.28***	-0.27**	0.53***
	3	Q10	0.23**	0.13*			-0.36***	-0.23**	-0.41***
		Q30	0.24**	0.31***			-0.48***	-0.29**	-0.63***
		Q60	0.25**	0.23**			-0.48***	-0.27**	-0.68***

*Appendix 3: three-way ANOVA (site*River*Q30 flow class) results for water quality variables in the Williams and Manning River estuaries between 2015 and 2019. Df = degrees of freedom, Sum Sq = sum of squares, Mean Sq = mean of squares.*

¤	Test¤	df¤	Sum·sq¤	Mean·Sq¤	F-value¤	p-value¤	¤
DOC¤	**Site¤**	2¤	0.24¤	0.119¤	0.459¤	0.633¤	¤
¤	**River¤**	1¤	11.85¤	11.85¤	45.679¤	<0.001*¤	¤
¤	**Q30¤**	3¤	0.36¤	0.120¤	0.461¤	0.71¤	¤
¤	**Site*River¤**	2¤	0.09¤	0.045¤	0.172¤	0.84¤	¤
¤	**Site*Q30¤**	6¤	1.07¤	0.178¤	0.688¤	0.66¤	¤
¤	**River*Q30¤**	3¤	1.03¤	0.344¤	1.326¤	0.268¤	¤
¤	**Site*River*Q30¤**	6¤	0.83¤	0.139¤	0.536¤	0.78¤	¤
¤	**Residuals¤**	163¤	42.29¤	0.259¤	¤	¤	¤
Turbidity¤	**Site¤**	2¤	0.50¤	1.25¤	0.609¤	0.545¤	¤
¤	**River¤**	1¤	44.29¤	44.29¤	108.057¤	<0.001*¤	¤
¤	**Q30¤**	3¤	2.82¤	0.94¤	2.29¤	0.804¤	¤
¤	**Site*River¤**	2¤	0.03¤	0.02¤	0.029¤	0.96¤	¤
¤	**Site*Q30¤**	6¤	5.17¤	0.86¤	2.102¤	0.056¤	¤
¤	**River*Q30¤**	3¤	1.29¤	0.43¤	1.046¤	0.37¤	¤
¤	**Site*River*Q30¤**	6¤	1.19¤	0.20¤	0.484¤	0.8199¤	¤
¤	**Residuals¤**	163¤	66.81¤	0.41¤	¤	¤	¤
NOx_c	**Site¤**	2¤	0.011¤	0.0057¤	0.253¤	0.7765¤	¤
¤	**River¤**	1¤	0.267¤	0.267¤	11.8¤	0.00075*¤	¤
¤	**Q30¤**	3¤	0.097¤	0.0324¤	1.43¤	0.235¤	¤
¤	**Site*River¤**	2¤	0.052¤	0.0259¤	1.14¤	0.3215¤	¤
¤	**Site*Q30¤**	6¤	0.043¤	0.007¤	0.316¤	0.92¤	¤
¤	**River*Q30¤**	3¤	0.013¤	0.004¤	0.189¤	0.903¤	¤
¤	**Site*River*Q30¤**	6¤	0.212¤	0.035¤	1.557¤	0.163¤	¤
¤	**Residuals¤**	163¤	3.694¤	0.022¤	¤	¤	¤
FRP¤	**Site¤**	2¤	0.0725¤	0.0362¤	4.225¤	0.163¤	¤
¤	**River¤**	1¤	0.397¤	0.397¤	46.340¤	<0.001*¤	¤
¤	**Q30¤**	3¤	0.06¤	0.0205¤	2.395¤	0.0703¤	¤
¤	**Site*River¤**	2¤	0.003¤	0.0015¤	0.176¤	0.8386¤	¤
¤	**Site*Q30¤**	6¤	0.0547¤	0.009¤	1.062¤	0.3878¤	¤
¤	**River*Q30¤**	3¤	0.0298¤	0.0099¤	1.158¤	0.3276¤	¤
¤	**Site*River*Q30¤**	6¤	0.0363¤	0.0060¤	0.704¤	0.6466¤	¤
¤	**Residuals¤**	163¤	1.395¤	0.0086¤	¤	¤	¤
Chl-a¤	**Site¤**	2¤	0.85¤	0.43¤	0.648¤	0.524¤	¤
¤	**River¤**	1¤	36.83¤	36.83¤	56.058¤	<0.001*¤	¤
¤	**Q30¤**	3¤	2.34¤	0.78¤	1.187¤	0.317¤	¤
¤	**Site*River¤**	2¤	0.62¤	0.31¤	0.470¤	0.626¤	¤
¤	**Site*Q30¤**	6¤	3.10¤	0.52¤	0.785¤	0.583¤	¤
¤	**River*Q30¤**	3¤	2.68¤	0.89¤	1.36¤	0.257¤	¤
¤	**Site*River*Q30¤**	6¤	0.38¤	0.06¤	0.096¤	0.997¤	¤
¤	**Residuals¤**	163¤	107.08¤	0.66¤	¤	¤	¤
DO¤	**Site¤**	2¤	0.02¤	0.0099¤	0.192¤	0.824¤	¤
¤	**River¤**	1¤	0.252¤	0.252¤	4.867¤	0.2881*¤	¤
¤	**Q30¤**	3¤	0.233¤	0.077¤	1.502¤	0.216¤	¤
¤	**Site*River¤**	2¤	0.037¤	0.187¤	0.362¤	0.697¤	¤
¤	**Site*Q30¤**	6¤	0.523¤	0.87¤	1.684¤	0.1280¤	¤
¤	**River*Q30¤**	3¤	0.412¤	0.137¤	2.653¤	0.0504¤	¤
¤	**Site*River*Q30¤**	6¤	0.258¤	0.04293¤	0.829¤	0.549¤	¤
¤	**Residuals¤**	163¤	8.42¤	0.05179¤	¤	¤	¤

Appendix 4: Algal genera found in the Williams River estuary between January and August 2018 that make up the orders displayed in Figure 3.5. Maximum biovolume (mm^3/L) and percentage of samples of each genus are also presented

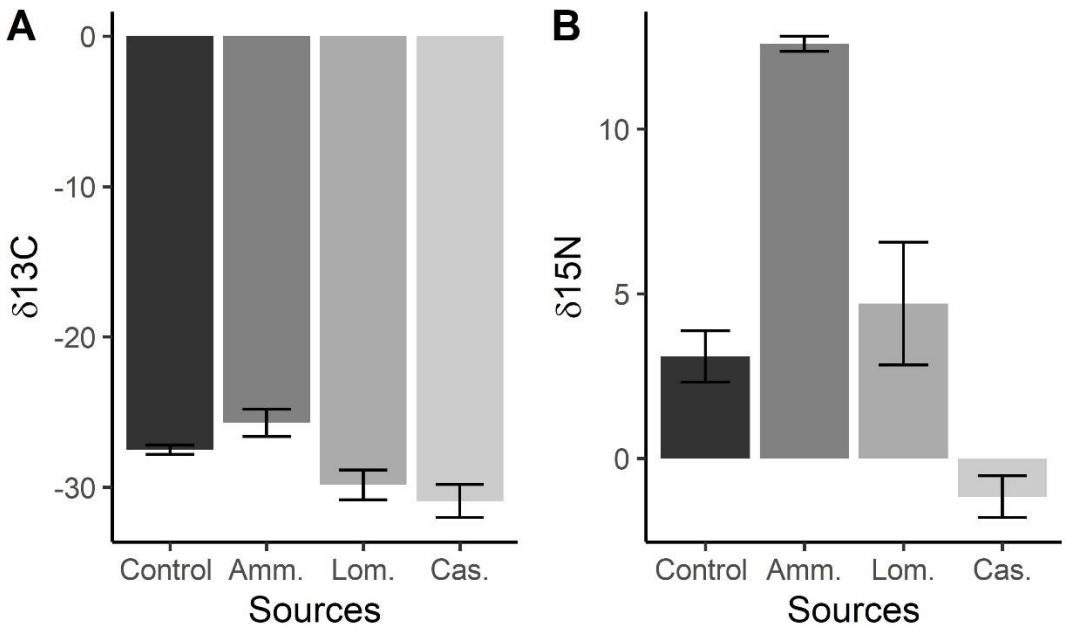
Group	Order	Max Biovolume	%	Group	Order	Max Biovolume	%
Green	*Ankistrodesmus*	0.02	24	**Diatoms**	*Cyclotella*	0.11	96
	Chlamydomonas	0.03	56		*Navicula*	0.01	56
	Closterium	0.03	24		*Sydnedra*	0.24	48
	Cosmarium	0.98	76		*Aulacoseira*	0.02	4
	Crucigenia	0.07	68		*Melosira*	0.28	24
	Dictyosphaerium	0.003	8		*Gyrosigma*	0.01	4
	Oocystis	0.10	16		*Nitzschia*	0.06	24
	Scenedesmus	0.86	64		*Cymbella*	0.02	8
	Staurastrum/ Staurodesmus	0.33	12		*Cocconeis*	0.001	4
	Pediastrum	0.05	16	**Cyanobacteria**	*Microcystis*	0.008	8
	Ankyra	0.005	8		*Aphanocapsa*	5.11	80
	Chlorella	0.38	74		*Dolichospermum cf. nova*	0.03	12
	Tetraspora	0.05	8		*Planktolyngbya*	0.14	12
	Nephrocytium	0.10	8	**Euglinoids**	*Euglena/ Phacus*	8.53	76
	Selenastrum	0.04	8		*Trachelomonas*	0.65	60
	Monoraphidium	0.05	56	**Cryptomonads**	*Cryptomonas*	2.87	92
	Chlorogonium	0.11	4		*Croomonas*	2.76	100
	Botryococcus	0.32	44	**Other**	*Mallomonas*	0.99	44

	Eudorina	0.10	4	*Strombidium*	0.26	4
Dinoflagellates	*Ceratium*	0.6	4			
	Peridinium	1.48	36			

Appendix 5: Biovolumes (mm^2/L) of algal orders in the nutrient amendment experiment to obtain autotrophic algal end members.

Species	Control	Amended
Mallomonas sp.	0.98	0
Chlamydomonas sp.	0.05	0.01
Strombidium sp.	0.38	0
Chroomonas sp.	0.087	2.27
Chryptomonas sp.	0.27	0
Cyclotella sp.	0	0.16
Peridinium sp.	0	0
Chroococus sp.	0	0.37
Total Biovolume	1.76	2.81

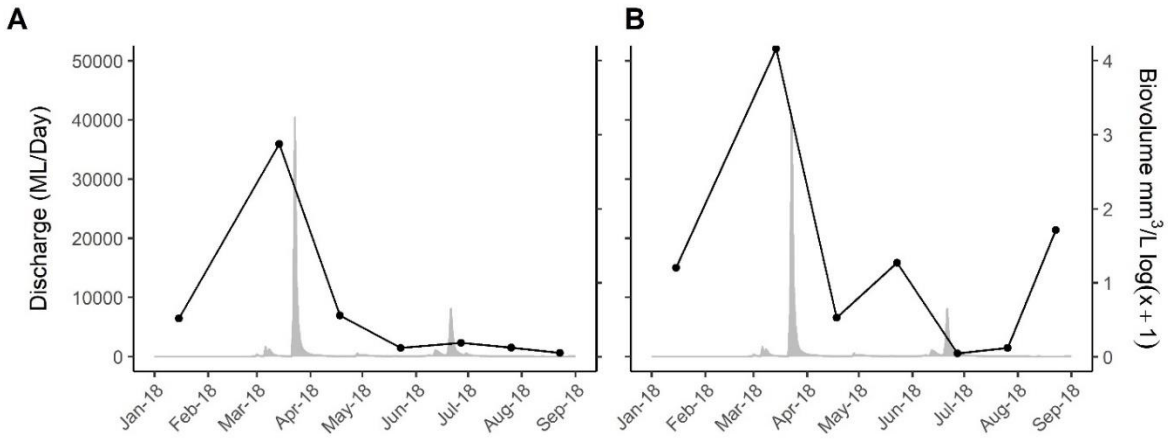
Appendix 6: A) δ 13C and B) δ 15N signatures of suspended particulate organic matter for the control and amended (Amm.) treatments in the nutrient amendment experiment and for terrestrial sources, Lomandra longifolia (Lom.) and Casuarina glauca (Cas.) collected from the region. Error bars represent standard deviation.



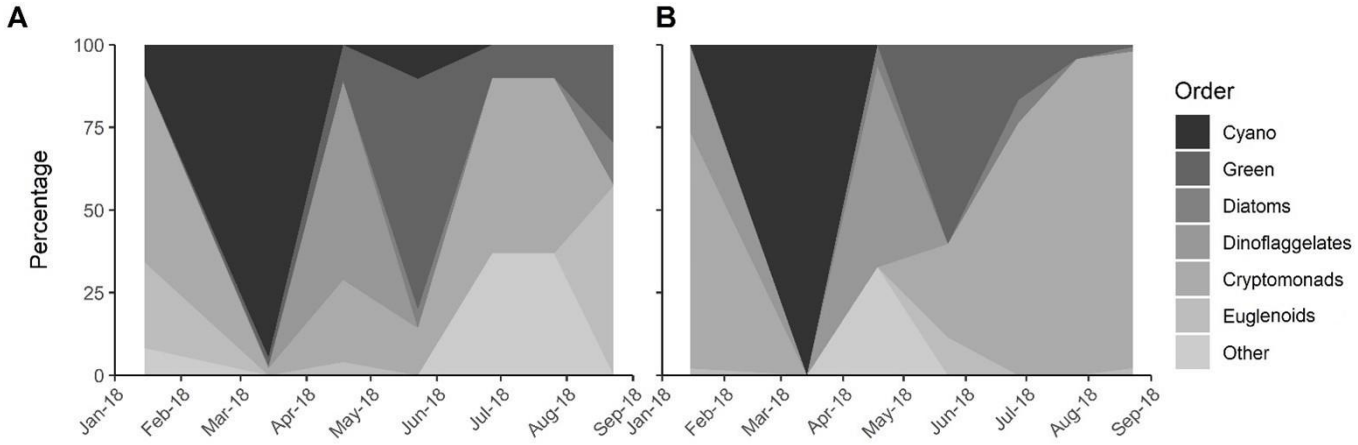
*Appendix 7: two-way ANOVA (site*month) results for chlorophyl a (Chl a), dissolved organic carbon (DOC), turbidity and suspended particulate organic matter (sPOM) δ13C in the Williams River between January and September 2018 . Df = degrees of freedom, Sumsq = sum of squares, Mean Sq = mean of squares.*

	df	Sum sq	Mean Sq	F-value	p-value
Chl *a* – Site	1	0.363	0.363	0.69	0.42
Chl *a* – Month	1	1.251	1.251	2.38	0.15
Chl *a* – Site*Month	1	0.109	0.110	0.21	0.65
Residuals	10	5.257	0.526		
DOC – Site	1	0.095	0.096	0.153	0.70
DOC – Month	1	0.173	0.173	0.28	0.61
DOC – Site*Month	1	0.034	0.034	0.06	0.82
Residuals	10	6.23	0.623		
Turbidity – Site	1	0.1	0.15	0.001	0.98
Turbidity – Month	1	162.3	162.3	0.632	0.45
Turbidity – Site*Month	1	0.8	0.76	0.003	0.96
Residuals	8	2054.4	256.8		
sPOM 13C – Site	1	0.53	0.526	0.119	0.74
sPOM 13C – Month	1	3.29	3.288	0.747	0.41
sPOM 13C – Site*Month	1	3.67	3.670	0.834	0.38
Residuals	10	44.02	4.402		

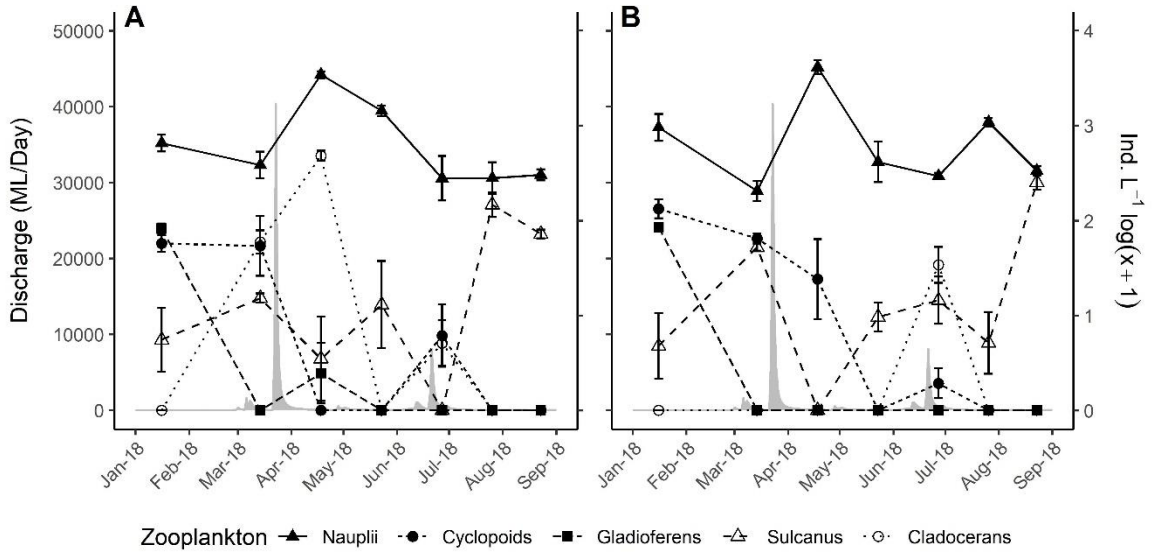
Appendix 8: Discharge (shaded peaks, ML/Day) and transformed (log(x+1)) total algal biovolume (mm^3/L) for sites 1 and 2 (A and B respectively) over the study period in the Williams River estuary.



Appendix 9: Percentage of total biovolume (%) for algal orders for sites 1 and 2 (A and B respectively) over the study period in the Williams River estuary. "Cyano" represents cyanobacterial orders and "Other" represents a combination of Mallomonas and Strombidium genera (Appendix 4).



Appendix 10: Discharge (shaded peaks, ML/Day) and transformed (log(x+1)) abundance of mesozooplankton (ind/L) for sites 1 and 2 (A and B respectively) over the study period in the Williams River estuary. Error bars represent standard error of the mean.



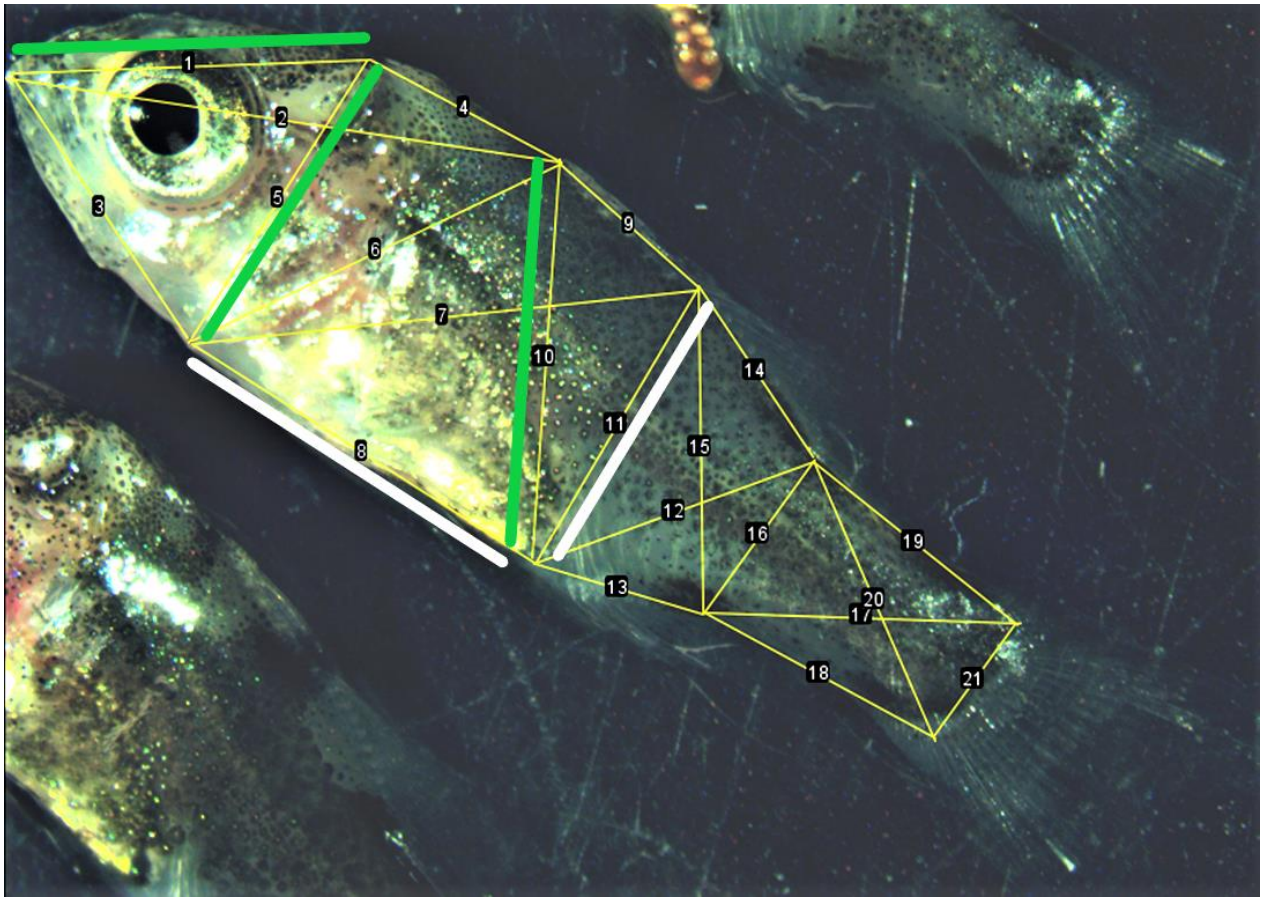
Appendix 11: Survival rates of each treatment. Larger standard deviation in the T2 0 mg/L samples are due to swim bladder infections in 1 tank. This T2 control tank was impacted two days before sampling so growth depensation effects were disregarded.

	Survival %		
Treatment	T1	T2	T3
0 mg/L	97.8 (1.3)	76.8 (13.8)	79.7 (1.4)
5 mg/L	99.8 (0.1)	89.2 (2.4)	80.2 (4.0)
10 mg/L	98 (1.2)	84.9 (5.1)	85.3 (1.5)

Appendix 12: Landmarks used in morphometric analysis taken from Cavalcanti et al., (1999)

Landmark Number	Landmark Position
1	anterior tip of the snout on the upper jaw
2	the most posterior aspect of the neurocranium (beginning of scalednape)
3	origin of pelvic fin
4	origin of spinous dorsal fin
5	origin of anal fin
6	origin of soft dorsal fin
7	insertion of anal fin
8	insertion of 2nd dorsal fin
9	insertion of 1st ventral caudal fin ray;
10	insertion of 1st dorsal caudal fin ray

Appendix 13: Truss based morphological measurements on a juvenile Australian bass (Macquaria novemculeata) from the 10 mg/L treatment at the conclusion of the experiment. Numbers indicate the order in which measurements (thin yellow lines) were taken between the landmarks given in Appendix 12 from Cavalcanti et al. (1999). Measurements are denoted in text using the prefix "X" e.g., X12, is the measurement between the of the anal fin and the insertion of the 2nd dorsal fin. Thick green lines indicated measurements which contributed to the differences in fish morphology between treatments in PC1 of Figure 5.4, while thick white lines indicate the same for PC2.



Appendix 14: two-way ANOVA results for fish weights and lengths. df = degrees of freedom, Sum Sq = sum of squares, Mean Sq = mean of squares.

	df	Sum sq	Mean Sq	F-value	p-value
Weight - Treatment	3	0.1258	0.04194	71.234	<0.001
Weight - Time	2	0.0229	0.0114	19.431	<0.001
Weight – Treatment*Time	4	0.0141	0.00353	5.992	<0.001
Residuals	839	04939	0.00059		
Standard Length - Treatment	3	37.4	12.467	15.024	<0.001
Standard Length - Time	2	8.8	4.4	5.302	<0.001
Standard Length – Treatment*Time	4	3.3	0.823	0992	0.411
Residuals	839	696.2	0.830		
Fork Length - Treatment	3	51.5	17.16	15.333	<0.001
Fork Length - Time	2	72.5	36.25	32.395	<0.001
Fork Length – Treatment*Time	4	4.9	1.22	1.089	0.361
Residuals	839	938.9	1.12		
Total Length - Treatment	3	82.9	27.632	18.982	<0.001
Total Length - Time	2	49.6	24.819	17.049	<0.001
Total Length – Treatment*Time	4	7.2	1.79	1.231	0.296
Residuals	839	1221.4	1.456		