The Dynamics of Carbon and Nitrogen Stable Isotope Analysis of Aquatic Organisms within the Grand River Watershed

By

Heather Anne Loomer

A thesis presented to the University of Waterloo in fulfillment of the thesis requirement for the degree of Master of Science in Biology

Waterloo, Ontario, Canada, 2008

© Heather Anne Loomer 2008
Author’s Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.
Abstract

Stable isotope analysis is a tool employed in ecological studies to provide information on the movement of elements and energy through a system. The stable carbon and nitrogen isotope analysis of aquatic organisms has been commonly used to address questions related to energy transfer between organisms and to identify the reliance of aquatic organisms on different sources of organic matter within the system. Within the rivers, stable isotope analysis has been used to describe food webs and connect conditions within the watershed with the river. The Grand River watershed is a predominantly agricultural watershed which receives inputs from ~26 MWWTP and is managed for flow by multiple large reservoirs and weirs. The stable isotope values of aquatic organisms within this watershed were analyzed from samples collected between May and September, 2007. Sites were selected in relation to three different municipal waste water treatment plants (MWWTP) in the centre of the watershed and along a 200 km stretch of the main stem of the Grand River. Results show that stable isotope analysis can be used to differentiate organisms collected from different sites and which represent different trophic levels within the river system under select conditions. Sites which are influences by inputs from organic matter or nutrients within distinct isotope values can be distinguished easily if the input is large and the isotope values are significantly distinct from background values. For smaller inputs changes in stable isotope values were not observed relative the background variability in the system. In this case, sites should be selected to allow for the characterization the variation in isotope values already occurring within the river. Samples collected later in the growing seasons have more distinctive
isotope values are between sites. At sites where seasonal variation is greater, the organisms collected may not show a clear separation between trophic levels. A lack of knowledge regarding the time period represented by the tissues of the organisms challenges interpretation these results. It is concluded that stable isotope values of aquatic organisms reflected the condition of this watershed. For nitrogen increasing loads from point sources were accompanied by increasing isotope values. Stable isotope values decreased over the river reach where recovery in river condition occurs as a result of ground water inputs. The influence of individual large MWWTP and reservoirs was observable and the management of the MWWTP and reservoir appears to affect the changes in isotope values which are observed.
Acknowledgements

At the end of this path I look back to the beginning to reflect on the journey and all the blood, sweat, and tears that go along with it. I have always found that the best lessons are often the most painful to learn, and under this pre-text I feel I must have learned some significant lessons about science, research, and myself in these capacities. To this there are many people who have contributed to this thesis without which it would not have been accomplished. As I started to write down all the people who have been directly or indirectly involved in this project the acknowledgements multiplied exponentially proving the completion of any task is never done a single person.

I would like to acknowledge the hard work of the Environmental Stable Isotope Lab at the University of Waterloo and give a special thank you to Bill Marks for his helpful advice and cheerful presence. There are a number of motivated researchers and students working in the University of Waterloo and on the Grand River with whom I have been able to work along side sharing data, discussions, and laughs. I would like to acknowledge all those involved in the Aquatic Ecology Group, “Day in the Length of the Grand” project, the first floor Biology grads, and all of the past and present members of the Servos lab. There are so many of you who have made this process memorable.

Within this group there are a few people who I would like to thank specifically by name. To Richard Elgood and Ken Oakes, who within different labs provide a welcoming presence and provided the frame work on which good work can be accomplished. I appreciate not only what you do but how you do it. To Kelly Lippert and Ryan Spafford, with whom I shared many long hours in smelly waders, I feel I have had a unique glimpse of who you are and will always hold a respect for you that comes from seeing the
multiple facets of someone’s personality. To Jen Ings, who has been a good friend and has provided the voice of experience from someone one step ahead along a shockingly similar path. To Brendan Knight, who has always provided me with a good ear, good advice, and a helping hand in the field and lab. Finally a special thank you to those of you who volunteered your time to help me collect my samples, processing them in the lab, or providing insight: Jill Heinrich, Jen Kormos, Lukshman “Lucky” Ragunathan, and Gerald Tetreault. I have shared so many smiles, out-of-tune melodies, and advice with all of you and it is impossible not to think of all of you with fondness.

I have been fortunate to have the involvement of three wonderful researchers in my project. As a mentor, Dr. Mark Servos demonstrates an infectious enthusiasm for research and ambition to keep moving to the next challenge. As a supervisor, he has effectively walked the fine balance between providing a helping hand and letting me learn to walk on my own, for this I am grateful. On my committee I have had Dr. Sherry Schiff and Dr. William Taylor, whose input has always been valuable and insightful. While I can not give any of you what you have given me, I can hope I will have learned from your example and will be able to pass this example on.

A special thank you to Mark Jeffrey and our four legged fiends who have managed to keep me anchored during this two year roller coaster ride,

That isn’t to say, I don’t love roller coasters…
Table of Contents

List of Tables ........................................................................................................................................ viii

List of Figures ...................................................................................................................................... ix

Chapter 1: Introduction ...................................................................................................................... 1
  The Use of Stable Isotopes to Describe Food Webs ....................................................................... 2

Chapter 2: The effect of different municipal wastewater treatment plant effluents on carbon and nitrogen stable isotope values of aquatic organisms within the Grand River watershed ............. 10
  Introduction ........................................................................................................................................ 12
  Methods ................................................................................................................................................ 13
  Results .................................................................................................................................................. 18
  Discussion ........................................................................................................................................... 30

Chapter 3: Trends in the stable carbon and nitrogen isotope values of riffle dwelling aquatic organisms along a 200 km river reach in the Grand River .......................................................... 39
  Introduction ........................................................................................................................................ 41
  Materials and Methods ....................................................................................................................... 42
  Results .................................................................................................................................................. 49
  Discussion ........................................................................................................................................... 64

Chapter 4: Conclusions ..................................................................................................................... 72

References ........................................................................................................................................... 74

Appendices .......................................................................................................................................... 78
  Appendix A .......................................................................................................................................... 78
  Appendix B .......................................................................................................................................... 79
  Appendix C .......................................................................................................................................... 80
List of Tables
Table 1: The municipality and associated population, treatment plant processes, and the ratio of ammonia to nitrate in the final effluent of the three MWWTP around which aquatic food web sampling was conducted............................................................... 15
Table 2:: The \( p \) values for the statistical tests run on the Primary consumer and darter datasets collected in May, July and September from sites above (-US #) and below (-DS#) the Waterloo (W--#), Kitchener (K--#), and GUelph (G--#) MWWTP with the Grand River watershed, Ontario. The \( p \) values were calculated from the Kruskal Wallis comparisons for each of the datasets and from comparisons (T-tests equal or unequal variances assumed) between adjacent sites......................................................... 27
Table 3: The river km where the sites sampled in June and September 2007, the municipal waste water treatment plants, and the dams are located on the main stem of the Grand River, Ontario ..................................................................................... 44
Table 4:: The \( p \) values for kruskal wallis tests performed on the primary consumer invertebrate and darter datasets and the t-tests between adjacent sites within each of the primary consumer invertebrates and darter data sets collected from the Grand River, Ontario during June and September, 2007................................................................. 52
Table 5: The average range of the stable carbon and nitrogen isotope values for each of the taxonomic groups of primary consumer invertebrates collected in September, 2007. The average was calculated from the range observed at each site along the main stem of the Grand River where the taxons were collected. The \( R^2 \) and \( p \) values for linear regressions between the nitrogen range and the carbon range for each of the taxonomic groups................................................................................................ 56
List of Figures

Figure 1: Sites in the Grand River watershed, Ontario where invertebrates and fish were collected and analyzed for carbon and nitrogen isotope ratios during May-September, 2007. The locations of the municipal waste water treatment plants relative to the sampling locations is shown. ............................................................. 14

Figure 2: Plots of $\delta^{15}$N by $\delta^{13}$C signatures for organisms collected from the Grand River watershed above (WUS 1 & WUS 2) and below (WDS 1) the Waterloo MWWTP in May (solid figures), July (grey figures) and September (open figures). The points for the invertebrates collected in September and all of the darters are represented by the mean (± standard error) and the invertebrates from May and July are single points from a pooled sample. ........................................................................................................ 20

Figure 3: Plots of $\delta^{15}$N by $\delta^{13}$C signatures for organisms collected from the Grand River watershed above (KUS 1) and below (KDS 1 & KDS 2) the Kitchener MWWTP in May (solid figures), July (grey figures) and September (open figures). The points for the invertebrates collected in September and all of the darters are represented by the mean (± standard error) and the invertebrates from May and July are single points from a pooled sample. ................................................................................................ 21

Figure 4: Plots of $\delta^{15}$N by $\delta^{13}$C signatures for organisms collected from the Grand River watershed above (GUS 1 & GUS 2) and below (GDS 1) the Guelph MWWTP in May (solid figures), July (grey figures) and September (open figures). The points for the invertebrates collected in September and all of the darters are represented by the mean (± standard error) and the invertebrates from May and July are single points from a pooled sample. .................................................................................................................................. 22

Figure 5: The mean (± standard error) nitrogen isotope signatures of primary consumers (bottom graphs – square symbols) and darters (top graphs – circle symbols) on the Speed (left graphs) and Grand (right graphs) rivers with respect to MWWTP outfalls in the cities of Waterloo, Kitchener, and Guelph. The solid symbols and solid line correspond with samples collected in May, the grey and dashed line in July, and the open and dotted line in September 2007 .................................................................................................................. 24

Figure 6: The mean (± standard error) carbon isotope signatures of primary consumers (bottom graphs – square symbols) and darters (top graphs – circle symbols) on the Speed (left graphs) and Grand (right graphs) rivers with respect to MWWTP outfalls in the cities of Waterloo, Kitchener, and Guelph. The solid symbols and solid line correspond with samples collected in May, the grey and dashed line in July, and the open and dotted line in September 2007 .................................................................................................................. 25

Figure 7: The median value and range of isotope values for carbon and nitrogen in the darters and primary consumers collected from sites surrounding the Waterloo and Kitchener MWWTP on the Grand River, Ontario in May and September of 2007. 28

Figure 8: The median value and range of isotope values for carbon and nitrogen in the darters and primary consumers collected from sites surrounding the Guelph MWWTP on the Speed River, Ontario in May and September of 2007. .......... 29

Figure 9: The 16 sites sampled for riffle dwelling invertebrate primary consumers and darters during June and September 2007 long the first 200 km of the Grand River. The subwatersheds are shaded in grey and the three major tributaries (the Conestogo, the Speed, and the Nith) are indicated by the letters A, B and C
Chapter 1: Introduction

Stable isotope analysis has a number of applications in environmental science and can be performed on different elements (hydrogen, nitrogen, oxygen, carbon, and sulfur) to answer a variety of research questions related to element cycling (Cifuentes et al., 1988; Bedard-Haughn et al., 2003). In general, stable isotope analysis can be used to track the occurrence of a specific process (Minagawa and Wada, 1984), to track an element from a given source as it moves through the system under study (France, 1995b), or a combination of both (Anisfeld et al., 2007).

The movement of elements and energy through aquatic food webs have been described using carbon and nitrogen stable isotope analysis of the tissues of these organisms (Peterson and Fry, 1987). The patterns in carbon and nitrogen isotope values allow for the differentiation between trophic levels and the identification of the source of the organic matter which support particular organisms. This has allowed questions related to migratory animals (Hobson et al., 1999), the effects of species introductions (Kidd et al., 1999), and the movement of biomagnifying contaminants though food webs (Atwell et al., 1998; Vander Zanden and Rasmussen, 2001) to be addressed.

Stable carbon and nitrogen isotope analysis of aquatic organisms have been used to identify inputs and changes in the cycling of these elements due to different forms of human activity (Hicks, 1997; deBruyn and Rasmussen, 2002; Anderson and Cabana, 2005). Of particular interest are the observed changes in nitrogen signatures influenced by municipal waste water treatment plants (MWWTP) and agricultural areas (Fry and Allen, 2003; Steffy and Kilham, 2004; Leavitt et al., 2006). These results have lead to the suggestion that nitrogen stable isotope values of organic components within aquatic
systems could be used to track these nitrogen inputs and associated contaminants as they move through the system (Spies R B et al., 1989; Pruell et al., 2006).

The application of stable carbon and nitrogen isotope analysis have occurred to a limited extent in rivers and rarely in rivers heavily influenced by human activity. It is therefore, unclear what limits exist on the application of stable carbon and nitrogen isotopes to describe food webs and reflect human activity within these types of systems.

**The Use of Stable Isotopes to Describe Food Webs**

Isotopes are different forms of the same element with a different number of neutrons and the stable isotopes are a subset which does not undergo radio-active decay. The ratio of different isotopes of a single element is a sample’s isotopic composition. The isotopic composition of a sample can be quantified by standardizing the ratio of heavy to light isotopes against an international standard. The result is a delta (δ) value which can be used to communicate differences in isotope compositions of different samples between and within studies and research groups.

\[
\delta_{\text{Sample}} = \left[ \frac{R_{\text{Sample}}}{R_{\text{Standard}}} - 1 \right] \times 1000 \quad \text{where} \quad R = \frac{X_{\text{Heavy Isotope}}}{X_{\text{Heavy Isotope}} + X_{\text{Light Isotope}}}
\]

[Equation 1]

The stable isotope composition can be altered by different reaction kinetics between each isotope (Bigeleisen, 1952). As such, the degree to which the ratio is altered can be indicative of a specific process which has occurred (Bedard-Haughn et al., 2003).

Fractionation is the change in isotope composition between the substrate and product of a process/reaction. It can be described by an alpha value (α) or an enrichment factor (ε). An alpha value is the ratio between the δ value of the substrate and product.

\[
\alpha_{s-p} = \frac{\delta_{\text{Substrate}}}{\delta_{\text{Product}}}
\]
The enrichment factor is the difference between the $\delta$ values of the substrate and product.

$$ \varepsilon_{s-p} = \delta_{\text{substrate}} - \delta_{\text{product}} $$

The stable isotope signature can also be altered by the input of one element with a ratio that is different from that which is already within the system (Schlacher et al., 2005). As such, the overall change in signatures can be used to indicate the occurrence and often the proportion of the new input to the system (Savage, 2005).

The organic matter produced by different photosynthetic cycles and habitats has been shown to have carbon and nitrogen stable isotope values which can fall within distinctive ranges (Peterson and Fry, 1987; France, 1995b). The tissues of organisms which are consuming organic matter from a specific habitat will reflect the isotope composition of their food. Between an organisms and its food the carbon isotopes values have been shown to be approximately 1 ‰ heavier and nitrogen stable isotopes values have been shown to be approximately 3.4 ‰ heavier (Deniro and Epstein, 1978; 1981; Minagawa and Wada, 1984). It is then possible to measure a number of organisms within a system, such as a lake, and identify where or on what particular groups of organisms are feeding and who is eating who within those groups. For example, the separation in carbon signatures between pelagic and littoral zones allows for the isotopic separation of organisms between the two habitats in lakes (France, 1995b). The nitrogen signatures can then determine the trophic levels within the two habitats (Vander Zanden and Rasmussen, 1999).
The processes responsible for the difference in the isotope values of the organic matter on which the organisms are feeding are complex and not clearly understood. Within the aquatic system organic matter can enter the system from external sources or be produced within the aquatic environment. Organic material produced externally can be terrestrial organics from the riparian zones such as leaf litter or grasses. The isotopic composition of this form of organic matter is determined by the isotopic composition of the inorganic carbon and nitrogen substrates available and the size of the fractionation which occurs during photosynthesis. Most of the variability in the carbon isotope composition of terrestrial producers has been attributed to differences in photosynthetic cycles and the physiological condition of the individual producer (Oleary, 1981).

Organic matter from sources external to the aquatic environment can originate from anthropogenic sources, such as sewage or industrial outfalls. Organics from point sources such as a sewage outfall are easier to track given the organics from the outfall are measured and distinctive from the isotopic composition of organics already within the system (deBruyn and Rasmussen, 2002). These sources can be variable in their isotopic composition as a result of where they originated and processes which could have affected them before being released into the aquatic environment. Although not always anthropogenic in origin, the input of organics with a distinctive isotope composition from a separate aquatic system such as a tributary, lake, or reservoir could also represent an organic input to a study site which could complicate interpretations. Measurement of the organics released from this system would be necessary to track the dependence of organisms on these sources of organics.
The products of primary production within the aquatic system have less of the variability in carbon isotope values explained than those produced terrestrially (France, 1995a). This is likely because the isotopic composition of the inorganic substrates available for primary production within aquatic systems is more variable and substrate is more likely to be limited (Hecky and Hesslein, 1995; Leggett et al., 1999). Limitation of substrate has been one explanation given to explain the variability in stable isotope composition of aquatically produced organic matter, particularly with respect to carbon isotope values (France, 1995b; MacLeod and Barton, 1998). Variability in the isotopic composition of the inorganic substrate is given as a second explanation for the observed variability in isotope values (Finlay, 2004). For nitrogen, this second explanation has been pursued by a number of researchers due to the distinctiveness of anthropogenic inorganic nitrogen (Kaushal et al., 2006; Anisfeld et al., 2007). Many studies have successfully tracked sewage plumes through harbors and coastal areas with stable nitrogen isotope analysis of macrophytes (Gartner et al., 2002; Savage and Elmgren, 2004; Savage, 2005).

Through similar logic the isotope values of aquatic organisms have been used to track nitrogen pollution (Cabana and Rasmussen, 1996; Fry and Allen, 2003; Vander Zanden et al., 2005). The difficulty with this is determining if the primary food source for the organism was produced within the aquatic environment or if it is organic matter which is input. This has implications for the design of the study and the conclusions it is possible to draw from isotope data. If organic matter is consumed than it is relatively simple to measure the organics input and measure the organisms and determine if the isotope compositions are similar. However, if it is autotrophic material which is consumed there
are further sources of variation which can influence our ability to determine if inorganic nutrients from a particular source can be observed in the organisms.

The $\delta^{15}N$ value of the autotrophic organic matter is determined by the interactions between the form (ammonia/ammonium, nitrate, or nitrogen gas) which is assimilated, the amount of fractionation which occurs during assimilation, and the $\delta^{15}N$ values of the assimilated form. The different inorganic nitrogen forms can be fractionated differently during assimilation and the form which is assimilated can vary between and within individual producers (Fogel and Cifuentes, 1993). While it is believed that the form assimilated is determined by the ability of the most energetically favorable form to meet the nitrogen demands (Fogel and Cifuentes, 1993), this is difficult to measure in the field setting. Each form of inorganic nitrogen available in the system can have a different $\delta^{15}N$ value which is influenced by the input and export of the form from the pool (Kirshenbaum et al., 1947; Kuuppo et al., 2006). Therefore, just as organics can be input from other aquatic systems or anthropogenic input so can the inorganic forms. However, the export of inorganic nitrogen by biological or physical processes can also show selectivity for one of the isotopes and alter the $\delta^{15}N$ value of the available substrate (Aravena et al., 1993).

Similar complexities exist for the processes affecting carbon isotope values of aquatic carbon. The variation in aquatic carbon signatures comes from interactions in form, the signatures of the inorganic carbon assimilated, and fractionation factors during photosynthesis (Fogel et al., 1992). While $\text{CO}_2$ is thought to be the preferred form of carbon assimilated by aquatic primary producers due to energetic costs (Fogel et al., 1992; Hecky and Hesslein, 1995) some macrophytes have been shown to assimilate
HCO₃⁻ (Keeley and Sandquist, 1992). The signature of inorganic carbon is affected by the input of inorganic carbon from a number of natural sources (carbonate rocks, the atmosphere, and respired organics) and possibly from anthropogenic sources (i.e. MWWTP). The input of each of these sources has the potential to affect the isotope signatures of the carbon which is assimilated. The fractionation during photosynthesis has been shown to vary in relation to substrate availability and has been given as an explanation for the separation in carbon signatures between littoral and pelagic zones of lakes and with varying rates of photosynthesis (Hollander and McKenzie, 1991; France, 1995b; Hecky and Hesslein, 1995).

In addition to the processes which can alter the isotope values of the autotrophic organic matter, there are also different sources of organic matter from which the aquatic organisms can choose. Knowledge regarding the feeding habits and methods as determined by the physiology of the organism can be useful in identifying those organisms which are more likely to be consuming different food sources (Merrit and Cummins, 1996). However, it is possible that organisms can consume bio-films which can contain both autotrophic material and trapped organic matter or particulate mater which is composed of particulates from a MWWTP and waste organics from upstream sites. Seasonal changes in the total and relative abundance of different sources of organic matter can result in changes in the source of organic matter consumed across seasons.

Furthermore uncertainty lies in the physiological processes which affect the isotope composition of the organism itself (Jardine et al., 2006). The processes which result in the net heavier isotope value of the orgasm over its diet are not fully understood and likely input a portion of variation in the observed results. The relative importance of size
of the time periods represented by the isotope values in the different tissues is another area which can have significant effects on observations (Leggett et al., 2000). This is particularly true when the stable isotope values of a single source of organic material varies (Leavitt et al., 2006) or if an organisms is consuming different food sources during different periods.

Regardless of the complication associated with the interpretation of patterns in stable isotope values of aquatic organisms, studies have shown that the stable isotope values of organisms do reflect the conditions in the system. In general, nitrogen isotope values have been observed to get proportionally heavier with increasing nitrogen loads in lakes (Cabana and Rasmussen, 1996; Vander Zanden et al., 2005), rivers (Anderson and Cabana, 2005), and coastal areas (McClelland et al., 1997). Individual point source nitrogen inputs alter the δ\(^{15}\)N values of organisms within the plume (deBruyn and Rasmussen, 2002; Steffy and Kilham, 2004; Schlacher et al., 2005). Carbon isotope values are inconsistently influenced by point source inputs such as MWWTP (Spies R B et al., 1989; deBruyn and Rasmussen, 2002; Vizzini and Mazzola, 2006). They are affected by changes in the riparian zone land use of rivers (Hicks, 1997; Gray et al., 2004).

**Research Objectives**

The following research is predominantly observational and designed to answer two simple questions. Can stable isotope analysis of food webs be used to trace the input of different MWWTP effluents into a largely agricultural watershed cumulatively influenced by different MWWTP? And, how do stable isotope values of
riffle dwelling aquatic organisms change over a 200 km river stretch as the river moves through reservoirs, assimilates waste 9 different MWWTP, and increases in size.
Chapter 2: The effect of different municipal wastewater treatment plant effluents on carbon and nitrogen stable isotope values of aquatic organisms within the Grand River watershed

This Chapter will be submitted as a manuscript to the Canadian Journal of Fisheries and Aquatic Sciences. The contributing authors are:

Loomer H.A\textsuperscript{1}, Oakes K.D. \textsuperscript{1}, Schiff S\textsuperscript{2}, Taylor W\textsuperscript{1}, and Servos M.R\textsuperscript{1},

\textsuperscript{1} Department of Biology, University of Waterloo, Waterloo ON, N2L 3G1
\textsuperscript{2} Department of Earth and Environmental Sciences, University of Waterloo Waterloo ON, N2L 3G1
Chapter Summary

The input of municipal wastewater treatment plant (MWWTP) effluent to aquatic systems has been found to alter isotope values of aquatic organisms at sites influenced by the effluent. Within the Grand River watershed input from ~26 MWWTPs occurs and it would be useful to identify sites influenced by individual effluent based on the isotope values of the food webs at the site. Three different MWWTPs were chosen within the highly developed Grand River watershed (serving Guelph, Waterloo, and Kitchener, Ontario) and sites were sampled above and below the outfalls in May, July, and September 2007. Results show that the isotope signatures of aquatic organisms downstream of the Kitchener MWWTP were distinct from other sites in the sampled river stretch. The Guelph and Waterloo MWWTP were less distinctive and attributing changes in isotope values to the input of MWWTP was not possible due to increasing isotope values with distance downstream. Within this type of watershed studies should be designed to account and evaluate the role of individual MWWTP effluent on pre-existing downstream trends in isotope values within the river. The isotope values of the primary consumers varies across seasons and this seasonal variability affected the used of stable isotope data to describe food webs within each site. The incorporation of elements from MWWTP effluent is more observable in September. It is recommended that further work should address the biogeochemical cycling of elements in response to the input of MWWTP effluent and how the stable isotope values of aquatic organisms are affected.
Introduction

Stable isotope analysis of aquatic organisms within a system has been used to describe the flow of energy through a food web. However, the $\delta$ values which can be observed within the food web are set by the $\delta$ values of the organic matter available to the food web. Between sites, the organic matter can have different $\delta$ values which make it possible to differentiate between aquatic organisms from different sites based on their $\delta$ values.

Organic matter can have a distinct isotopic fingerprint from other sites within a system because it originates from a different source such as particulates from MWWTP or terrestrial or in the cases of autotrophic aquatic organic matter because of fluctuations in the $\delta$ values and availability of the inorganic substrate (Fogel and Cifuentes, 1993; deBruyn and Rasmussen, 2002; Finlay, 2004). The input of organics and dissolved nutrient from municipal waste water has been shown to affect the range of $\delta$ values observed within food webs between sites in rivers and coastal areas (Steffy and Kilham, 2004; Schlacher et al., 2005; Vizzini and Mazzola, 2006). The implications of these observations are that sites supported by energy and nutrients from MWWTP can be differentiated based their $\delta$ values.

In rivers impacted by a single MWWTP this application has been documented (deBruyn and Rasmussen, 2002). However, many rivers are cumulatively impacted by different MWWTP and non-point sources of nutrients which have also been shown to affect $\delta^{15}$N values within food webs (Steffy and Kilham, 2004; Anderson and Cabana, 2005). Increased total nitrogen loads from point and non-point sources have been
associated with increasing $\delta^{15}$N values observed in aquatic organisms and primary producers in rivers, lakes, and coastal areas (Cabana and Rasmussen, 1996; McClelland et al., 1997; Anderson and Cabana, 2005).

The Grand River, Ontario receives inputs from approximately 26 different MWWTP of varying sizes and has a watershed with approximately 70% of the land devoted to agriculture. Within this watershed it is unclear if the $\delta$ values of food webs within the plume of a MWWTP will be distinctive from upstream sites or from those within the plumes of different treatment plants. The objective of this research was to characterize the range of carbon and nitrogen $\delta$ values observed in food webs up and downstream of three different MWWTP, across seasons within the Grand River in 2007.

**Methods**

*Treatment Plant and Site Selection*

The Grand River watershed is located in southern Ontario and is the largest drainage basin on the northern shore of Lake Erie. This watershed supports a population of approximately 925,000, and has intensive agricultural activity (~70% agricultural activity). As a consequence, nutrient levels are consistently elevated and oxygen levels frequently fall below a guideline of 4 mg/L at night during the summer season (Cooke, 2006).
Figure 1: Sites in the Grand River watershed, Ontario where invertebrates and fish were collected and analyzed for carbon and nitrogen isotope ratios during May-September, 2007. The locations of the municipal waste water treatment plants relative to the sampling locations are shown.

Within the Grand River watershed, three waste water treatment plants were chosen based on differences between the treatment plants (Table 1). Each of the plants treats a different amount of waste water and produce effluent of differing quality or composition.
Table 1: The municipality and associated population, treatment plant processes, and the composition in the final effluent of the three MWWTP around which the sampling of aquatic organisms was conducted.

<table>
<thead>
<tr>
<th>Treatment Plant Characteristics</th>
<th>Waterloo</th>
<th>Kitchener</th>
<th>Guelph</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population Served</td>
<td>105,100</td>
<td>185,000</td>
<td>82,000</td>
</tr>
<tr>
<td>Treatment processes</td>
<td>Secondary-conventional activated sludge</td>
<td>Secondary-conventional activated sludge</td>
<td>Tertiary-nitrification and phosphorous removal</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Effluent Characteristics</th>
<th>Waterloo</th>
<th>Kitchener</th>
<th>Guelph</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ave. Suspended Solids (kg/d April-Sept 2007)</td>
<td>881</td>
<td>465</td>
<td>76</td>
</tr>
<tr>
<td>Ave. Biological Oxygen Demand (kg/d April-Sept 2007)</td>
<td>152</td>
<td>656</td>
<td>86</td>
</tr>
<tr>
<td>Ave. Organic N (kg/δ April-Sept 2007)</td>
<td>-not reported</td>
<td>- not reported</td>
<td>73</td>
</tr>
<tr>
<td>Ave. Ammonia Load (kg/d April-Sept 2007)</td>
<td>188</td>
<td>1560</td>
<td>22</td>
</tr>
<tr>
<td>Ave. Nitrate Load (kg/d April-Sept 2007)</td>
<td>238</td>
<td>99</td>
<td>- not reported (~814 calculated from 2007 Yearly Ammonia/ Nitrate)</td>
</tr>
<tr>
<td>Total N/ person (kg/d April-Sept 2007)</td>
<td>&gt;4.05(10^3)</td>
<td>&gt;8.9(10^3)</td>
<td>&gt;1.1(10^3)</td>
</tr>
<tr>
<td>Yearly Ammonia/Nitrate</td>
<td><strong>2007:</strong> 0.99</td>
<td><strong>2007:</strong> 15</td>
<td><strong>2007:</strong> 0.027</td>
</tr>
<tr>
<td></td>
<td><strong>2006:</strong> 0.39</td>
<td><strong>2006:</strong> 18</td>
<td><strong>2006:</strong> 0.028</td>
</tr>
</tbody>
</table>

1(Kitchener Wastewater Treatment Plant, 2007; Waterloo Wastewater Treatment Plant, 2007; WPCP, 2007)
2(Environment Canada, 2008)
For this study, nine sites were selected based on their proximity to the three waste water treatment plant outfalls, habitat, and site accessibility (Figure 1). Sites were selected above (GUS 2) and below (GDS 1) the Guelph MWWTP on the Speed River. A further upstream site (GUS 1) was chosen on the Eramosa River, a major tributary of the Speed River. On the Grand River sites were chosen above (WUS 2 & KUS 1) and below (WDS 1 & KDS 1) the Waterloo and Kitchener MWWTP. Two sites were added to investigate the larger stretch of this river. One was a less impacted site upstream (WUS 1) and the other was an additional downstream Kitchener site (KDS 2). During September sampling another site (WUS 3) was added directly above the Waterloo MWWTP on the opposite bank of the river.

**Food Web Collections**

Aquatic invertebrates were collected in May, July, and September of 2007 from riffle and pool areas at each site by kick and dip netting. Invertebrates were field-sorted to the family level (according to Merrit and Cummins, 1999) and held on ice (1-3 h) prior to storage at -20 ºC until further identification to genus could be done under a dissecting scope. Distilled water was used in the cleaning process, and any extraneous organic material and calcareous structures (i.e. shells) were carefully removed. Each sample was dried at 60 (± 5) ºC for 24-48 hours and then ground to a fine homogeneous powder with a mortar and pestle.

All organisms of the same genus or lowest identifiable taxonomic group (Merrit and Cummins, 1996) were pooled for May and July. Individual organisms (3-5) within
each genus or lowest taxonomic group were analyzed for September’s samples. The lowest taxonomic groups analyzed were decided based on the occurrence of these groups at most sites. In May, these groups were: Ephemerellidae, Stenonema spp., Ascellus spp., and Chironomidae. In July, the groups used were: Elmidae, Hydropsychidae, Sphaeridae, Physella spp., Ascellus spp., and Chironomidae. In September, Elmidae, Hydropsychidae, Simulidae, Physella spp., Sphaeridae, and Ascellus spp were the groups collected. The predatory invertebrates Argia spp., Engallagma spp., Hetaerina spp., and Calopteryx spp. were analyzed in September as well.

The fish were collected during the May and September 2007 sampling periods. A backpack electro-shocking unit (Smith-Root 12A-POW) was used in the riffle areas for collection of greenside darters (Etheostoma blennioides) and rainbow darters (Etheostoma caeruleum) (Scott and Crossman, 1998). Only fish with total lengths ranging from 4.6-5.7 cm were used to minimize variation attributable to shifts in trophic level with size. Fish were euthanized by severance of the spinal cord according to protocols approved by our local Animal Care Committee (AUP 04-24). Total length and weight were recorded. A skinless sample of dorsal-epaxial muscle was collected adjacent the first dorsal fin and stored at -20ºC until dried at 60 (± 5) ºC for 24-48 hours and ground into a fine homogeneous powder with mortar and pestle.

**Stable Isotope Analysis**

The finely ground powder prepared from the aquatic invertebrate and fish collections was weighed (0.2 ± 0.05 mg) into tin cups and analyzed for stable isotope signatures (δ¹³C and δ¹⁵N) and % elemental composition using a Delta Plus Continuous
Flow Stable Isotope Ratio Mass Spectrometer (Thermo Finnigan / Bremen-Germany) coupled to a Carlo Erba Elemental Analyzer (CHNS-O EA1108 - Italy). Analysis was performed by the Environmental Isotope Lab at the University of Waterloo (Drimmie and Heemskerk, 2005). A subset of sampled were run in replication (N=24) and the difference (mean ± standard error) between replicates for carbon and nitrogen was 0.11 ± 0.02 ‰ and 0.17 ± 0.02 ‰, respectively. The carbon isotope signature of all invertebrate samples was normalized for lipid content based on the equation

$$\delta^{13}C_{corrected} = -3.32 + (0.99) \cdot (C:N)$$ (Post *et al.*, 2007)

[Equation 4]

**Statistics and Data Presentation**

Due to a lack of homogeneity of variance within the data sets, a Kruskal-Wallis test was performed on each of the groups defined by trophic level (darter or primary consumer) and season (May, July, or September). Comparisons were run on those data sets with significant results using independent T-tests between adjacent sites. Seasonal differences for each site were determined between the May and September darter groups using independent T-tests. For all tests the alpha value was set at 0.05. T-tests assumed equal or unequal variances, depending on the results of the Levene’s statistic. The error bars associated with the averages presented in all figures represent the standard error of the mean. Statistical analyses were preformed with SPSS v.16, SPSS INC, Chicago and graphs were generated with Sigmaplot v.9.01, Systat Software INC, San Jose.

**Results**

The plot of nitrogen by carbon isotope values for all organisms sampled from single sites allowed for visual inspection of plots (Figure 2, Figure 3, & Figure 4).
While all sites were different in the invertebrate taxons which were observed some overall patterns can be observed. The carbon values for the primary consumers taxons were lighter in carbon and nitrogen in May and become progressively heavier during July and September. The size of the range in carbon values observed within each site over the season is approximately 4 ‰. The size of the range in nitrogen isotope values observed within the sites was not consistent between sites and those sites which had larger ranges also had less separation between the δ values of the primary consumers and the darters and a greater increase in δ values was observed in July and September.
Figure 2: Plots of $\delta^{15}$N by $\delta^{13}$C signatures for organisms collected from the Grand River watershed above (WUS 1 & WUS 2) and below (WDS 1) the Waterloo MWWTP in May (solid figures), July (grey figures) and September (open figures). The points for the invertebrates collected in September and all of the darters are represented by the mean ($\pm$ standard error) and the invertebrates from May and July are single points from a pooled sample.
**Figure 3:** Plots of δ¹⁵N by δ¹³C signatures for organisms collected from the Grand River watershed above (KUS 1) and below (KDS 1 & KDS 2) the Kitchener MWWTP in May (solid figures), July (grey figures) and September (open figures). The points for the invertebrates collected in September and all of the darters are represented by the mean (± standard error) and the invertebrates from May and July are single points from a pooled sample.
greenside darter - *Etheostoma blennioides* (September)

rainbow darters - *Etheostoma caeruleum* (September)

darters - *E. caeruleum & E.blennioides* (May)

*Engallagma spp.* (September)

*Hetaerina spp.* (September)

*Calopteryx spp.* (September)

*Hydropsychiidae* (September)

*Elmidae* (July)

*Sphaeridae* (September)

*Sphaeridae* (July)

*Physella spp.* (July)

*Chironomidae* (July)

*Chironomidae* (May)

*Ascellus spp.* (September)

*Ascellus spp.* (July)

*Ascellus spp.* (May)

*Ephemerellidae* (May)

*Stenonema spp.* (May)

Figure 4: Plots of $\delta^{15}\text{N}$ by $\delta^{13}\text{C}$ signatures for organisms collected from the Grand River watershed above (GUS 1 & GUS 2) and below (GDS 1) the Guelph MWWTP in May (solid figures), July (grey figures) and September (open figures). The points for the invertebrates collected in September and all of the darters are represented by the mean ($\pm$ standard error) and the invertebrates from May and July are single points from a pooled sample.
To further understand the trends observed in the nitrogen by carbon plots, a plot of the darter and primary consumers $\delta^{13}$C and $\delta^{15}$N signatures against river distance across seasons were generated (Figure 5 & Figure 6). An increase in isotope values for both carbon and nitrogen was observed with downstream river km with the exception of $\delta^{15}$N at KDS 1. The between site differences and downstream patterns increased in size as the season progressed such that the largest downstream increases were observed in September and the smallest in May (Figure 5 & Figure 6).
Figure 5: The mean (± standard error) nitrogen isotope signatures of primary consumers (bottom graphs – square symbols) and darters (top graphs – circle symbols) on the Speed (left graphs) and Grand (right graphs) rivers with respect to MWWTP outfalls in the cities of Waterloo, Kitchener, and Guelph. The solid symbols and solid line correspond with samples collected in May, the grey and dashed line in July, and the open and dotted line in September 2007.
Figure 6: The mean (± standard error) carbon isotope signatures of primary consumers (bottom graphs – square symbols) and darters (top graphs – circle symbols) on the Speed (left graphs) and Grand (right graphs) rivers with respect to MWWTP outfalls in the cities of Waterloo, Kitchener, and Guelph. The solid symbols and solid line correspond with samples collected in May, the grey and dashed line in July, and the open and dotted line in September 2007.
The Kruskal Wallis tests showed significant between site differences for all data sets ($p = <0.001$ to $0.008$) except the nitrogen signatures for the May primary consumers ($p = 0.400$) (Table 2). Comparisons between adjacent sites showed that significant differences were more often observed in the September darter data, least often in the May primary consumers, and the rest fell in between (Table 2).

A comparison between darters collected in May and September showed that seasonal differences were not consistent across sites. The sites downstream of the Kitchener, and Guelph MWWTP showed seasonal differences in both carbon and nitrogen [KDS 1, KDS 2, and GDS 1 ($p =$ <0.001, 0.026, and <0.001 for carbon $p =$ <0.002, 0.001, 0.003, and 0.001 for nitrogen respectively)]. The upstream site for Kitchener (KUS 1) also showed a seasonal change in nitrogen ($p <$ 0.001). The closest upstream site to the Guelph MWWTP (GUS 2) showed a seasonal change in carbon ($p =$ 0.001).

To assess how distinct sites were in the isotope values within the sampled reach of the Grand and Speed Rivers the range of values observed at each site were plotted (Figure 7 & Figure 8). Sites are less distinct from each other in the primary consumer datasets compared to the darter datasets and for both groups in the Grand and Speed Rivers sites were most distinct in September.
**Table 2:** The $p$ values for the statistical tests run on the Primary consumer and darter datasets collected in May, July and September from sites above (-US #) and below (-DS#) the Waterloo (W--#), Kitchener (K--#), and Guelph (G--#) MWWTP with the Grand River watershed, Ontario. The $p$ values were calculated from the Kruskal Wallis comparisons for each of the datasets and from comparisons (T-tests equal or unequal variances assumed) between adjacent sites.

<table>
<thead>
<tr>
<th>Sites</th>
<th>Primary Consumer Invertebrates</th>
<th>Darters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>May</td>
<td>July</td>
</tr>
<tr>
<td></td>
<td>$\delta^{15}$N</td>
<td>$\delta^{13}$C</td>
</tr>
<tr>
<td>$p$ values data set</td>
<td>.400</td>
<td>.008</td>
</tr>
<tr>
<td>WUS 1 vs. WUS 2</td>
<td>.752</td>
<td>.004</td>
</tr>
<tr>
<td>WUS 2 vs. WDS 1</td>
<td>.230</td>
<td>.489</td>
</tr>
<tr>
<td>WUS 2 vs. WUS 3</td>
<td>.002</td>
<td>.383</td>
</tr>
<tr>
<td>WUS 3 vs. WDS 1</td>
<td>.002</td>
<td>.383</td>
</tr>
<tr>
<td>WDS 1 vs. KUS 1</td>
<td>.909</td>
<td>.020</td>
</tr>
<tr>
<td>KUS 1 vs. KDS 1</td>
<td>.056</td>
<td>.001</td>
</tr>
<tr>
<td>KDS 1 vs. KDS 2</td>
<td>.941</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>GUS 1 vs. GUS 2</td>
<td>.146</td>
<td>.040</td>
</tr>
<tr>
<td>GUS 2 vs. GDS 1</td>
<td>.610</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>
Figure 7: The median value and range of isotope values for carbon and nitrogen in the darters and primary consumers collected from sites surrounding the Waterloo and Kitchener MWWTP on the Grand River, Ontario in May and September of 2007.
Figure 8: The median value and range of isotope values for carbon and nitrogen in the darters and primary consumers collected from sites surrounding the Guelph MWWTP on the Speed River, Ontario in May and September of 2007.
Discussion

Interpretation of between site differences in δ values of aquatic organisms is affected by the time period in which the organisms were collected. Those organisms collected in May were less distinct in their isotope values between sites in contrast to those collected in September (Figure 7 & Figure 8). The influence of MWWTP effluent on δ values of aquatic organisms was better observed in the fall than in the spring indicating that the time period sampled affects the results observed. Regardless of the mechanism driving the seasonal variability in the results, the implication for interpretation of the results of this study is that results from each season need to be interpreted separately.

For analysis of the results of this study we have broadly grouped the components of our food webs into darters and primary consumers which represented two trophic levels. Invertebrate predators are not included in these groups. The between site patterns in δ values for the primary consumers and the darters follow similar patterns in both isotopes. While the variability in the primary consumers is often greater, this is not surprising considering the diversity of functional feeding groups included in this group. The results from the darter group show less variation in δ values likely because they represent a less diverse taxonomic assemblage and because both species are riffle dwelling feeding on organisms within that ecological niche. As such, the darters are the most precise data sets from with which to make comparisons between sites.

A concern with comparing a higher trophic level such as the darters across sites is that differences in the δ values of the fish may result from changes in trophic level and not from changes in the δ values of the organic matter supporting the food web within the
site or microhabitat. The riffle dwelling invertebrate predator which was collected was a Zygoptera genus, *Argia spp.* Inspection of the isotope data shows that at sites where *Argia spp.* were collected their $\delta^{15}$N values did plot between those of the darters and some predominantly riffle dwelling primary consumers such as *Cnephia spp.* or Hydrophychidae. This may be because they form an intermediate trophic level, they are consuming algal and detrital material as well as primary consumer invertebrates, or they are consuming a similar food source but there are differences in the trophic enrichment of nitrogen which occurs between invertebrates and fish.

When the riffle dwelling individual taxons of primary consumers within a site were compared across seasons, increases in carbon and nitrogen $\delta$ values were observed (Figure 2, Figure 3, & Figure 4). At sites where Hydrospychidae were collected in May and September (WUS 1, WDS 1, KDS 1, KDS 2) higher carbon and nitrogen $\delta$ values were observed in September with the exception of WDS 1. At WDS 1 the $\delta$ values of the Hydropsychids were similar in May and September and were similar in carbon but approximately 3 – 4 ‰ lighter in nitrogen isotope values than the darters (Figure 2). Other taxons which are not predominately riffle dwelling (*Ascellus spp.*) also show this seasonal increase in $\delta$ values. Determining if the invertebrate predators represented an intermediate trophic level between the darters and the primary consumers was difficult because it was not possible to determine which primary consumer taxon(s) were consumed by the darters due to the seasonal changes in $\delta$ values. Intensive sampling of the riffle areas for organic matter and community composition of invertebrates may have provided some insight into this relationship. The $\delta$ values of the darters are compared
between sites in this study but interpretations are made with caution given the lack of resolution with respect to trophic dynamics between the invertebrates and the darters.

In this study, attributing the changes in $\delta$ values of aquatic organisms at sites downstream of MWWTP inputs to the assimilation of nutrients, organic or inorganic, originating from MWWTP effluents was affected by the size and direction of the change in carbon and nitrogen $\delta$ values. The $\delta$ values of the organisms living within the plume of the Kitchener treatment plant were lighter in nitrogen and heavier in carbon which gave them a unique orientation in the nitrogen by carbon plot (Figure 7). Because these sites were distinct from other sites sampled on this stretch of the Grand River, it is concluded that these changes in $\delta$ values reflects the reliance of food webs on nutrients derived from the MWWTP. Although, the $\delta$ values of darters within the Waterloo and Guelph MWWTP plumes were heavier than those observed at the nearest upstream sites (Figure 5 & Table 2), when these values were plotted in the nitrogen by carbon plot they both plotted in association with the uninfluenced sites in the study (Figure 7 & Figure 8). When the nitrogen and carbon $\delta$ values are looked at separately this association can be described as an increasing trend from both isotopes with distance downstream.

Comparisons with changes in $\delta$ values between upstream sites provide a method to evaluate the role of MWWTP effluent has on the $\delta$ values in the plumes. Although, sites in this study were not selected to allow a robust evaluation and characterization of the pre-existing downstream trends in $\delta$ values before the MWWTP effluent entered the river, visual inspection of the data and the results from statistical tests between adjacent sites are useful. As discussed previously the dataset which is analyzed affects interpretations and the darters will be discussed because they represent the most precise
dataset. In May, the increasing trend with distance downstream for nitrogen and carbon can be observed in the data set; although, significant differences only occurred between the carbon values at GUS 1 and GUS 2. In contrast, comparisons between adjacent sites for both carbon and nitrogen from the September data sets were significant. Between sites GUS 1 and GUS 2 the river increases in size with the joining of the Speed and the Eramosa Rivers and the forested riparian zone changes to a channelized urban stream which moves through a series of impoundments in the city of Guelph. These sites do not form a good control and while differences in the $\delta$ values of organisms living within the plume can be observed relative to the $\delta$ values of the upstream sites, it is not possible to attribute these changes to the MWWTP effluent.

The $\delta$ values of darters collected within the Waterloo MWWTP effluent plume in May were not significantly different for nitrogen but were for carbon. Between WUS 1 and WUS 2 both nitrogen and carbon were significantly different. However, the input from the Conestogo tributary may have been a confounding factor and in September another upstream site was added which was approximately 1 km upstream of the outfall on the other side of the river (WUS 3). While carbon $\delta$ values were not different between the adjacent sites surrounding the Waterloo treatment plant in September, nitrogen $\delta$ values were different between WUS 3 and WDS 1 but not between WUS 2 and WUS 3. In this case, it is possible that $\delta$ values of darters collected within the plume of the Waterloo MWWTP were affected by the effluent and it can be concluded that nutrients from the MWWTP are supporting the darters collected within the plume. A similar significant difference during September in the nitrogen $\delta$ values of the primary consumers collected from WUS 3 and WDS 1 and a lack of difference between WUS 2
and WUS3 occurred. The repetition of this pattern in the second trophic level further supports the conclusions drawn from the darter datasets.

It is clear from this study that the selection of sites within the river affects how the data can be interpreted. One concern when choosing sites was whether or not fish would move between sites. The home range of these fish appeared to be small enough that movement between sites was not a problem because the darters at WUS 3 were more similar to the ones 5 km upstream at WUS 2 then the ones within the plume 1 km downstream. Across all sites similar patterns in the δ values of the invertebrates sampled were observed, supporting the conclusion that darters did not travel between sites.

Previous studies have shown that increasing anthropogenic nitrogen inputs are associated with increasing nitrogen δ values in aquatic organisms and primary producers (Cabana and Rasmussen, 1996; Anderson and Cabana, 2005; Vander Zanden et al., 2005). Within this system the increase in nitrogen δ values with distance downstream could also be viewed as an increase in δ15N values with an increase in anthropogenic nitrogen inputs, indicating that a similar pattern exists in this system. The occurrence of this pattern requires that studies need to be designed to control for this and assess the role of the individual MWWTP on this increasing pattern. Specific questions should address the scale of the increasing pattern and whether or not it would still exist if the MWWTP effluent were not entering the system.

The biogeochemical carbon and nitrogen cycles within a river have many different components, of which, the food web is a single unit. As a component of a river’s biogeochemical cycle, the organisms are a useful measurement because the isotope values of their tissues reflect a longer time period than other measures, such as dissolved
concentrations and their respective δ values. However, interpretation of this data is limited by a lack of understanding of the processes which determine the δ values observed of the individual organisms.

The stable isotope composition of an organism’s tissues is affected predominantly by the isotopic composition of their food (Deniro and Epstein, 1978; 1981; Minagawa and Wada, 1984). This finding forms the foundation of the application of stable isotopes in the description of food webs (Peterson and Fry, 1987). However, the physiological processes involved in the assimilation, recycling, and elimination of elements from an organism’s tissues are complex and results in uncertainties for the application of stable isotope analysis of food webs, reviewed in (Jardine et al., 2006). A major knowledge gap in this study is the time frame represented by the δ values and the relative weighing of different time periods on the observed δ values. Given the seasonal variability observed in this study, an understanding of the time period represented by the tissues is necessary.

Further source of uncertainty in this study is the source and stable isotope values of the organic matter upon which the aquatic organisms are feeding. Primary production is expected to be the dominant process in the study reach; however, the input of particulate organics from the MWWTPs and riparian zone vegetation are also present and could be an alternative source. Given the variability in the data sets either the organisms are feeding on different sources of organics or the δ values of a single source fluctuates across seasons and between sites. The increasing pattern from carbon and nitrogen observed with distance downstream and across seasons has been observed previously in autotrophic material for carbon (Finlay, 2004) and nitrogen (Anderson and Cabana, 2005).
The effluent released from the Guelph and Waterloo MWWTP is higher in inorganic nutrients than particulates (Table 1) and it is likely that nutrients from MWWTP are entering the food webs through aquatic autotrophic organic matter. The larger load of particulates from the Kitchener MWWTP may be responsible for the distinctness of the $\delta$ values at KDS 1. However, the increased release of particulates is a function of differences in the treatment process (Metcalf and Eddy, 2003) and treatment processes such as denitrification have been shown to affect $\delta^{15}N$ values of nitrates (Savage and Elmgren, 2004; Anisfeld et al., 2007). Within other treatment plants the $\delta$ values of the different nitrogen forms (particulate, ammonia, and nitrate) have been shown to differ from each other (Tucker et al., 1999; Gartner et al., 2002; Kuuppo et al., 2006) and temporally (Gartner et al., 2002). Therefore, the distinctness of the $\delta^{15}N$ values of organisms at KDS 1 may result from the consumption of autotrophic material which used lighter inorganic nitrogen from the MWWTP effluent.

The carbon $\delta$ values of MWWTP and of organisms living within the plume are less frequently reported (Schlacher et al., 2005; Leavitt et al., 2006) and studies which have report values for particulate matter (Spies R B et al., 1989; deBruyn and Rasmussen, 2002). Depending on the $\delta$ values at the reference site, observed changes in $\delta$ values may or may not occur resulting from a lack of distinctness in the carbon $\delta$ values of the effluent. The carbon values downstream of the Kitchener plant are heavier that the nearest upstream site and this increase is attributed to the input of the Kitchener MWWTP effluent. The $\delta$ values of the organisms downstream of the Waterloo and Guelph sites are also heavier than the nearest upstream site; although, attributing these differences to the input of the MWWTP effluent is difficult. Characterization of carbon
cycling within the plant and in response to the input of MWWTP effluent would clarify the mechanisms responsible for the observed changes in isotope values of aquatic organisms.

Further work in this area should assess the isotope values and quantity of different sources of organic matter to determine if the observed results can be attributed to the consumption of organism from the MWWTP or the consumption of autotrophic material dependent on nutrients from the MWWTP. This would provide information on the pathway organics are entering the food webs and which component of the river biogeochemical cycle the $\delta$ values of aquatic organism’s tissues are most closely affected by.

The results of this study have implication for the application of stable isotopes to describe food webs within heavily impacted river systems, such as the Grand River. The uniqueness of the sites within the river may be useful in identifying fish movement and feeding patterns in the larger stretch of the river. However, difficulty associated with defining the primary consumer trophic level (discussed previously) limits the application of stable isotopes to describe food webs at the scale of an individual site. Comparisons of the range of $\delta$ values observed within the food web provide information on the cycling of elements within the river and with increased understanding of the relationships between the food web isotope values and the larger biogeochemical cycle within the river, this could function as a measure of river health.

**Conclusions**

This study was one of the few which has looked at the use of stable isotopes in food webs influenced by individual MWWTP within a cumulatively impacted river and
shows the δ values observed at sites downstream of MWWTP can be distinctive from upstream sites given appropriate site selection and dataset analysis. Between sites unaffected by MWWTP effluents, δ values are not constant and study designs need to account for the downstream changes which appear to occur as a result of cumulative loads and other activities which alter element cycling within the river. The distinctiveness of sites with respect to δ values has implications for the application of stable isotopes to describe the movement and feeding patterns of transient fish within this system. However, the seasonal variability in δ values complicates the interpretation of food webs within individual sites. As a component of the river’s larger biogeochemical cycle, the δ values within the food web are affected and, therefore a reflection of, the element cycling. To facilitate the interpretation of an organism’s stable isotope values with respect to the rest of the food web or as an indication of nutrient cycling in the river, an understanding of the interconnections between the larger biogeochemical cycles and the food web is required, particularly in areas influenced by MWWTP effluent.
Chapter 3: Trends in the stable carbon and nitrogen isotope values of riffle dwelling aquatic organisms along a 200 km river reach in the Grand River

This study was completed in association with a larger project looking at the biogeochemistry along the length of the Grand River across seasons. The sites were selected and water samples were collected by those involved in this project and the ammonia and nitrate concentrations were run by Justin Harbin and Richard Elgood from the lab of Dr. Sherry Schiff in the Department of Earth Sciences at the University of Waterloo.

Those associated with this project were:
Araven, Ramon
Chen, Gao
Chomick, Krista
Elgood, Richard
Hood, Jennifer
Hutchins, Ryan
Harbin, Justin
Kuntz, Tim
Loomer, Heather
Mohamed, Mohamed
Rosamond, Madeline
Schiff, Sherry
Servos, Mark
Smith, Ralph
Snider, David
Taylor, William D
Thuss, Eric
Thuss, Simon
Tomkins, Trevor
Venkiteswaran, Jason
Warner, Barry

The load estimates were calculated from data reported by the MWWTP’s on the upper and middle Grand River to the Ministry of the Environment and collected from the tributaries by the Provincial Water Quality Monitoring Network.

This Chapter will be submitted as a manuscript to the Canadian Journal of Fisheries and Aquatic Sciences. The contributing authors are:

Loomer H.A¹, Schiff S², and Servos M.R¹

¹ Department of Biology, University of Waterloo, Waterloo ON, N2L 3G1
² Department of Earth and Environmental Sciences, University of Waterloo Waterloo
Chapter Summary

Stable carbon and nitrogen isotope values of aquatic organisms have been applied to both describe the flow of energy between coexisting organisms and the influences of watershed activity on elements cycling in rivers. The Grand River watershed is a flow regulated system which is cumulatively influenced by urban and agricultural developments. Within this watershed stable isotope analysis of aquatic organisms was applied to describe how different trophic levels of riffle dwelling organisms reflect activity within the watershed. Carbon and nitrogen isotope values reflected the expected separation in trophic levels across sites and allowed for the separation organisms from different sites in the river based on their isotope values. The carbon isotope values reflected the presence of individual large reservoirs on the river but not the smaller dams. Increasing nitrogen isotope values were observed in relation to increasing anthropogenic nitrogen loads and decreased in areas where ground water inputs are believed to aid in the recovery of the river from urban developments.
Introduction

Stable isotope analysis is a tool used in ecological studies to track the movement of elements from individual sources and to identify the occurrence of processes in the environment (Mariotti et al., 1981; Anisfeld et al., 2007). When stable isotope analysis is applied to the tissues of aquatic organisms the flow of energy through the food web can be described (Peterson and Fry, 1987) or the reliance of the food web on nutrients or organics from specific sources can be identified (Deniro and Epstein, 1978; France, 1995b). The later has been used to indicate changes in the cycling of elements within the river do to activity within the watershed (Fry and Allen, 2003; Steffy and Kilham, 2004).

Stable isotope analysis of aquatic organisms has been used to show the connections between the activity in the watershed and the cycling of elements in the aquatic system. Studies have observed changes in $\delta^{15}$N values which correspond with agricultural activity in the watershed (Fry and Allen, 2003; Anderson and Cabana, 2005; Vander Zanden et al., 2005) or increasing urban populations in lakes (Cabana and Rasmussen, 1996) and coastal areas (McClelland et al., 1997). Carbon values have also been observed to change between forested and un-forested stretched or watersheds (Hicks, 1997; Fry and Allen, 2003; Gray et al., 2004) and with increasing river size in uninfluenced systems (Finlay, 2004).

The Grand River watershed in southern Ontario is cumulatively influenced by agricultural and urban development, municipal waste water treatment plants (MWWTP), and various dams and reservoirs. The stable isotope values of aquatic organisms from different trophic levels were analyzed in this watershed to determine if stable isotope
analysis can provide information on the effects of watershed activity on element cycles within the river and to provide insight into the potential applications and limitations of stable isotope analysis to describe food webs within this kind of system.

**Materials and Methods**

*Study Site*

The Grand River in southern Ontario is one of the largest drainage basins for Lake Erie and represents an area of 6965 km$^2$ over a decline of 362 m in elevation. Land use is predominantly agricultural (~78%) with growing urban developments (~3-5%) in the middle of the watershed. The water quality observed within the watershed covers a range of classifications, with reductions occurring in agriculturally dominated tributaries and downstream of major urban areas (Cooke, 2006).

In June and September of 2007, the Grand River was sampled at 16 sites within a 200 km stretch, beginning in the head waters, for riffle dwelling primary consumer invertebrates and small fish. The sites were chosen to provide identify the changes in isotope values as the river moves through agricultural areas, dams, urban development and receives input from MWWTP and major tributaries (Figure 9& Table 3).
Figure 9: The 16 sites sampled for riffle dwelling invertebrate primary consumers and darters during June and September 2007 along the first 200 km of the Grand River. The sub-watersheds are shaded in grey and the three major tributaries (the Conestogo, the Speed, and the Nith) are indicated by the letters A, B and C respectively. All MWWTP on the main branch of the Grand River are marked with the solid triangles.
**Table 3:** The river km where the sites sampled in June and September 2007, the municipal waste water treatment plants, and the dams are located on the main stem of the Grand River, Ontario.

<table>
<thead>
<tr>
<th>River Km</th>
<th>Relevance</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>Site 1</td>
<td></td>
</tr>
<tr>
<td>9.2</td>
<td>MWWTP 1</td>
<td>Lagoon treatment, continuous release (population served 1,400)</td>
</tr>
<tr>
<td>24.6</td>
<td>Site 2</td>
<td></td>
</tr>
<tr>
<td>34.9</td>
<td>Dam 1</td>
<td>Luther Marsh; flood control and flow augmentation</td>
</tr>
<tr>
<td>35.6</td>
<td>Site 3</td>
<td></td>
</tr>
<tr>
<td>42.7</td>
<td>Site 4</td>
<td></td>
</tr>
<tr>
<td>46.3</td>
<td>Dam 2</td>
<td>overflow weir</td>
</tr>
<tr>
<td>46.8</td>
<td>MWWTP 2</td>
<td>(population served 1,489)</td>
</tr>
<tr>
<td>50.4</td>
<td>Site 5</td>
<td></td>
</tr>
<tr>
<td>71.2</td>
<td>Dam 3</td>
<td>Shand Dam; flood control and flow augmentation</td>
</tr>
<tr>
<td>72.6</td>
<td>Site 6</td>
<td></td>
</tr>
<tr>
<td>77.9</td>
<td>MWWTP 3</td>
<td>(population served 6,050)</td>
</tr>
<tr>
<td>80.4</td>
<td>Dam 4</td>
<td>overflow weir</td>
</tr>
<tr>
<td>83.2</td>
<td>MWWTP 4</td>
<td>(population served 3,583)</td>
</tr>
<tr>
<td>83.2</td>
<td>Dam 5</td>
<td>overflow weir</td>
</tr>
<tr>
<td>86.2</td>
<td>Site 7</td>
<td></td>
</tr>
<tr>
<td>99.7</td>
<td>Site 8</td>
<td></td>
</tr>
<tr>
<td>107.6</td>
<td>Tributary 1</td>
<td>Conestogo River</td>
</tr>
<tr>
<td>120.6</td>
<td>Site 9</td>
<td></td>
</tr>
<tr>
<td>121.6</td>
<td>MWWTP 5</td>
<td>(population served 66,627)</td>
</tr>
<tr>
<td>136.4</td>
<td>Site 10</td>
<td></td>
</tr>
<tr>
<td>141.5</td>
<td>MWWTP 6</td>
<td>(population served 164,000)</td>
</tr>
<tr>
<td>147.0</td>
<td>Site 11</td>
<td></td>
</tr>
<tr>
<td>148.6</td>
<td>Tributary 2</td>
<td>Speed River</td>
</tr>
<tr>
<td>150.1</td>
<td>MWWTP 7</td>
<td>(population served 18,727)</td>
</tr>
<tr>
<td>154.5</td>
<td>Dam 6</td>
<td>overflow weir</td>
</tr>
<tr>
<td>155.1</td>
<td>Site 12</td>
<td></td>
</tr>
<tr>
<td>157.0</td>
<td>MWWTP 8</td>
<td>(population served 60,000)</td>
</tr>
<tr>
<td>165.7</td>
<td>Site 13</td>
<td></td>
</tr>
<tr>
<td>177.3</td>
<td>Dam 7</td>
<td>overflow weir</td>
</tr>
<tr>
<td>177.5</td>
<td>Site 14</td>
<td></td>
</tr>
<tr>
<td>178.0</td>
<td>Tributary 3</td>
<td>Nith River</td>
</tr>
<tr>
<td>180.0</td>
<td>MWWTP 9</td>
<td>(population served 7,700)</td>
</tr>
<tr>
<td>182.9</td>
<td>Site 15</td>
<td></td>
</tr>
<tr>
<td>191.2</td>
<td>Site 16</td>
<td></td>
</tr>
</tbody>
</table>

**Ammonia, Nitrate and CO\textsubscript{2} Concentrations**

Water samples were collected two or three times between sunrise and the afternoon a clear day in June and in September 2007. The ammonia, nitrate, and CO\textsubscript{2} concentrations were measured. Ammonia concentration was run using a Technicon Auto analyzer and an automated salicylate procedure at Wilfrid Laurier University, Waterloo, Ontario and nitrate concentration was determined by ion chromatography and was measured using a Dionex ICS-90 at the University of Waterloo, Waterloo, Ontario. CO\textsubscript{2} concentrations were determined using headspace equilibration and were measured on a Varian CP-3800 GHG analyzer. i.e. gas chromatograph with TCD, FID and ECD detectors.

**Ammonia and Nitrate Load Estimates**

The Water Survey of Canada monitors levels and flow rates in Canadian waterways and have 38 flow gauges within the Grand River watershed. Along the samples stretch of the Grand River, 6 flow gauges (02GA 001,003, 016, 034,041, 048) exist in close proximity to 6 of the sites (1, 6, 8, 11, 12, 16). The relationship been flow and watershed area at these six sites was quantified with linear regression ($R^2 = 0.89, p <0.0001$) and the equation was used to approximate the flows at the other 10 site.

\[ y = 0.77 + 0.0055x \]  \hspace{1cm} [Equation 1]

The observed nitrate loads (kg/day) at each of the sites were calculated from the concentrations observed during the two surveys (N=4) and the average daily flows on the day of sampling.

This study does not attempt to calculate robust load estimates of nitrogen input to the system but it does include some preliminary estimates to aid in the interpretation of
the isotope data. For non-point sources, annual export coefficients calculated in mixed agricultural catchments (Winter et al., 2002) were applied to the sub-catchment surrounding the main stem of the Grand River and the daily average was then calculated assuming an equal distribution of the load across seasons.

Load estimates of nitrate and ammonia (kg/day) were calculated from point sources, MWWTP and larger tributaries along the sampled stretch of the Grand River were calculated. Concentrations and flow data reported by the treatment plants and the Ontario provincial water quality monitoring network (PWQMN) and the Water Survey of Canada between April and September (treatment plants) and May and September (tributaries) of 2007 was used to calculate these estimates. The nitrogen concentrations in the final effluent of the treatment plants and at the mouths of the tributaries were measured once monthly and flow data was measured continuously through the month and averaged to calculate a monthly nitrogen load. The loads for each month were then used to calculate an average for the growing season. The flow data for the tributaries was not always collected at the same points where nitrogen concentrations were and further upstream gauge stations were used to calculate the estimates. The 1st, 3rd, and 4th MWWTP did not have nitrate concentrations available and the estimates of nitrate loads are somewhat incomplete in the upper portion of the river.

Aquatic Organism Collection

Primary consumer invertebrates (Sphaeridae, Simulidae, Hydropsychidae, Elmidae, & *Ascellus* spp.) were collected in June and September of 2007 from riffle areas at each site by kick and dip netting. They were sorted at the family level (according to
Merrit and Cummins 1999) in the field and held on ice (1-5 hours) while other sites were sampled and put in the freezer (-20 ºC) at the end of each day.

Greenside darters (*Etheostoma blenniodes*) and rainbow darters (*Etheostoma caeruleum*) (Scott and Crossman 1998) were collected during September 2007 with an electro-shocking backpack unit in the riffle areas of each site. The fish were sacrificed by severance of the spinal cord, held on ice (1-5 hours) while other sites were sampled, and put in the freezer (-20 ºC) at the end of each day. The fish were later measured for length, weight, and the epaxial muscle was removed and frozen (-20ºC).

**Aquatic Organism Sample Preparation**

The invertebrate samples were kept frozen until in field identification could be verified, further identification to genus could be done, and samples could be cleaned of excess organic matter under a dissecting scope. Distilled water was used in the cleaning process. The taxonomic identification and the number of organisms in a sample were recorded and the sample was put in a new micro-centrifuge tube and dried at 60(± 5) ºC for 24-48 hours. Invertebrates collected in September were analyzed as individual organisms to capture the inter-organism variation within each site. Each sample was then ground to a fine homogeneous powder with mortar and pestle. The fish muscle tissue was dried at 60(± 5) ºC for 24-48 hours and ground into a fine homogeneous powder with mortar and pestle.

**Stable Isotope Analysis**

The powder was weighed (0.2 ± 0.05 mg) into tin cups and analyzed for stable isotope signatures (δ¹⁵N and δ¹³C). This was done by a Delta Plus Continuous Flow Stable Isotope Ratio Mass Spectrometer (Thermo Finnigan / Bremen-Germany) coupled
Analysis was performed by the Environmental Isotope Lab at the University of Waterloo (Drimmie and Heemskerk, 2005). A random subset of samples was run in replicate and the average (±SE) difference between the isotope values for the same sample was 0.21±0.047 and 0.27±0.048 ‰ for nitrogen and carbon, respectively. The carbon values of the invertebrates were normalized for lipid content with equation 2.

Lipid normalized was performed on all samples with C:N ratios greater than 3.5 which was all of the invertebrate samples in this study.

\[ \delta^{13}C_{\text{corrected}} = -3.32 + (0.99) \cdot (\text{C:N}) \] (Post et al., 2007)[Equation 4]

**Statistical Analysis and Presentation**

The relationship between the isotope values of the invertebrate primary consumers and the darters was investigated for the sampled stretch of the river with a linear regression between the isotope values of the two trophic levels. This regression was run on the average isotope values of the darters and the average of the primary consumers collected in June and September. Equal weighting of each taxonomic group and sample period was given during calculation of the primary consumer average.

The inter-organism variability in isotope values was assessed in the primary consumer aquatic organisms collected in September to determine how representative the pooled samples reported in June were. To do this, the range of carbon and nitrogen isotope values for each taxonomic group and site was calculated. The average range (±SE) of carbon and nitrogen was calculated for the entire dataset and for each taxonomic group. The sites at where the largest and smallest ranges in carbon and nitrogen isotope values were observed were reported for each taxonomic group. A linear regression was
run between the range of the carbon and nitrogen to determine if a larger range of carbon predicted a larger range in nitrogen within a specific taxonomic group.

Because there was not a single taxonomic group which was collected at all of the study sites, the organisms from different collection periods were grouped into two trophic levels, primary consumer invertebrate and predatory darter. Due to a lack of homogeneity of variance in all three data sets comparisons between all the groups were performed with nonparametric statistics, Kruskal Wallis test. Comparisons were performed on those data sets which were significantly different with independent T-tests between the adjacent sites, equal or unequal variance was assumed depending on the results of the Levene statistic. For all tests the alpha values was set at 0.05. All descriptive and analytical statistics were performed with SPSS v.16 (SPSS INC, Chicago) and all graphs and the regression were generated with Sigmaplot v 9.0 (Systat Software INC, San Jose).

**Results**

For both isotopes the pattern in values with distance downstream was similar for the primary consumers collected in June and September and the darter collected in September (Figure 10 & Figure 11). The darters and invertebrates collected in September showed a reduced range of isotope values within sites (Figure 10 & Figure 11) which was reflected by a greater number of significant between adjacent site differences (Table 4).
Figure 10: The $\delta^{13}C$ values for organisms collected from the riffle areas at 16 sites along the first 200 km of the Grand River in June (top) and September (bottom). The results for the June data set (top) were run as pooled samples and do not have an error estimate associated with them. In the September data set (bottom) the invertebrates are represented by the median values ($\pm$ the 90th & 10th percentile) and the darters are represented by the average ($\pm$ standard error).
Figure 11: The $\delta^{15}$N values for organisms collected from the riffle areas at 16 sites along the first 200 km of the Grand River in June (top) and September (bottom). The results for the June data set (top) were run as pooled samples and do not have an error estimate associated with them. In the September data set (bottom) the invertebrates are represented by the median values ($\pm$ the 90$^{th}$ & 10$^{th}$ percentile) and the darters are represented by the average ($\pm$ standard error).
Table 4: The p values for kruskal wallis tests performed on the primary consumer invertebrate and darter datasets and the t-tests between adjacent sites within each of the primary consumer invertebrates and darter data sets collected from the Grand River, Ontario during June and September, 2007.

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Primary Consumers</th>
<th>Darters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>June</td>
<td>Sept</td>
</tr>
<tr>
<td>p of the dataset</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1 vs. 2</td>
<td>.089</td>
<td>.235</td>
</tr>
<tr>
<td>2 vs. 3</td>
<td>.894</td>
<td>.042</td>
</tr>
<tr>
<td>3 vs. 4</td>
<td>.621</td>
<td>.156</td>
</tr>
<tr>
<td>4 vs. 5</td>
<td>.115</td>
<td>.120</td>
</tr>
<tr>
<td>5 vs. 6</td>
<td>.568</td>
<td>.277</td>
</tr>
<tr>
<td>6 vs. 7</td>
<td>.061</td>
<td>.222</td>
</tr>
<tr>
<td>5 vs. 7</td>
<td>.568</td>
<td>.277</td>
</tr>
<tr>
<td>7 vs. 8</td>
<td>.693</td>
<td>.900</td>
</tr>
<tr>
<td>8 vs. 9</td>
<td>.737</td>
<td>.901</td>
</tr>
<tr>
<td>9 vs. 10</td>
<td>.075</td>
<td>.785</td>
</tr>
<tr>
<td>10 vs. 11</td>
<td>.848</td>
<td>.262</td>
</tr>
<tr>
<td>11 vs. 12</td>
<td>.359</td>
<td>.838</td>
</tr>
<tr>
<td>12 vs. 13</td>
<td>.672</td>
<td>.241</td>
</tr>
<tr>
<td>13 vs. 14</td>
<td>.099</td>
<td>.058</td>
</tr>
<tr>
<td>14 vs. 15</td>
<td>.006</td>
<td>.304</td>
</tr>
<tr>
<td>15 vs. 16</td>
<td>.117</td>
<td>.390</td>
</tr>
</tbody>
</table>
The total range of carbon and nitrogen stable isotope values from both trophic levels and sample periods observed at each site were plotted in nitrogen by carbon plot (Figure 12). The orientation of sites within this plot showed that isotope values allowed for the differentiation of organisms collected from different sites. Those sites which were more closely located on the river showed more of an overlap in isotope ranges.
Figure 12: The maximal and minimal carbon and nitrogen stable isotope values observed in all the aquatic organisms collected at each of the 16 sites sampled along the first 200 km of the main stem of the Grand River in June and September, 2007.
The inter-organism variability for each of the taxonomic groups sampled in the September primary consumer invertebrate data set was reported (Table 5). For all taxonomic groups at all sites (N=46) the average range (±SE) for carbon and nitrogen was, 1.61±0.144 and 1.33±0.177 ‰, respectively. The taxonomic group which showed the largest range of isotope values was Hydropsychidae (N=11) with ranges of 2.33±0.30 and 2.16±0.48 for carbon and nitrogen, respectively. The linear regressions which were run between the range of carbon and nitrogen for each taxonomic group showed significant relationships in the taxons Simulidae, and Ascellus spp., $R^2 = 0.75$ and 0.70, respectively (Table 5).
Table 5: The average range of the stable carbon and nitrogen isotope values for each of the taxonomic groups of primary consumer invertebrates collected in September, 2007. The average was calculated from the range observed at each site along the main stem of the Grand River where the taxons were collected. The $R^2$ and $p$ values for linear regressions between the nitrogen range and the carbon range for each of the taxonomic groups.

<table>
<thead>
<tr>
<th>Taxonomic Group (# of sites)</th>
<th>Average Range (SE)</th>
<th>Max Range (Site # max range occurred)</th>
<th>Min Range (Site # min range occurred)</th>
<th>Linear Regression between C &amp; N ranges</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotope</td>
<td>C</td>
<td>N</td>
<td>C</td>
<td>N</td>
</tr>
<tr>
<td>Sphaeridae (12)</td>
<td>1.06(.12)</td>
<td>0.76(.20)</td>
<td>1.90 (12)</td>
<td>2.78 (9)</td>
</tr>
<tr>
<td>Simulidae (7)</td>
<td>1.10(.32)</td>
<td>0.66(.15)</td>
<td>2.37 (6)</td>
<td>1.22 (6)</td>
</tr>
<tr>
<td>Hydropsychidae (11)</td>
<td>2.33(.30)</td>
<td>2.16(.48)</td>
<td>3.60 (5)</td>
<td>5.49 (7)</td>
</tr>
<tr>
<td>Elmidae (9)</td>
<td>2.03(.33)</td>
<td>1.71(.42)</td>
<td>3.39 (2)</td>
<td>4.65 (10)</td>
</tr>
<tr>
<td>Ascellus spp. (7)</td>
<td>1.40(.33)</td>
<td>1.21(.30)</td>
<td>2.53 (12)</td>
<td>2.70 (12)</td>
</tr>
</tbody>
</table>

* Insufficient power
A significant regression was calculated between the average carbon isotope value of the darters collected from September against the average signature of the primary consumers collected in June and September ($R^2=0.91$, $p<0.001$) (Figure 13). The slope of this regression was 1.0 and, the y intercept was 1.1. A similar linear regression was calculated from the average primary consumer (June and September) and darter (September) $\delta^{15}N$ values and showed a significant relationship between these data sets ($R^2=0.92$, $p<0.0001$) with a slope very close to one (0.93) (Figure 13). The intercept of the regression line is 5.7 ($\pm0.8$) but the increased deviation from the regression line is greater for the heavier nitrogen values and the inconsistent variance in the dataset reflects this ($p=0.0078$).
Figure 13: A regression between the average carbon isotope signature of primary consumers collected in June and September of 2007 and the average signature of the darters collected in September 2007 from 16 sites along the first 200 km of the Grand River, Ontario. The open point represents site 10 and the gray point represents site 3.
The carbon signatures of the organisms showed a peak at site 3, downstream of the Luther Marsh (Dam 1). This peak was greatest in the September invertebrates and darters and smallest, although observable, in the June invertebrates. The trough was observed downstream of the Shand Dam (Dam 3) in the September and June invertebrates; although, darters were not observed at this site. The isotope values became progressively heavier with increasing watershed area from site 6 until site 12, after which the signatures appear to plateau with minor variations between sites which corresponded with an area upstream of 800 and 1500 km², respectively.

The observed CO₂ concentrations ranged from 45 - 963 and 186 – 785 umol/L in June and September. Minimal concentrations were observed at site 8 and at site 15 during mid-day in June and September. Maximal concentrations were observed at sites 4 and 8 before sunrise in June and September. A peak or trough in CO₂ concentration was not observed corresponding to the peak observed in the organisms at site 3, downstream of Luther Marsh (Dam 1), or the trough at site 6, downstream of the Shand Dam (Dam 3).
Figure 14: The average CO2 concentration observed during June (closed circles, solid line) and September (open circle, dotted line) surveys of 16 sites along the first 200 km of the main stem of the Grand River, Ontario. The average was calculated from concentrations measured 2-3 times between sunrise and mid the afternoon of the same day.
The $\delta^{15}N$ signatures of the organisms remained similar the first 50 km of the river (site 1- site 4). The $\delta^{15}N$ values of the organisms increased with distance downstream over the next 100 km of the river (site 5- site 12). In the last 50 km (site 13 –site 16) the values decreased.

The observed concentration of nitrate ranged from 0 to 3.67 mg/L. The lowest concentrations were observed in the head waters between sites 1 and 5. The concentrations then began to increase and reached a peak at between sites 12 and 13. In the last ~50 km of the river, the concentration decreases slightly or not at all. In contrast the ammonia concentrations observed were between (0.0116 and 0.0826) at all sites with the exception of sites 6, 11, and 12, where it was observed as high as 0.145, 2.41, 0.598 mg/L and respectively. The highest ammonia concentration, observed at site 11, was downstream of the MWWTP 5 which is correspondingly the largest ammonia load (1604 kg/day) from point sources to the river. The loads from the point sources ranged from 1.021 – 1604 and 1.116 – 554.7 kg/day for ammonia and nitrate, respectively, and the largest load of nitrate was from the Conestogo River.
Figure 15: The load estimates of ammonia (top) and nitrate (middle) to the main stem of the Grand River from point sources, MWWTP and large tributaries, between April and September, 2007. The average (range) ammonia (grey) and nitrate (open) concentrations at each of the 16 sites sampled along the first 200 km of the main stem of the Grand River in June and September, 2007.
Figure 16: The average (±SE) δ¹⁵N values of darters collected in September 2007 (open circles) and the cumulative observed load of nitrate (±SE) collected in June and September 2007 (solid circle) from sites along the first 200 km of the Grand River and the cumulative load (kg/d) of dissolved inorganic nitrogen (grey inverted triangles) from point sources to the main stem of the Grand River between April and September of 2007 plotted against the log of the watershed area upstream.
Discussion

The riffle dwelling invertebrates which were sampled in this study fall into three different functional feeding groups, grazers, filter-feeders, and detritovoirs (Merrit and Cummins, 1996). Because different functional feeding groups consume different forms of organic matter, it is possible for their isotope signatures to vary between groups given a divergence in δ values between the sources of organic matter exists. The functional feeding group most represented across sites in the June and September invertebrate collections were filter feeders. Analysis of differences between functional feeding groups was not possible due to the limited occurrence of other groups across sites. However, visual inspection of the data shows no clear trend in separation of functional feeding groups in both isotopes and sampling periods (Figure 2 & 3).

The inter-organism variability in the September primary consumer invertebrate dataset showed that specific taxons were more (Hydropsychidae) or less (Sphearidae) variable in isotope values and this variability was related between the two isotopes in some taxons (Simulidae) and not in others (Elmidae) (Table 5). The variability in isotope values within the taxons used in this study may be a result of different geniuses and species, instars and sizes, or food sources between organisms. In the taxonomic groups which were the most variable in both carbon and nitrogen isotopes a significant relationship between the size of the variability in carbon and nitrogen was not observed (Hydropsychidae and Elmidae). Rather, the taxons which showed the least variability within the taxon showed the strongest relationship between the size of variation in carbon and nitrogen (Simulidae and Ascellus spp.). This is an interesting observation which may be related to the food consumed by these taxons and the distribution of these taxons in the
river. It is clear from this data that a better understanding of the factors which affect invertebrate isotope values is necessary.

The use of pooled samples is useful because it allows for the analysis of a larger number of organisms and results in an average isotope value for the group. However, an understanding of the variability within organisms which comprise the group and the source of the variability is important for the application of stable isotopes to describe food webs. Given, the between site variability in isotope values the variation in δ values does not appear to greatly affect our interpretations (Table 4). In systems where between site differences are less, this may be a factor which should be considered.

The relationship between the average carbon and nitrogen δ values of the invertebrates and the darters is significant, R² = 0.90 & 0.92 and p = <0.0001 & <0.0001 respectively. The intercept value ± standard error for each of the regressions represents the overall difference in isotope values between the primary consumer invertebrates and the darters (N= 5.7 ± 0.8, and C= 1.1± 2.5). The agreement of these values with previously reported differences between trophic levels for carbon (0.8 ± 1.1)(Deniro and Epstein, 1978) and nitrogen (3.4 ± 1.1, range1.3-5.4 (Minagawa and Wada, 1984); 3.0 ± 2.6, range -0.5 and 9.2 (Deniro and Epstein, 1981)) which indicates that the organisms sampled represent two trophic levels within the riffle areas along the sampled stretch of river. For nitrogen the increased deviation surrounding the regression line when isotope values are greater, could be an artifact of increased variability in isotope values of organic matter at these sites as observed in chapter 2. For this study, the differences in the range of isotope values observed at each site (Figure 12) can be attributed to differences in the δ values of the organic matter within the riffle area.
The regressions were calculated between the $\delta$ values of darters collected in September and primary consumer invertebrates collected in June and September. When the averages were calculated, equal weighing was given to each time period which does not occur in organisms due to the turnover of tissues within an organism (Tieszen et al., 1983). The occurrence of differences in $\delta$ values of primary consumers between samples periods was not common except at site 3 in the carbon values. The average darter carbon isotope values was higher than expected and may be a reflection of the increased influence of the later period on the carbon isotope values (predicted=-24.28, actual=-22.74; Figure 13). Between June and September seasonal differences at the other 15 sites were not large. The sites were more distinct from each other later in the season due to a decrease in the variability in $\delta$ values of the primary consumers (Figure 10, Figure 11 & Table 4). The results from chapter 2 show more dramatic seasonal changes when organisms were collected earlier in the season. This highlights the importance of understanding the time frame represented by an organisms tissues and the time period in which samples are collected.

To understand the processes which determine the isotope values in the food webs it is necessary to understand the source of the organic matter on which the food web is dependent. Unfortunately this study did not include a robust characterization of the isotope values of the organic matter in the riffle areas and conclusions have to be made with caution. However, because this portion of the river is un-shaded by surrounding tree cover and light is able to penetrate the benthic zones, the study reach has the characteristics of a middle order stream and autotrophic production is expected to be dominant (Vannote et al., 1980). Given that the fluctuation and range of carbon isotope
values corresponds with previous observations of autotrophic material in rivers (France, 1995a; Hicks, 1997; Finlay, 2004), the influence of autotrophic organic matter on the isotope values of the aquatic organisms is suggested.

Previous studies have shown that the carbon isotope values of autotrophic material from riffle areas become heavier with increasing watershed area (Finlay, 2004). This increasing pattern was attributed to two interconnected processes, the increase in the δ values of the photosynthetic substrate, CO₂, and a reduced CO₂ availability which reduces the size of fractionation during photosynthesis (Finlay, 2004). Within this system, the elevated concentrations of CO₂ indicate that the carbon isotope value of autotrophic organic material is not largely affected by a variation in fractionation factors as a result of substrate availability. The fluctuations in δ values of autotrophic material in this system would be a function of the δ values of the CO₂.

The elevated CO₂ values which would indicate the P:R ratio is >1 and respiration is the dominant process in the sampled stretch of the river. This is contrary to expectations and does not account for the presence of other CO₂ inputs to the river such as ground water and MWWTP effluent. These inputs would not only affect the CO₂ concentrations but also the δ values of the CO₂ and inherently the isotope values of the autotrophic material in the river. Further work on carbon cycling and the sources and signatures of organic matter within the river is required to understand the processes which determine carbon isotope values of aquatic material and the organisms which it supports.

The nitrogen δ values follow an increasing trend with increasing watershed area until site 12 where they shift to decreasing values. Previous studies have found a positive
association between increasing nitrogen loads and $\delta$ values of primary producers or aquatic organisms (Cabana and Rasmussen, 1996; McClelland et al., 1997; Anderson and Cabana, 2005). Until site 12 this pattern appears to be occurring and the cumulative influence of anthropogenic nitrogen pollution can be observed.

The load estimates calculated from the point source inputs were calculated from single monthly measurements which may do not effectively capture the variability in the effluent and this needs to be considered when interpreting these results. These estimated provide some broad indications of the loads to the system but they did not account for the input of particulate nitrogen to the system and this should to be considered when discussing them in terms of the nitrogen budget in the system. A comparison between the total DIN load from point sources and the observed cumulative nitrate load within the river shows that these are approximately equal. The implications from this are that nitrogen from non-point sources such as atmospheric input, ground water, and surface run-off and organics for natural or anthropogenic sources are retained by biological processes within the river. Based on export coefficients calculated in previous studies in mixed agricultural watersheds (Winter et al., 2002) the daily nitrogen load to this system could be as high as 1174 and 4218 kg/day. This source of nitrogen to the system in comparison to the cumulative dissolved nitrogen estimates from the point sources (approximately 5000 kg/day) could represent a significant contribution to the nitrogen budget of this system and further research is warranted.

The point source load estimates for dissolved inorganic nitrogen were separated by the two dominant forms ammonia and nitrate and compared with observed concentrations of ammonia and nitrate to show the effects of the ammonia and nitrate
inputs on the respective concentrations within the river. The input of MWWTP 6 (Kitchener) is the only plant which the input of ammonia can be observed to change the ammonia concentrations in the river. In the river it was the nitrate concentrations which appeared to be influenced by the input of nitrogen from point sources. These results further show the occurrence of biological cycling within the river and may indicate that those processes which use ammonia are not saturated while those which use nitrate are. The nitrate concentrations do not appear to increase after site 12 when the isotope values in the organisms begin to decrease.

The stability in the nitrate concentrations and the decrease in δ values which is observed after site 12 may be an indication of the recovery of the river. This stretch of river is titled the exceptional water reach because of the increase in biotic diversity and water quality over this stretch (Scott and Imholf, 2005). An input of ground water to the river and a rapid decrease in elevation occurs between sites 12 and 16. There was no change in the carbon δ values of the organisms over this stretch and a stable pattern in isotope values was observed instead of the increasing pattern which was observed with distance downstream along other stretches. Whether or not these observations are related is a question with remains to be answered.

The flow in this river is regulated such that high flows in the spring are retained and low flows in the summer are augmented. This has implications for the cycling of elements along a downstream gradient. The effects of this on carbon values were observed at site 3 where seasonal variability and elevated carbon values were observed relative to other sites on the river. This site is downstream of inputs from the Luther March which appears to be affecting carbon cycling at this point in the river based on the
δ values of the organisms. Whether these changes in δ values of the aquatic organisms result from input of CO₂ or organic matter from the Marsh which is isotopically distinct remains to be determined. The δ values at site 6 downstream of the Shand Dam were lower than many other sites and similar to sites 1 and 2. The Shand Dam is unique because it is a bottom draw dam and takes the water from the hypolimnion or mesolimnion of the reservoir, Belwood Lake, to supplement flows during periods of low flow. Interestingly, the effects of the other impoundments and dams located on the river were not observed in the carbon isotope values or the organisms. The different observations may be a reflection of differences in the operation of each dam and the retention time within each reservoir. Luther Marsh and the Belwood Lake are reservoirs which are managed for flood control and water level augmentation when needed while the other dams and impoundments are overflow weirs (GRCA, 2000).

The presence of the dams on the river may also influence the carbon budget in the river. In the shallow riffle areas sampled the P:R ratio is expected to be >1 but elevated CO₂ concentrations were observed, discussed previously. The occurrence of slower flowing and deeper pools or reservoirs created by the impoundments and dams along the river may create stretches where P:R is <1 and the produced CO₂ is then carried downstream to influence downstream concentrations. As such, the observed CO₂ concentrations be reflecting cumulative effects.

A similar change in nitrogen values was not observed in relation to the dams. However, dams and reservoirs have implications for the cumulative load of nitrogen observed at a site. The export of nitrogen from a reservoir can be a large export of nitrogen from the river (Garnier et al., 2000; Bosch and Allan, 2008). Factors such as the
retention time within the reservoir are likely to affect the load of nitrogen to downstream sites. Unfortunately, the results from this study do not provide any additional information as to the effects of dams on nitrogen cycling within the river.

**Conclusions**

The carbon and nitrogen stable isotope values of riffle dwelling aquatic organisms can be used to differentiate between trophic levels and sites in the Grand River, Ontario. The isotope values of the aquatic organisms reflect the activity within the watershed and carbon and nitrogen provide information about different watershed conditions. The between site differences in isotope values identified largest reservoirs as influences on carbon isotope values and an association between the cumulative input of nitrogen from point source inputs and nitrogen isotope values. Carbon isotope values of the aquatic organisms are believed to be reflecting the isotope values of the CO$_2$. The elevated CO$_2$ concentrations in the river indicate that inputs from in-stream respiration, ground water, or the MWWTP are occurring but there is no distinct pattern in either the concentration or the isotope data to provide more insight in this area. The recovery of the river which is attributed to the input of ground water was associated with decreasing nitrogen isotope values. Preliminary nitrogen load estimates indicate that a significant portion of nitrogen input to the river is retained but that nitrate loads almost equivalent to the cumulative DIN loads from point source input are observed in the river.
Chapter 4: Conclusions

This work was one of the few which has looked at the use of stable isotope analysis of aquatic organisms with in a system with a large number of anthropogenic disturbances. The results from this work indicate that the cumulative influences of these disturbances can influence the isotope values of organisms from individual sites. Studies need to be designed to characterize the effects of upstream loads in order to assess the influence of a single input on the isotope values of aquatic organisms.

The input of effluent from the Kitchener MWWTP to the river has been shown to have a significant influence on the stable isotope values of the aquatic organisms. The input from the Luther March was also shown to strongly affect isotope values and it is concluded that this input is also having an influence on the river. Stable isotope analysis of aquatic organisms is not used to assess a negative or positive impact but to identify areas which further study is warranted. Some other sites in this river where interesting patters are occurring are through the recovery reach where ground water input is occurring and downstream of the Shand dam.

Within the Grand River it is concluded that nitrogen loads from point sources in the middle of the watershed overwhelm the river’s capacity to retain them during the growing period. The implications of this are that during periods when nitrogen is less effectively retained by the river, the nitrogen loads moving downstream will be even greater. Stable isotope and concentration data indicates that before the point sources commence the nitrogen loads to the river are not exceeding its capacity to retain the loads.
It was shown that it is possible to distinguish between organisms collected from different river sites based on their carbon and nitrogen isotopic values. This has implications for the application of stable isotopes to describe the feeding patterns and migration of higher trophic levels within the system. Of particular importance would be to identify those fish with greater exposure to potentially toxic river sites such as MWWTP effluents. Further work should investigate the stable isotope values of higher trophic levels.

Further work on stable isotope analysis of aquatic organisms within this kind of system should consider the limitation on the conclusions which could be drawn from this study. Conclusions drawn from this work were limited by three areas of uncertainty: the time period represented by the tissues of the organisms, the isotope values of the different sources of organic matter, and the inorganic nutrient cycling within each site. The results from this work indicate that the organisms from the late summer are reflecting a discreet period of the summer and during this period they are supported predominantly by autotrophic material. Given this scenario, the isotope values of aquatic organisms from September are greatly affected by the inorganic cycling of carbon and nitrogen within the river. Further work in this system should validate and expand on the details of this explanation.
References


Appendices

Appendix A

The organisms and number of samples (P=Pooled sample) analyzed for stable carbon and nitrogen (Chapter 3) isotope values at each of the 16 sites along the Grand River in June (J) and September (S), 2007.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>P</td>
<td>4</td>
<td>P</td>
<td>5</td>
<td>P</td>
<td>6</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>4</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>4</td>
<td>P</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>P</td>
<td>4</td>
<td>P</td>
<td>9</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>P</td>
<td>5</td>
<td>P</td>
<td>5</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>P</td>
<td>5</td>
<td>P</td>
<td>5</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>P</td>
<td>6</td>
<td>P</td>
<td>5</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>P</td>
<td>4</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>P</td>
<td>4</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>P</td>
<td>4</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>P</td>
<td>4</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
</tr>
<tr>
<td>11</td>
<td>5</td>
<td>P</td>
<td>4</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>P</td>
<td>5</td>
<td>P</td>
<td>5</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>P</td>
<td>5</td>
<td>P</td>
<td>5</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>5</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>8</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
</tr>
<tr>
<td>16</td>
<td>5</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>8</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
<td>3</td>
<td>P</td>
</tr>
</tbody>
</table>

78
### Appendix B

The $p$ values for the Kruskal–wallis and post-hoc independent T-tests performed on the primary consumer and darter data sets from Chapter 2-3.

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Sites</th>
<th>Primary Consumer Invertebrates</th>
<th>Darters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>May $\delta^{15}N$</td>
<td>June $\delta^{15}N$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\delta^{13}N$  &amp; $\delta^{13}C$ &amp; $\delta^{13}N$  &amp; $\delta^{13}C$ &amp; $\delta^{13}N$  &amp; $\delta^{13}C$ &amp; $\delta^{13}N$  &amp; $\delta^{13}C$</td>
<td></td>
</tr>
<tr>
<td>$p$ values for data set</td>
<td>.400</td>
<td>.008</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>2</td>
<td>WUS 1 vs. WUS 2</td>
<td>.752</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>2</td>
<td>WUS 2 vs. WDS 1</td>
<td>.230</td>
<td>.004</td>
</tr>
<tr>
<td>2</td>
<td>WUS 2 vs. WUS 3</td>
<td>&lt;.001</td>
<td>.084</td>
</tr>
<tr>
<td>2</td>
<td>WUS 3 vs. WDS 1</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>2</td>
<td>WDS 1 vs. KUS 1</td>
<td>.909</td>
<td>.020</td>
</tr>
<tr>
<td>2</td>
<td>KUS 1 vs. KDS 1</td>
<td>.056</td>
<td>.001</td>
</tr>
<tr>
<td>2</td>
<td>KDS 1 vs. KDS 2</td>
<td>.941</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>2</td>
<td>GUS 1 vs. GUS 2</td>
<td>.146</td>
<td>.040</td>
</tr>
<tr>
<td>2</td>
<td>GUS 2 vs. GDS 1</td>
<td>.610</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>$p$ values for data set</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>3</td>
<td>1 vs. 2</td>
<td>.089</td>
<td>.235</td>
</tr>
<tr>
<td>3</td>
<td>2 vs. 3</td>
<td>.894</td>
<td>.042</td>
</tr>
<tr>
<td>3</td>
<td>3 vs. 4</td>
<td>.621</td>
<td>.156</td>
</tr>
<tr>
<td>3</td>
<td>4 vs. 5</td>
<td>.115</td>
<td>.120</td>
</tr>
<tr>
<td>3</td>
<td>5 vs. 6</td>
<td>.568</td>
<td>.277</td>
</tr>
<tr>
<td>3</td>
<td>6 vs. 7</td>
<td>.061</td>
<td>.222</td>
</tr>
<tr>
<td>3</td>
<td>5 vs. 7</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>3</td>
<td>7 vs. 8</td>
<td>.693</td>
<td>.900</td>
</tr>
<tr>
<td>3</td>
<td>8 vs. 9</td>
<td>.737</td>
<td>.901</td>
</tr>
<tr>
<td>3</td>
<td>9 vs. 10</td>
<td>.075</td>
<td>.785</td>
</tr>
<tr>
<td>3</td>
<td>10 vs. 11</td>
<td>.848</td>
<td>.262</td>
</tr>
<tr>
<td>3</td>
<td>11 vs. 12</td>
<td>.359</td>
<td>.838</td>
</tr>
<tr>
<td>3</td>
<td>12 vs. 13</td>
<td>.672</td>
<td>.241</td>
</tr>
<tr>
<td>3</td>
<td>13 vs. 14</td>
<td>.099</td>
<td>.058</td>
</tr>
<tr>
<td>3</td>
<td>14 vs. 15</td>
<td>.006</td>
<td>.304</td>
</tr>
<tr>
<td>3</td>
<td>15 vs. 16</td>
<td>.117</td>
<td>.390</td>
</tr>
</tbody>
</table>

79
**Appendix C**
The observed pH and temperature (°C) at study sites in the Grand River watershed (Chapter 3) during the June and September sampling sessions of 2007.

<table>
<thead>
<tr>
<th>Site</th>
<th>June</th>
<th>Sept.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>Temp (°C)</td>
</tr>
<tr>
<td>1</td>
<td>7.85</td>
<td>17.2-21.6</td>
</tr>
<tr>
<td>2</td>
<td>7.78</td>
<td>17.4-23.1</td>
</tr>
<tr>
<td>3</td>
<td>7.78</td>
<td>18.0-20.8</td>
</tr>
<tr>
<td>4</td>
<td>7.59</td>
<td>19.0-23.4</td>
</tr>
<tr>
<td>5</td>
<td>8.13</td>
<td>17.81-21.4</td>
</tr>
<tr>
<td>6</td>
<td>8.01</td>
<td>20.53-22.0</td>
</tr>
<tr>
<td>7</td>
<td>8.18</td>
<td>19.5-21.4</td>
</tr>
<tr>
<td>8</td>
<td>8.00-8.5</td>
<td>19.2-23.4</td>
</tr>
<tr>
<td>9</td>
<td>8.11</td>
<td>20.7-22.1</td>
</tr>
<tr>
<td>10</td>
<td>8.5-8.8</td>
<td>20.0-23.4</td>
</tr>
<tr>
<td>11</td>
<td>7.55-8.7</td>
<td>20.0-23.0</td>
</tr>
<tr>
<td>12</td>
<td>8.26</td>
<td>21.0-20.7</td>
</tr>
<tr>
<td>13</td>
<td>7.90</td>
<td>19.7-22.1</td>
</tr>
<tr>
<td>14</td>
<td>7.86</td>
<td>20.73-22.7</td>
</tr>
<tr>
<td>15</td>
<td>7.89</td>
<td>20.1-24.1</td>
</tr>
<tr>
<td>16</td>
<td>7.97</td>
<td>19.9-23.4</td>
</tr>
</tbody>
</table>