THE EFFECTS OF COMMON CARP ON ZOOPLANKTON COMMUNITIES USING MORPHOLOGICAL AND MOLECULAR TECHNIQUES

A thesis

submitted in fulfilment

of the requirements for the degree

of

Doctor of Philosophy in Biological Sciences

at

The University of Waikato

by

STEVE WOODS

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2018

ABSTRACT

Understanding the factors causing a decline of water quality is important to ecologists, but particularly challenging given the complexity of natural systems. New Zealand's freshwater ecosystems are under threat from increased eutrophication, and in the Waikato region this is intensified with the presence of common carp (*Cyprinus carpio*). The overall aim of this thesis was to capture the environmental and biological changes of a natural pond environment when carp were introduced at a high density, and to develop and test a DNA based approach of assessing zooplankton communities for quick and easy monitoring of lakes.

The ecological aspect of this thesis explored how carp influenced the physical and chemical variables, as well as the biological communities of a shallow lake environment. Suspended sediments and nitrogen increased in the presence of carp. The zooplankton communities changed through time and were associated with small suspended sediments, but there was no observed influence on the zooplankton community driven by carp. This study illustrates how a new introduction of carp may have little impact on an already degraded system, such as those commonly seen in the Waikato region.

The genetic component of this thesis aimed to determine the suitability of the 28S nuclear gene region as an ecological tool used in the routine monitoring of lakes. A reference library was created with 336 zooplankton individuals, including 60 rotifer, nine cladoceran, and seven copepod taxa. Sequence success was high (79%) and support for

identification was generally high at the species level. These findings showed that the 28S region could be used to sample entire zooplankton communities taken from natural environments.

Next generation sequencing was used to sequence the entire zooplankton communities and test the effectiveness of 28S to capture the abundant and less abundant taxa, and ultimately determine its use as a tool for ecologists. Samples were identified and counted prior to sequencing to validate the results. High and moderate abundant zooplankton taxa successfully generated sequences and were correctly identified but primer biases were apparent with the low abundant taxa. There was no evidence to support that metabarcoding can provide an estimation of abundance, as the number of sequences generated was correlated with body size. However, metabarcoding appears to be able to determine the trophic state of lake ecosystems based on the composition of the rotifer community.

Overall findings show how adding additional stressors to an already degraded lake may not result in the predicted outcome. This shows that the work needed to restore or remediate the system is not as simple as removing the one stressor predicted to be causing the majority of issues (e.g. carp), but reducing all stressors that are associated with the degradation of the lake (e.g. nutrients, sediments). This thesis also shows the benefits of including DNA based techniques in ecological monitoring.

ACKNOWLEDGMENTS

Firstly, I would like to thank my supervisors, Ian Hogg, Conrad Pilditch, Ian Duggan, and Jonathan Banks. Your patience of reviewing and input into this thesis is greatly appreciated. Thank you for providing me many learning opportunities during my time at the University of Waikato. I gratefully acknowledge the financial support of the Commonwealth Scholarship for providing me the means to complete this work. Thank you as well to the students and staff at the university that assisted in field and lab work, and data analysis. Special thanks to Stacey Meyer for helping with the last little bits of getting this completed.

This thesis was equal parts scientific and personal growth. My friends in New Zealand that welcomed me and made me feel at home, the impact you have made on my time spent in your country will never be forgotten. Particular thanks to Brooke Baker for showing unending love and patience during the most trying times. You kept me going mentally and physically. Also thanks to the hockey players and my therapy dogs for destressing me.

Thanks to the people in Canada that stood by me for the past few years. Mark & Vanessa, Taryn, Laura B, and Laura W, we may not share the same blood but you are my family and I love you all. Finally, the most important people, and my biggest source of love and encouragement. Mom, Dad, Kevin (and Roz, Madden), Erin (and Panache), Kaylin, thank you for supporting me and teaching me how to be a better person. You

have no idea how much you all mean to me and how much I love you.

Thanks for showing me what’s actually important in life.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: THESIS INTRODUCTION

Introduction

‘Eutrophication’ describes the shift of natural lake ecosystems from a nutrient poor (oligotrophic) status towards a nutrient rich (eutrophic) state. This transition is most often the result of nutrients such as phosphorus and nitrogen entering the system from either natural sources (e.g. weathering of rocks, geothermal activity), or human activities (e.g. agriculture, urban development) (Bush, 2000). The addition of nutrients to the system provides the foundation where primary producers, such as phytoplankton and submergent macrophytes, start to increase in densities (Bush, 2000). Run-off with high concentrations of nitrogen and phosphorus flow from the land, into surface and ground waters (Carpenter et al., 1998; Vitousek et al., 1997). Such nutrient enrichment usually results in the stimulation of primary producers, typically in the form of algae, which eventually results in a decrease in water quality. The process of accelerated eutrophication (e.g. over tens of years) is invariably undesirable, and of great concern for lake managers (Smith, 2003).

Eutrophication is of global concern (Bennett et al., 2001), affecting both freshwater and coastal marine ecosystems (Smith, 2003). In New Zealand, Hamill and Lew (2006) found that of 153 New Zealand lakes, over half were eutrophic. The Waikato Region of New Zealand (see Figure 1.1) is a prime agricultural region, and of all the lakes monitored, the only one designated oligotrophic was Lake Taupō, the largest lake in the Southern Hemisphere. In 2010, the trends of New Zealand lakes were revisited, showing that 44% of monitored lakes were eutrophic, and that

Trophic Lake Index (TLI) was positively correlated with pastoral land cover (Verburg et al., 2010).

Nutrient enrichment from agricultural activities, such as fertilizer runoff and animal excretions, as well as urban development are primary sources. However, nutrient inputs to shallow lakes are often enhanced by other factors including the presence of non-indigenous fish species within the water body. Common carp (*Cyrpinus carpio*), and other benthivores can amplify the problem of eutrophication. Carp are widespread globally, and considered one of the worst invasive species (Lowe et al., 2000). The first record of the destructive nature of carp was seen in a small lake in Wisconsin in 1929 (Cahn, 1929). The lake was transformed from a productive fishery consisting of largemouth bass (*Micropterus salmoides*), northern pike (*Esox lucius*), walleye (*Sander vitreus*), and panfish, to one dominated by carp (Cahn, 1929). At densities of as little as 100kg/ha, carp can begin to have dramatic effects on their environment (Vilizzi et al., 2015; Zambrano and Hinojosa, 1999). As adults, their omnivorous diets and benthic feeding habitats uproot plants and disturb the sediments, which then enter the water column. As young, carp also feed directly on zooplankton, which feed on algae and have the potential to reduce algal abundance (Scott and Crossman, 1973). One of the major problems of carp as part of the eutrophication process is that they promote a rapid shift (a ‘tipping point’) in the ecosystem, moving it from one dominated by macrophytes and clear water, to a turbid-water state, dominated by phytoplankton (Scheffer et al., 1993; Vilizzi and Copp, 2015). Once an ecosystem has shifted to this alternative stable state, the ability to reverse

the aquatic ecosystem back to its original clean water state requires much more effort (Scheffer et al., 2001), and is especially difficult when nutrient levels remain high in the catchment (Tátrai et al., 2005).

Much of the research on the effects of carp has been focused on overseas populations (see review by Vilizzi and Copp, 2015). In New Zealand, few studies have been experimental (e.g. Rowe, 2007; Daniel and Morgan, 2011), and are instead observational or part of already established routine monitoring programs (e.g. Waikato Regional Council, 2014). This is unfortunate as non-indigenous species in New Zealand pose significant threats to freshwater ecosystems (Rowe and Smith, 2001). Non-indigenous species, and especially carp, appear to accelerate the process of eutrophication, leading to hyper-eutrophic conditions (Rowe, 2007). Within the Waikato region, carp are particularly common, and have degraded shallow lakes, leaving them in the algal dominated state and devoid of macrophytes (Daniel and Morgan, 2011).

Carp introduce both bottom-up, and top-down effects on zooplankton. For example, bottom-up effects include adult feeding behaviour, which increase suspended sediments in the water column and interfere with feeding of zooplankton (Kirk and Gilbert, 1990; Kirk, 1991a). An example of top-down effects includes the direct predation on zooplankton by juvenile carp (Scott and Crossman, 1973). Zooplankton are a key trophic component of freshwater ecosystems, predating on phytoplankton and providing food for higher trophic levels such as planktivorous fish. Previous research has indicated the need to examine

the relationships between water quality and zooplankton communities (Jeppesen et al., 2011). As zooplankton are the intermediary step between primary producers and higher trophic levels, their communities respond quickly to both top-down and bottom-up influences in their environment. Studying the effects that non-indigenous pest species have on zooplankton communities is pertinent to understanding the damage that these species are having on the Waikato lakes.

Prior to beginning my thesis research in 2012, studies focusing on the effects carp have on zooplankton communities had been undertaken either on small spatial scales (e.g. Khan et al., 2003) or on short temporal scales (e.g. Wahl et al., 2011). Predation (e.g. Khan et al., 2003), suspended sediments (e.g. Lougheed et al., 1998) and phytoplankton community changes (e.g. Tatrai et al., 2005) have been proposed as influencing factors on zooplankton communities. Thus, there is still uncertainty of the influence carp have on zooplankton communities. Because of the top-down and bottom-up potential, this is not entirely surprising. Accordingly, I undertook a long-term (3 year) large-scale (whole-ecosystem) approach to more thoroughly assess the effects of pest fish on lake ecosystem response. Although my study was conducted in New Zealand, the knowledge gained from these studies are widely applicable as many zooplankton taxa are cosmopolitan.

One of the current limitations for studying zooplankton is the need for routine collection of samples from affected areas, returning them to an appropriate laboratory facility and then morphologically identifying and

enumerating species. This relies heavily on relevant taxonomic expertise, which is usually limited or often unavailable (Boero, 2001; Wheeler, 2004). The introduction of DNA-based identification methods (Hebert et al., 2003) has provided an alternative to the morphologically-based approaches. This method facilitates the routine identification of taxa without the need for continuous consultation with taxonomic experts. For example, in 2017, the Barcode of Life Datasystems (BOLD) database (Ratnasingham and Hebert, 2007), had close to 5.5 million sequences, providing references to over 175,000 animal, 65,000 plant and 20,000 fungal species. The time and cost of identification has been reduced with a DNA-based approach, and has the added benefit of identifying morphologically similar taxa, such as copepod nauplii (Fontaneto, 2014). The traditional COI region can identify and reveal taxonomic relationships between the rotifers, cladocerans, and copepods effectively (e.g. Fontaneto, 2014), but multiple primers are often required, and the success rate is often low.

Current DNA-based research is approaching a time where sampling of entire zooplankton communities is possible through DNA-based techniques. However, the next-generation sequencing (NGS) platforms currently available are limited in the size of fragment suitable for sequencing (~400bp; van Dijk et al., 2014), shorter than the standard COI barcoding region. What is needed is a shorter region that has the propensity to identify specimens to a species-level, but is widely applicable to the entire zooplankton community. Ribosomal DNA is more conserved, but has intermittent variable sections that appear to be promising for species identification (De Ley et al., 2005; Hirai et al., 2013; Sonnenberg

et al., 2007). One of the major obstacles to DNA-based research is finding a truly universal primer pair that can equally sequence all taxa. Ribosomal DNA, compared to mitochondrial DNA is more reserved and allows for the creation of universal primer pairs. However, these sections are understudied compared to the COI region, and must be validated to ensure that the sequence divergences are large enough to distinguish between taxa, and assign them to their designated taxonomy.

Metabarcoding, using NGS methodologies (Taberlet et al., 2012), takes entire samples, bypassing the need to sort and count individuals, and runs them through a high-throughput sequencing machine. Individual tags are applied to each sample, allowing for multiple whole-lake samples to be run at the same time. This technology opens the door to managers looking to monitor lakes, tracking environmental or anthropogenic induced changes and capturing spatial and temporal variability (Brannock et al., 2016; Chain et al., 2016; Pearman and Irigoien, 2015). However, these metabarcoding studies still rely on the creation of reference libraries built by single sequencing. The pitfall in using a ribosomal DNA region is that they are understudied, and do not have the backbone of a large reference library that is associated with the COI region. Few studies have attempted to analyze zooplankton communities using metabarcoding (e.g. Chain et al., 2016; Pearman and Irigoien, 2015). However, a complete reference library is often lacking, and species level identification is not always possible. To translate DNA-based techniques into applicable information on the status of an aquatic ecosystem, the identification to species level is often required. Further, the sequence data should be able to distinguish

the dominant taxa, essentially outputting the same information as a traditional morphological count.

Organization of Thesis

The main aim of my thesis was to first test the effects common carp (*Cyprinus carpio*) on lake ecosystem dynamics (e.g. nutrients, water clarity, zooplankton communities). The latter half of the thesis aimed to develop a genetic-based method to work around the traditional microscope work needed to conduct routine morphological assessments of zooplankton.

This thesis consists of three research chapters. The first research chapter (Chapter 2) tests the effects of carp on zooplankton community dynamics in a large-scale (whole lake), long term (2.5-year) experiment. My objectives were to determine the consequences of carp presence on environmental variables and biological communities at the ecosystem scale. Specifically, how physical and chemical processes change with the introduction of carp, such as nutrient concentrations, suspended sediment concentrations, as well as changes in biological components. I was particularly interested in how these environmental variables influence the zooplankton community dynamics, such as a presence or absence of species, community composition, and disappearance of filter feeders.

The second research chapter (Chapter 3) tests the suitability of a more conserved ribosomal DNA marker (28S) to determine if it could be used to identify zooplankton individuals to the species level. The second objective was to create a complete reference library, covering all

zooplankton species at the study site, providing a foundation for a metabarcoding study.

The third research chapter (Chapter 4) tests the effectiveness of using the 28S gene region in Next Generation Sequencing (NGS) approaches to assess community composition and contrasts this with traditional, morphological-based approaches.

The thesis concludes with a general conclusion chapter (Chapter 5) and provides suggestions for future research.

Figure 1.1. Study area in New Zealand showing the Waikato region (in darker green), which was the focus of the study (sourced from Waikato Regional Council; https://www.waikatoregion.govt.nz/community/about-the-waikato-region, Date accessed 12-08-2017)

Literature Cited

Bennett, E.M., Carpenter, S.R. and N.F. Caraco. 2001. Human impact on erodible phosphorus and eutrophication: a global perspective. BioScience, 51(3), 227-234.

Boero, F. 2001. Light after dark: the partnership for enhancing expertise in taxonomy. TRENDS in Ecology & Evolution, 15(5), 1pp.

Brannock, P.M., Ortmann, A.C., Moss, A.G. and K.M. Halanych. 2016. Metabarcoding reveals environmental factors influencing spatio-temporal variation in pelagic micro-eukaryotes. Molecular Ecology, 25, 3593-3604.

Bush, M.B. 2000. Ecology of a changing planet, Second Edition. Prentice-Hall, Inc. Upper Saddle River, NJ.

Cahn, A.R. 1929. The effect of carp on a small lake: the carp as a dominant. Ecology, 10(3), 271-274.

Carpenter, S.R., Caraco, N.F., Correll, D.L., Howarth, R.W., Sharpley, A.N. and V.H. Smith. 1998. Nonpoint pollution of surface waters with phosphorus and nitrogen. Ecological Applications, 8(3), 559-568.

Chain, F.J.J., Brown, E.A., MacIsaac, H.J. and M.E. Cristescu. 2016. Metabarcoding reveals strong spatial structure and temporal turnover of zooplankton communities among marine and freshwater ports.

Daniel, A.J. and D.K.J. Morgan. Lake Ohinewai pest fish removal. CBER Contract Report 120, Centre for Biodiversity and Ecology Research, The University of Waikato, 30pp.

De Ley, P., De Ley, I.T., Morris, K., Abebe, E., Mundo-Ocamp, M., Yoder, M., Heras, J., Waumann, D., Rocha-Olivares, A., Burr, A.H.J., Baldwin, J.G and W.K. Thomas. 2005. An intergrated approach to fast and informative morphological vouchering of nematodes for applications in molecular barcoding. Phil. Trans. R. Soc. B., 360, 1945-1958.

Fontaneto, D. 2014. Molecular phylogenies as a tool to understand diversity in rotifers. International Review of Hydrobiology, 99, 178-187.

Hamill, K. and D. Lew. 2006. Snapshot of lake water quality in New Zealand. Ministry for the Environment, Wellington, New Zealand, 59pp.

Hebert, P.D.N., Cywinska, A., Ball, S.L. and J.R. deWaard. 2003. Biological identifications through DNA barcodes. Proc. R. Soc. Lond., 270, 313-321.

Hirai, J., Shimode, S. and A. Tsuda. 2013. Evaluation of ITS2-28S as a molecular marker for identification of calanoid copepods in the subtropical western North Pacific. J. Plankton Res., 35(3), 644-656.

Jeppesen, E., Nõges, Davidson, T.A., Haberman, J., Nõges, T., Blank, K., Lauridsen, T.L., Søndergaard, M., Sayer, C., Laugaste, R.,

Johansson, L.S., Bjerring, R. and S.L. Amsinck. 2011. Zooplankton as indicators in lakes: a scientific-based plea for including zooplankton in the ecological quality assessment of lakes according to the European Water Framework Directive (WFD). Hydrobiologia, 676, 279-297.

Khan, T.A., Wilson, M.E. and M.T. Khan. 2003. Evidence for invasive carp mediated trophic cascade in shallow lakes of western Victoria, Australia. Hydrobiologia, 506-509, 465-472.

Kirk, K.L. and J.J. Gilbert. 1990. Suspended clay and the population dynamics of planktonic rotifers and cladocerans. Ecology, 71(5), 1741-1755.

Kirk, K.L. 1991a. Suspended clay reduces *Daphnia* feeding rate: behavioural mechanisms. Freshwater Biology, 25, 357-365.

Lougheed, V.L., Crosbie, B. and P. Chow-Fraser. 1998. Predictions on the effect of common carp (*Cyprinus carpio*) exclusion on water quality, zooplankton, and submergent macrophytesin a Great Lakes wetland. Can. J. Fish. Aquat. Sci., 55, 1189-1197.

Lowe, S., Browne, M., Boudjelas, S. and M. De Poorter. 2000. 100 of the world's worst invasive alien species. A selection from the Global Invasive Species Database. The Invasive Species Specialist Group, 12 pp.

Pearman, J.K. and X. Irigoien. 2015. Assessment of zooplankton community composition along a depth profile in the Central Red Sea. PLoS ONE, 10(7), 14pp.

Ratnasingham, S. and P.D.N. Hebert. 2007. BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). Molecular Ecology Notes, 7, 355-364.

Rowe, D.K. 2007. Exotic fish introductions and the decline of water clarity in small North Island, New Zealand lakes: a multi-species problem. Hydrobiologia, 583, 345-358.

Rowe, D.K. and J.P. Smith. 2001. The role of exotic fish in the loss of macrophytes and increased turbidity of Lake Wainamu, Auckland. NIWA Client Report: ARC02286, National Institute of Water & Atmospheric Research Ltd, Hamilton, NZ. 32pp.

Scheffer, M., Hosper, S.H., Meijer, M-L., Moss, B. and E. Jeppesen. 1993. Alternative equilibria in shallow lakes. TREE, 8(8), 275-279.

Scheffer, M., Carpenter, S., Foley, J.A., Folkes, C. and B. Walker. 2001. Catastrophic shifts in ecosystems. Nature, 413, 591-596.

Scott, W.B. and E.J. Crossman. 1973. Freshwater fishes of Canada. Bulletin 184. Fisheries Research Board of Canada, 966 pp.

Sonnenberg, R., Nolte, A.W. and D. Tautz. 2007. An evaluation of LSU rDNA D1-D2 sequences for their use in species identification. Frontiers in Zoology, 4(6), 12pp.

Smith, V.H. 2003. Eutrophication of freshwater and coastal marine ecosystems. A global problem. Environ. Sci. & Pollut. Res., 10(2), 126-139.

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C. and E. WIllerslev. 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. Molecular Ecology, 21, 2045-2050.

Tátrai, I., Mátyás, K., Korponai, J., Szabó, G., Pomogyi, P. and J. Héri. 2005. Response of nutrients, plankton communities and macrophytes to fish manipulation in a small eutrophic wetland lake. Internat. Rev. Hydrobiol., 90(5-6), 511-522.

van Dijk, E.L., Auger, H., Jaszczyszyn, Y. and C. Thermes. 2014. Ten years of next-generation sequencing technology. Trends in Genetics, 30(9), 418-426.

Verburg, P., Hamill, K., Unwin, M. and J. Abell. 2010. Lake water quality in New Zealand 2010: Status and trends. NIWA Client Report: HAM2010-107, National Institute of Water and Atmospheric Research Ltd. Hamilton, NZ.

Vilizzi, L., Tarkan, A.S. and G.H. Copp. 2015. Experimental evidence from causal criteria analysis for the effects of common carp *Cyprinus carpio* on freshwater ecosystems: a global perspective. Reviews in Fisheries Science and Aquaculture, 23, 253-290.

Vitousek, P.M., Mooney, H.A., Lubchenco, J. and J.M. Melillo. 1997. Human domination of earth's ecosystems. Science, 277, 494-499.

Waikato Regional Council. 2014. Waikato region shallow lakes management plan: Volume 2. Shallow lakes resource statement: Current status and future management recommendations. Waikato Regional Council, Hamilton, NZ, 221pp.

Wheeler, Q.D. 2004. Taxonomic triage and the poverty of phylogeny. Phil. Trans. R. Soc. Lond., 359, 571-583.

Zambrano, L. and D. Hinojosa. 1999. Direct and indirect effects of carp (*Cyprinus carpio* L.) on macrophyte and benthic communities in experimental shallow ponds in central Mexico. Hydrobiologia, 408/409, 131-138.

CHAPTER 2: THE ECOLOGICAL EFFECTS OF COMMON CARP ON ZOOPLANKTON COMMUNITY COMPOSITION AND ABUNDANCE: A LARGE-SCALE FIELD EXPERIMENT.

*to be published under the same title as: Woods, S., Hogg, I.D., Pilditch, C.A., Duggan, I.C.

Abstract

I undertook a long-term (2.5 year), whole-ecosystem experiment to examine the effects of common carp (*Cyprinus carpio*) on physical/chemical parameters and zooplankton communities in lotic habitats. Two adjacent ponds in the Waikato region of New Zealand were paired and using a BACI experimental design, I monitored for 15 months prior to treatment and following this, the experimental pond was stocked with carp (>400 kg/ha) and monitored for a further 14 months. There were significant increases in inorganic suspended sediments and nitrogen concentrations following the introduction of carp. Seasonal changes in zooplankton densities were predominantly associated with increased suspended sediments and smaller particle sizes in the water column. At the community level, a multivariate analysis suggested the overall composition of the zooplankton communities was relatively unchanged following carp addition. I conclude that the introduction of carp into a system that is already in a eutrophic state may have measurable effects on some environmental factors but is unlikely to have a large influence on the overall community composition of zooplankton.

Introduction

Common carp (*Cyprinus carpio*) are frequently implicated as a cause of environmental degradation in freshwater ecosystems. They are considered one of the most successful invasive species globally (Lowe et al., 2000), due to their tolerance of a wide range of environmental conditions (Crivelli, 1981; Scott and Crossman, 1973), early sexual maturity (Tempero et al., 2006), high fecundity (Swee and McCrimmon, 1966), and rapid growth (Tempero et al., 2006). These life history characteristics often result in extremely high population densities, and biomasses of over 1000 kg ha^{-1} in natural lake habitats (Hicks et al., 2005).

The life history traits of common carp, combined with an aggressive feeding behaviour in, and on benthic sediments, can initiate a cascade of linked interactions that affect several trophic levels and, ultimately, shift ecosystems to degraded alternative stable states (e.g. Bajer et al., 2009; Zambrano and Hinojosa, 1999). Cahn (1929) first noted that carp could instigate such shifts, resulting in turbid waters devoid of macrophytes. For shallow lakes, this results in a shift from a clear-water state dominated by submerged macrophytes, and large filter-feeding cladocerans, to a turbid-water state dominated by selective feeding rotifers and phytoplankton (Scheffer et al., 1993; 2001). Previous studies have estimated that when biomasses of carp exceed approximately 100 kg/ha, the system will start to shift towards a turbid-water state (e.g. Bajer et al., 2009; Vilizzi et al., 2015; Zambrano and Hinojosa, 1999). Carp directly influence the turbidity of the water column, and the disruption and uprooting of macrophytes,

through their foraging activities (Crivelli, 1983; Miller and Crowl, 2006). However, knowledge of the indirect effects of carp such as those imposed on the other trophic levels such as zooplankton, remains limited (e.g. Lougheed et al., 1998).

Zooplankton populations among lakes are influenced by bottom up processes such as changing phytoplankton communities (Gilbert, 1990), the top-down pressures of predators (Jeppesen et al., 1997), as well as changing environmental parameters such as increased suspended sediments; Kirk, 1991a). Accordingly, zooplankton populations can respond quickly to changes in their environment (e.g. Tátrai et al., 2005), making them useful indicators of environmental changes in lakes (e.g. Pace, 1986; Duggan et al., 2001; Jeppesen et al. 2011; Gannon and Stemberger, 1978; Haberman and Haldna, 2014). Larger filter feeding cladocerans can also have a strong influence on water clarity by removing phytoplankton from the water column. Efforts to improve water quality often use biomanipulation to promote zooplankton communities dominated by large cladocerans, rather than those dominated by smaller rotifers (Shapiro et al., 1975, Shapiro and Wright, 1984).

The presence of carp can influence zooplankton communities in several ways. For example, an increase in suspended sediment loads caused by carp feeding and mating behaviours (Parkos et al., 2003) may mechanically interfere with larger-bodied zooplankton such as cladocerans, decreasing ingestion rates (Arruda et al., 1983; Kirk and Gilbert, 1990; Kirk, 1991a). The amount of suspended sediment in the

water column is dependent on the size and density of the fish, with larger individuals in higher densities having a more pronounced effect (e.g. Parkos et al., 2003; Weber and Brown, 2009). Increases in nutrients through excretions and the release of trapped nutrients from the sediment (Lougheed et al., 1998; Parkos et al., 2003) indirectly influences zooplankton biomass through an increase in primary productivity within the system (Carpenter et al. 1996).

Previous studies examining the effects of carp on zooplankton community dynamics of lakes have been inconclusive. For example, some studies have shown an increase in zooplankton biomass (Parkos et al. 2003), some have shown a decrease in biomass (Lougheed et al. 1998), while others have shown no change (Wahl et. al, 2011). Cladoceran responses to the presence of carp have also been variable with some studies showing either significant decreases in population densities (Khan et al., 2003), no significant changes (Miller and Crowl, 2006; Matsuzaki et al., 2007; Wahl et al., 2011), or significant increases (Parkos et al., 2003; Chumchal and Drenner, 2004). These variable responses have been attributed to the effects of: 1) predation (Khan et al., 2003; Wahl et al., 2011); 2) phytoplankton quality or quantity (Chumchal and Drenner, 2004; Tatrai et al., 2005); and 3) suspended sediments (Lougheed and Chow-Fraser, 1998; Lougheed et al., 1998).

Many of the previous experimental studies have used enclosures, mesocosms, or microcosms to manipulate systems. While these smaller-scale experiments have provided some insight into potential ecosystem

responses, the greater environmental complexity of whole ecosystems would benefit from larger-scale field experiments (Carpenter, 1996; Schindler, 1998; Pace et al., 1998). Zooplankton can respond quickly to changing environmental conditions (e.g. Attayde and Hansson, 2001), and are good indicators of ecosystem change (e.g. Mohamed Anas et al., 2014; Sousa et al., 2008). Larger temporal-scale studies would provide further insights into potential long-term ecosystem responses. Accordingly, I undertook a long-term (2.5 year) whole-lake manipulation using a Before-After Control-Impact (BACI) design (*sensu* Stewart-Oaten et al. 1986), to determine the effect of carp on zooplankton populations. Specifically, I examined the effects of a high density of adult, non-gravid carp (400 kg ha^{-1}), to determine their influence on zooplankton. I predicted that the benthic feeding habits of carp, and high density of stocking, would increase suspended solids and nutrient concentrations within the water column. Further, I predicted that large-bodied cladocerans would decrease in densities through indirect effects of the increased suspended solids, and that the community would become dominated by rotifers. As I show, the response was counter to my prediction and required an alternative explanation.

Methods

This experiment was conducted in two small eutrophic ponds enclosed in the Hamilton Zoo, Hamilton, New Zealand (37^{o} 46' 27", 175^{o} 12' 51"). The ponds were chosen for their similar size, close proximity, restriction from public access, ease for water level manipulation, and similar water quality. The two ornamental ponds are exhibits for native and exotic waterfowl, with an average number of 33 (range 4-62) seen across both ponds throughout the study period. Mammals were also present nearby (e.g. rhinoceros, chimpanzees, goats). The ponds received runoff from this surrounding area. However, most water passed through a gravel filter bed, which removed nutrients and sediments. The ponds were connected through a small channel (35cm width) with a one-way flow of water from the control pond to the treatment pond. Calculations indicate that the treatment pond would take on average, 10 d to turnover, with a very generous flow of 100 L min^{-1}. There was a 1.2 metre vertical drop from the control pond to the treatment pond, and wire mesh on both outflows that prevented any movement of fish between ponds. The ponds were of relatively similar sizes with the control and treatment ponds being 0.15 ha and 0.12 ha in size, with maximum depths of 1.2 m and 1.5 m, respectively. This and a previous study conducted in 2005 showed similar water quality parameters and zooplankton communities between the two ponds (Fowler, 2006). At the start of the present study, both ponds had low densities of pest fish, with goldfish (*Carassius auratus*; 2.3 kg ha^{-1}) observed in the control pond and mosquito fish (*Gambusia affinis*) observed in both the control and treatment ponds. Native short-finned eels

(*Anguilla australis*) were also present in both ponds at low densities (<3 kg ha^{-1}). The substrate of both ponds had a high organic content and macrophytes were absent.

Given the lack of replicate ponds and the possibility of high inter-annual variability for the ponds, I used a “Before-After-Control-Impact” (BACI) study design (*sensu* Stewart-Oaten et al. 1986), with a single impact site, and a single control site. The ‘before’ period lasted from August 21, 2012 to November 25, 2013, and the ‘after’ period from November 25, 2013 to January 21, 2015. The upstream pond was designated as the ‘control’ pond, and the downstream pond was used as the treatment, or ‘impact’ pond. Three sites were sampled in each pond. One was located 2-3 m from the inflow, one in the middle, and one 2-3 m from the outflow. A regular monthly sampling schedule was employed for the duration of the study. However, a more intensive sampling effort was undertaken immediately following carp addition to detect any acute effects (days 3, 6, 14, 19, 26, post treatment). Regular monthly samples were resumed one month following the carp addition.

Feral common carp, captured from Lake Waikare, in the Waikato region of New Zealand, were added to the ‘treatment’ pond at a density of 400 kg ha^{-1} (n = 29 individuals). The mean length and weight of the added fish was 467 mm and 1.72 kg, respectively. All fish added were males and outfitted with two Suprelorin hormone injections (9.7 mg dolorein) anterior to the dorsal fin to minimise the possibility of accidental breeding within the system. Throughout the study, there was minimal fish mortality with only

one dead individual found. This individual was replaced the following day with a fish of comparable size. Fish were removed at the end of the study by draining the pond and dragging a seine net through the remaining water. All 29 added fish were recaptured. The mean length and weight of the fish at the end of the study was 520 cm and 3.24 kg, respectively, giving a density of 766 kg ha^{-1}. Surprisingly, other younger fish were present (n=84) in the pond at the end of the study, which may have indicated problems with the anti-reproduction treatments, or presence of uncaptured age 0 fish at the start of the study. According to Tempero et al. (2006), there were two age cohorts; age 1 (mean length 101 mm) and age 2 (mean length 235 mm). The age 2 cohort predates the time when fish were added to the experimental pond. When these smaller fish were added to the calculation, the final density of carp in the treatment pond was 985 kg ha^{-1}.

Each month, temperature, dissolved oxygen, pH, and conductivity were taken from each site using a 600QS Multiparameter Water Quality Sonde unit from the surface to bottom at 0.2 m intervals. Water transparency and depth was measured with a 20 cm diameter Secchi disk. Suspended sediments, particle sizes and phytoplankton were measured by capturing water (1-2 L for the former two, 250 mL for the latter) 10 cm below the water surface. Total suspended sediments were determined by filtering the sample through a pre-weighed 0.5 µm glass fibre filter and drying at 105 ^{o}C to constant weight. Filters were then burned at 550 ^{o}C, to constant weight, to determine organic content. The particle size samples were analyzed immediately upon returning to the laboratory on a Malvern

Mastersizer S (Malvern, UK). Since cladocerans were the largest zooplankton in the ponds, and their maximum diet threshold is 40 µm (Gophen and Geller, 1984; Kirk, 1991b), the samples were first filtered through a 37 µm mesh to remove zooplankton and large phytoplankton, and the upper limit of measurement on the instrument was set to 40 µm. Nutrients were taken from a 2 L sample collected 10 cm below the water surface and analyzed on a QuikChem® 8000 Flow Injected Analyser (FIA) using standard protocols. Climate data were obtained from the Hamilton Airport meteorological station located within 20 km of the study site. Hourly wind speeds, and daily temperatures (mean, max/min), and rainfall from 21 August 2012 to 21 January 2015 were used in the analyses.

Zooplankton were sampled by inserting a 5.68 L tube into the water column on a 45^{o} angle to ensure the sediment was not disturbed, bunging the ends and filtering the water through a 37 µm net. Samples were stored in 70% ethanol at 4 ^{o}C until processed. Samples were made up to a known volume, and 5 ml aliquots were processed until a minimum of 300 individuals were counted, or until the entire sample was completed. Identification was to the level of species (Shiel, 1995), except for *Anuraeopsis*, *Monommata*, *Proales*, *Chydorus* and *Mesocyclops* species, which were to the genus level, and Bdelloid rotifers to class.

Using Primer v6 (Clark and Gorley, 2006), a similarity matrix was constructed based on Euclidean normalised environmental variables. A BEST analysis was run to determine the variables that were the driving factors in the environmental variation throughout the study period. The

influential environmental variables, and how those changed between sampling months, and sampling sites, were analyzed using a distance-based linear model (DistLM; 9999 permutations), and the corresponding marginal tests within the model.

Individual environmental parameters, including physical and chemical variables, total and dissolved nutrients, suspended sediments, and particle sizes were analyzed using a two-way repeated measures analysis of variance (repeated-measures ANOVA). Differences between location (control vs. treatment) and time of year (sampling month) for each period of the study (before and after) were tested. Fishers LSD tests were used to compare the means.

Zooplankton community assemblages were analyzed using a Bray-Curtis dissimilarity matrix on log(x+1) transformed data. Rare species (< 3 individuals L^{-1} across the entire study) were removed from multivariate analyses to reduce the effect of species sampled by chance. Zooplankton community differences between ponds and treatment periods were tested with permutational multivariate analysis of variance (PERMANOVA; McArdle and Anderson, 2001), and significance was determined by 9999 permutations. Pair-wise comparisons were completed to further assess significant differences between ponds at each sampling period, in both the before and after periods. Similarity percentages (SIMPER) were used to determine taxa contributing most to the variation in the zooplankton communities in the control and treatment pond, before and after fish

addition. Zooplankton communities were visualized using multidimensional scaling (MDS) in Primer v6 (Clarke and Gorley, 2006).

Results

Zooplankton Community

A total of 113,136 zooplankton were counted from the study sites over the 2.5 yr experiment. Taxa included the phylum Rotifera (50 taxa), cladocerans (4 taxa), and copepods (cyclopoid and harpacticoid; Table 2.1). There was no evidence of a common species being extirpated from either pond over the course of the study. In addition, there was consistent overlap between the ponds, with no species excluded from either pond. Mean densities of total zooplankton were similar between the ponds before treatment (Figure 2.1); 696 ind L^{-1} and 848 ind L^{-1} in the control and treatment ponds, respectively. Mean densities across both ponds showed a slight increase at day 146 (Summer 2013) to 1071 ind L^{-1}, and a large increase at day 360 (Winter 2013) to 3534 ind L^{-1}. Mean zooplankton densities increased after fish addition to 2419 ind L^{-1} in the control pond and 3135 ind L^{-1} in the treatment pond. Immediately after fish addition (day 454, Spring 2013) there was a large spike in zooplankton densities, lasting from day 459 to 573 (Spring, Summer and Autumn of 2014) in both the control and treatment ponds. An additional peak occurred in the control pond at day 734, increasing to a density of 5002 ind L^{-1}. The maximum density of zooplankton occurred in the treatment pond on day 462 (Autumn 2014) at 9307 ind L^{-1}. These values decreased again on day 797 (Winter 2014) to densities around 1000 ind L^{-1} (Figure 2.2).

Table 2.1. Mean densities of identified zooplankton in the control and treatment pond, before and after fish addition; Low Abundance (L) =< 10 ind L^{-1}, Medium abundance (M) = 10-50 ind L^{-1}, High Abundance (H) = 50-100 ind L^{-1}, Very High Abundance (V) => 100 ind L^{-1}. **Represents extremely low abundance species (<3 ind).

	Control Before	**Treatment Before**	**Control After**	**Treatment After**
Rotifera				
Anuraeopsis sp.	L	L	L	M
Asplanchna brightwellii (Gosse, 1850)	L	M	M	M
Bdelloid spp.	L	M	M	H
Brachionus angularis (Gosse, 1851)	M	H	M	H
Brachionus budapestinensis (Daday, 1885)	L	L	L	L
Brachionus calyciflorus (Pallas, 1766)	M	M	V	V
Brachionus urceolaris (Müller, 1773)	L	L	L	L
Calamoecia lucasi (Brady, 1906)	L	L	L	L
Cephalodella catalina (Müller, 1786)	L	L	L	L
Cephalodella forficula (Ehrenberg, 1832)	L	L	L	L
Cephalodella gibba (Ehrenberg, 1830)	L	L	L	L
Cephalodella ventripes (Dixon-Nuttall, 1901)	L	L	L	L
Colurella uncinata (Müller, 1773)	L	L	L	L
Dicranophoroides caudatus (Ehrenberg, 1834)	L	L	L	L
Epiphanes macrourus (Barrois & Daday, 1894)	L	L	L	L
Euchlanis incisa (Carlin, 1939)	**	**	**	**
Euchlanis pyriformis (Gosse, 1851)	**	**	**	**
Euclanis deflexa (Gosse, 1851)	L	L	L	L
Filinia longiseta (Ehrenberg, 1834)	M	M	V	V
Filinia terminalis (Plate, 1886)	**	**	**	**
Gastropus hyptopus (Ehrenberg, 1838)	H	H	H	H
Itura myersi (Wulfert, 1935)	**	**	**	**
Keratella cochlearis (Gosse, 1851)	**	**	**	**
Keratella slacki (Berzins, 1963)	V	V	V	V
Keratella tecta (Gosse, 1851)	**	**	**	**
Keratella tropica (Apstein, 1907)	**	**	**	**
Lecane bulla (Gosse, 1851)	L	L	L	L
Lecane closterocerca (Schmarda, 1859)	L	L	L	L
Lecane flexilis (Gosse, 1886)	L	L	L	L
Lecane furcata (Murray, 1913)	L	L	L	L
Lecane inermis (Bryce, 1892)	**	**	**	**
Lecane luna (Müller, 1776)	L	L	L	L
Lecane lunaris (Ehrenberg, 1832)	L	L	L	L
Lepadella ovalis (Müller, 1786)	L	L	L	L
Lophocharis salpina (Ehrenberg, 1838)	L	L	L	L
Monommata sp.	L	L	L	L
Mytilina bisulcata (Lucks, 1912)	L	L	L	L
Platyias quadricornis (Ehrenberg, 1832)	L	L	L	L
Pleurotrocha petromyzon (Ehrenberg, 1830)	L	L	L	L
Polyarthra dolichoptera (Idelson, 1925)	M	M	V	V
Proales sp.	L	L	L	L
Squatinella mutica (Ehrenberg, 1832)	L	L	L	L
Synchaeta oblonga (Ehrenberg, 1832)	L	L	L	L
Synchaeta pectinata (Ehrenberg, 1832)	M	L	L	L
Synchaeta stylata (Wierzejski, 1893)	L	L	L	L
Testudinella patina (Hermann, 1783)	L	L	L	L
Trichocerca pusilla (Jennings, 1903)	**	**	**	**
Trichocerca similis (Wierzejski, 1893)	M	M	M	M
Trichocerca stylata (Gosse, 1851)	L	L	L	M

Table 2.1 (continued). Mean densities of identified zooplankton in the control and treatment pond, before and after fish addition; Low Abundance (L) =< 10 ind L^{-1}, Medium abundance (M) = 10-50 ind L^{-1}, High Abundance (H) = 50-100 ind L^{-1}, Very High Abundance (V) => 100 ind L^{-1}. **Represents extremely low abundance species (<3 ind).

	Control Before	**Treatment Before**	**Control After**	**Treatment After**
Cladocera				
Alona c.f. *affinis* (Leydig, 1860)	L	L	L	L
Bosmina meridionalis (Sars, 1904)	L	L	V	V
Chydorus sp.	L	L	L	L
Daphnia galeata (Sars, 1863)	M	H	M	M
Copepoda				
Mesocyclops sp.	M	M	M	M
Copepod nauplii	V	V	V	V
Harpacticoid nauplii	L	L	L	L
Ostracods				
Ostracod sp.	L	L	L	L

The most common zooplankton taxa (mean density >10 individuals L^{-1}) were *Asplanchna brightwelli*, Bdelloid spp., *Bosmina meridionalis*, *Brachionus angularis*, *Brachionus calyciflorus*, copepod nauplii, *Daphnia galeata*, *Filinia longiseta*, *Gastropus hyptopus*, *Keratella procurva*, *Keratella slacki*, *Mesocyclops* sp., *Polyarthra dolichoptera*, *Synchaeta pectinata*, and *Trichocera similis*. There were an equal proportion of rotifers, copepods and cladocerans until day 333, when the numbers of rotifers began to rise dramatically (Figure 2.1). This zooplankton community, dominated by rotifers, continued until day 608, when cladocerans became the dominant group in both ponds. The same trends were evident at the different sites in each pond. After fish were introduced there was a difference in rotifer densities in the control pond compared to the treatment pond (2737 ind L^{-1} vs. 636 ind L^{-1}). There was also a difference, albeit not as drastic, in the mean density of cladocerans; 505 ind L^{-1} in the control pond, and 243 ind L^{-1} in the treatment pond.

A significant difference was found in the zooplankton communities between the ponds during the before period ($p = 0.0007$, PERMANOVA, 9999 permutations), and the after period ($p = 0.0002$), and supported with the interaction effects between site and sampling period (before $p = 0.0001$, after $p = 0.0002$) (Table 2.2). However, post-hoc tests revealed no significant difference between the ponds at any of the individual sampling periods, in either the before or after periods. There were only subtle differences in the zooplankton communities between the control and treatment pond in the before period (SIMPER analysis, average Bray-Curtis dissimilarity before = 30.46, after = 22.09) (Table 2.3, Figure 2.3).

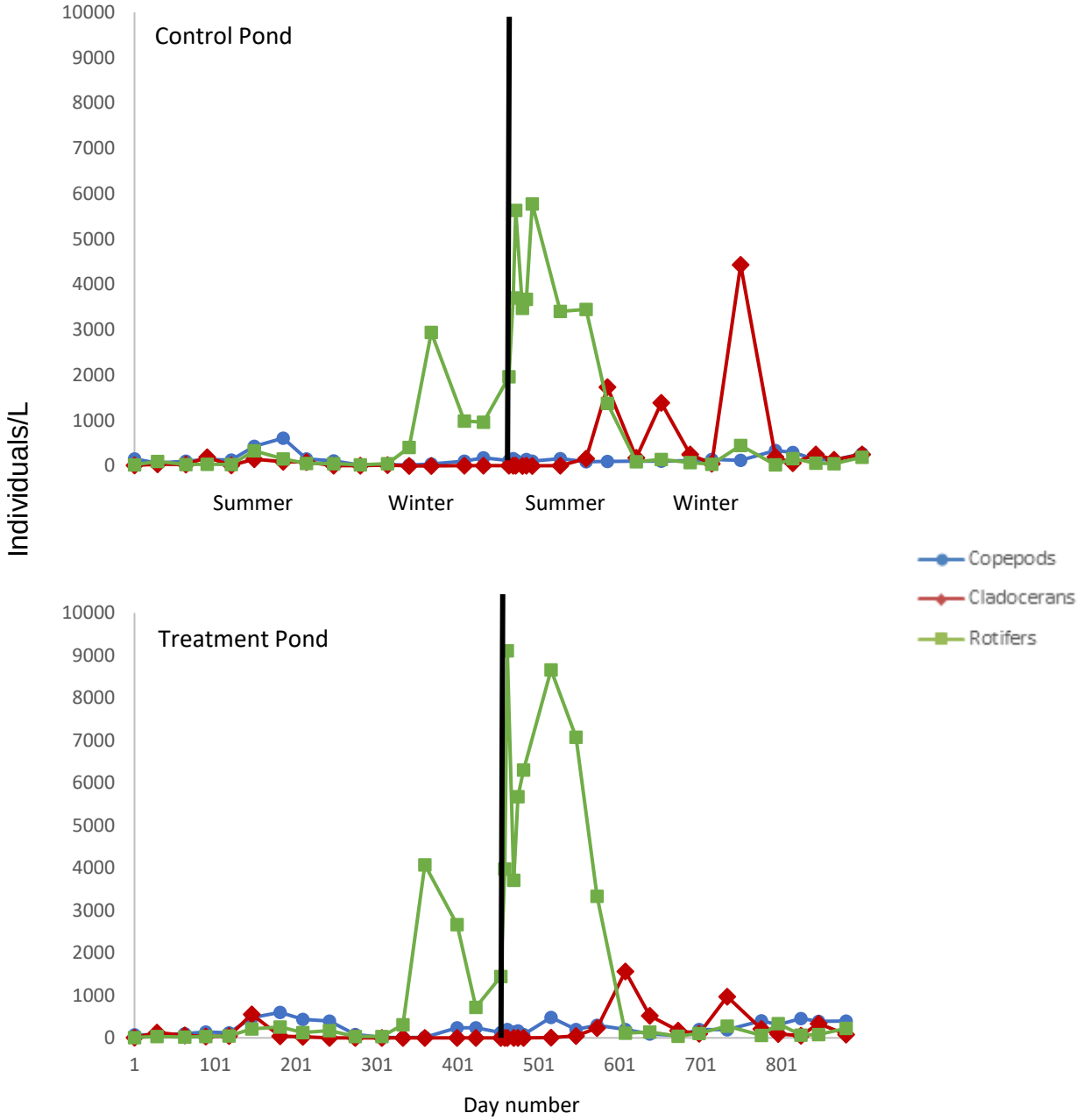


Figure 2.1. Zooplankton densities in the control pond and treatment pond. The solid line represents the date in which fish were added to the treatment pond.

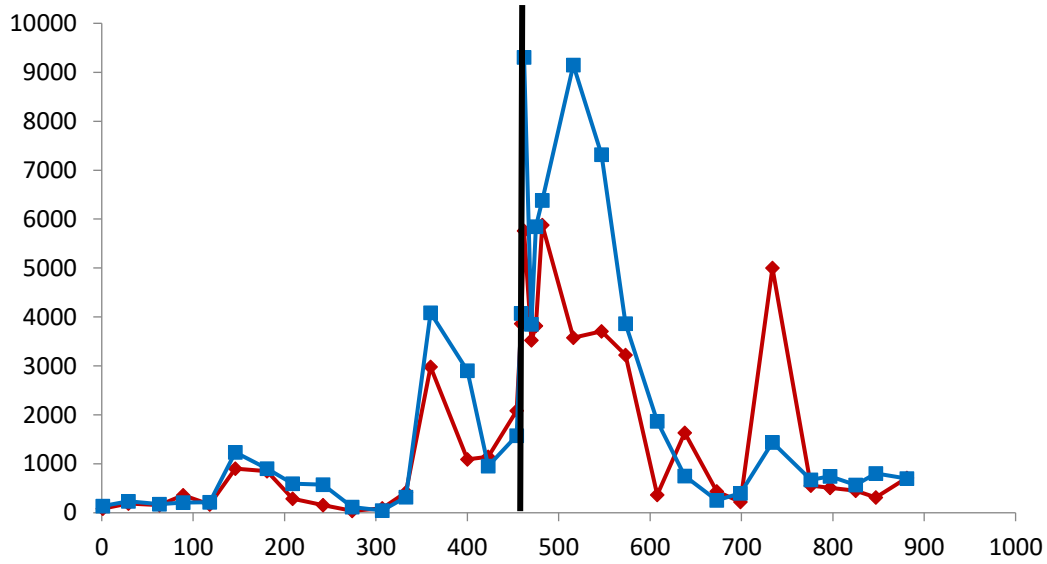


Figure 2.2. Total densities of zooplankton in the control pond (blue) and treatment pond (red). The solid line represents the date in which fish were added to the treatment pond.

The differences were predominantly driven by *Daphnia galeata*, *Mesocyclops* spp., copepod nauplii, and Bdelloid rotifers in the before period, and *Bosmina meridionalis*, Bdelloid rotifers, *Mesocyclops* spp., and *Polyarthra dolichoptera* in the after period.

Environmental Parameters

Two-way repeated measure ANOVAs were performed on all environmental parameters (Table 2.4) prior to carp addition to determine if there were any significant differences between the ponds. These analyses indicated that differences between the ponds were low. Significant differences were found for several variables. Dissolved oxygen, temperature, conductivity, NH_4^+, NO_2 were significantly higher in the treatment pond, and inorganic suspended sediments, NO_3^-, and NO_x were higher in the control pond (Table 2.4, Figure 2.4). However, most differences were small, suggesting that the biological relevance was likely to be minimal. No significant differences were detected for pH, PO_4, total suspended sediments, organic suspended sediments, total nitrogen, or total phosphorus. After fish addition, pH, conductivity, temperature, total nitrogen, inorganic sediments, organic sediments, and total sediments were significantly higher in the treatment pond. NO_2, NO_3^-, NO_x, and NH_4^+ were higher in the control pond (Table 2.4). Seasonal changes were observed for several parameters, such as water temperature, average wind speed and rainfall (Figure 2.5).

Table 2.2. PERMANOVA results for differences in zooplankton communities between the control and treatment ponds, before and after fish addition.

Before						
Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Pond	1	1094	1094	3.5623	0.0007	9950
Time	15	1.3132E5	8754.8	28.507	0.0001	9827
PondxTime	15	17349	1156.6	3.766	0.0001	9812
Res	62	19041	307.11			
Total	93	1.699E5				

After						
Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Pond	1	1091.2	1091.2	5.2239	0.0002	9953
Time	17	1.25E5	7352.9	35.2	0.0001	9853
PondxTime	17	5841.6	343.62	1.645	0.0002	9819
Res	72	15040	208.89			
Total	107	1.4697E5				

Table 2.3. SIMPER results (log x+1 transformed data) showing the predominant taxa contributing to the variation in the control and treatment ponds, before and after fish addition.

Species	Mean abundance	Mean abundance	Mean dissimilarity	Contribution %
Before	**Control**	**Treatment**		
Daphnia galeata	2.00	1.65	2.57	8.45
Mesocyclops spp.	1.77	2.07	2.11	6.92
Copepod nauplii	4.38	4.22	1.96	6.44
Bdelloid spp.	1.31	1.60	1.56	5.11
Synchaeta pectinata	1.05	0.54	1.39	4.55
Trichocerca similis	2.44	2.62	1.38	4.52
Keratella slacki	2.38	2.59	1.29	4.24
Filinia longiseta	1.13	1.31	1.19	3.91
Anuraeopsis spp.	0.32	0.57	1.10	3.60
Polyarthra dolichoptera	0.84	0.67	1.06	3.49
Pleurotrocha petromyzon	0.30	0.69	0.95	3.12
Synchaeta oblonga	0.47	0.44	0.76	2.49
Mean dissimilarity = 30.46				
After	**Control**	**Treatment**		
Bosmina meridionalis	3.59	2.97	1.94	8.78
Bdelloid spp.	2.57	3.13	1.82	8.24
Mesocyclops spp.	2.50	3.05	1.71	7.76
Polyarthra dolichoptera	4.08	4.54	1.38	6.25
Daphnia galeata	1.58	1.46	1.16	5.25
Keratella slacki	3.12	3.51	1.07	4.85
Copepod nauplii	4.62	5.04	1.03	4.66
Anuraeopsis spp.	0.70	0.75	0.94	4.26
Asplanchna brightwelli	1.60	2.06	0.83	3.76
Filinia longiseta	3.36	3.34	0.83	3.75
Brachionus angularis	1.21	1.48	0.81	3.67
Trichocerca stylata	0.47	0.78	0.74	3.33
Mean dissimilarity = 22.09				

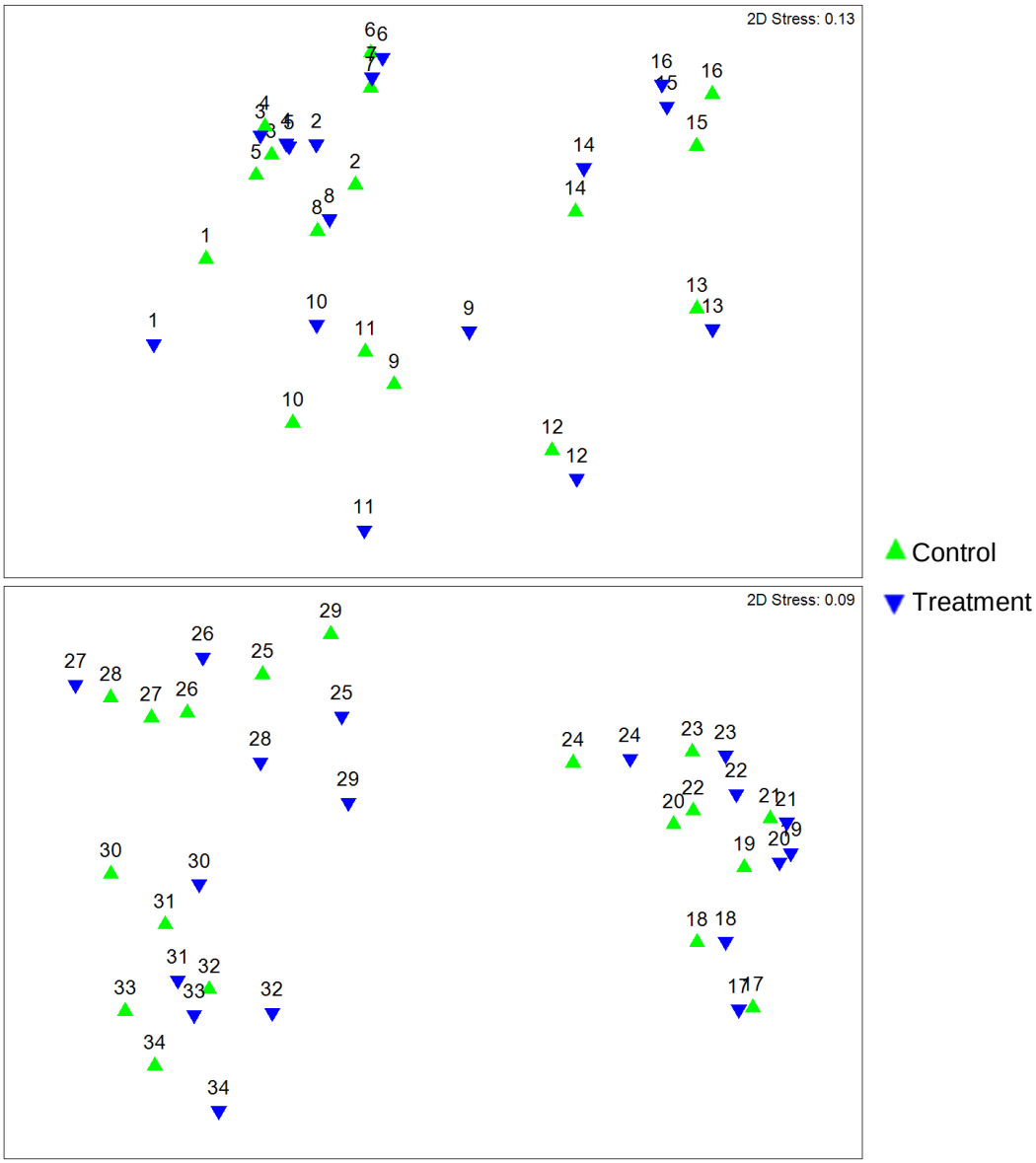


Figure 2.3. MDS plots of zooplankton communities sampled throughout the study. Upper plot represents the 'before' period, while the lower plot represents the 'after' period. Numbers represent sampling dates (1-16; Before, 17-34; After). Each point represents the combined samples from each date.

Table 2.4. Comparison of means and two-way repeated measure ANOVA results between the control and treatment ponds on physical and chemical variables, before and after treatment. Bold text indicates a significant difference.

	BEFORE		**AFTER**	
DO	**Control = 62.597**	Date – D.F. = 15, F = 39.37, P = 0.0000	Control = 58.113	Date – D.F. = 17, F = 23.03, P = 0.0000
	Treatment = 66.824	**Site – D.F. = 1, F = 4.01, P = 0.0487, N = 94**	Treatment = 64.450	Site – D.F. = 1, F = 3.25, P = 0.0750, N = 105
pH	Control = 6.8740	Date – D.F. = 15, F = 49.27, P = 0.0000	**Control = 6.9158**	Date – D.F. = 17, F = 23.23, P = 0.0000
	Treatment = 6.9373	Site – D.F. = 1, F = 3.03, P = 0.0856, N = 94	**Treatment = 7.0235**	**Site – D.F. = 1, F = 8.18, P = 0.0053, N = 105**
spCond	**Control = 198.54**	Date – D.F. = 15, F = 364.94, P = 0.0000	**Control = 205.43**	Date – D.F. = 17, F = 188.96, P = 0.0000
	Treatment = 200.78	**Site – D.F. = 1, F = 14.85, P = 0.0002, N = 94**	**Treatment = 209.67**	**Site – D.F. = 1, F = 15.36, P = 0.0002, N = 105**
Temp	**Control = 16.390**	Date – D.F. = 15, F = 492.36, P = 0.0000	**Control = 17.774**	Date – D.F. = 17, F = 882.49, P = 0.0000
	Treatment = 16.736	**Site – D.F. = 1, F = 12.19, P = 0.0008, N = 94**	**Treatment = 18.266**	**Site – D.F. = 1, F = 43.50, P = 0.0000, N = 105**
TN (mg/L)	Control = 1.1191	Date- D.F. = 11, F=46.64, P=0.0000	**Control = 1.3385**	Date – D.F. = 15, F = 13.34, P = 0.0000
	Treatment = 1.1266	Site - D.F. = 1, F = 0.09, P = 0.7604, N =70	**Treatment = 1.4764**	**Site – D.F. = 1, F = 7.40, P = 0.0080, N = 94**
TP (mg/L)	Control = 0.3240	Date - D.F. = 11, F = 253.28, P = 0.0000	Control = 0.4594	Date – D.F. = 15, F = 55.41, P = 0.0000
	Treatment = 0.3368	Site – D.F. = 1, F = 3.23, P = 0.0774, N = 70	Treatment = 0.4496	Site – D.F. = 1, F = 0.49, P = 0.4840, N = 94
LOGPO_4 (mg/L)	Control = 0.1012	Date – D.F. = 15, F = 16.46, P = 0.0000	Control = 0.4498	Date – D.F. = 15, F = 17.19, P = 0.0000
	Treatment = 0.971	Site – D.F. = 1, F = 0.62, P = 0.4352, N = 94	Treatment = 0.4650	Site – D.F. = 1, F = 0.26, P = 0.6125, N = 94
NH_4 (mg/L)	**Control = 0.1602**	Date – D.F. = 15, F = 36.09, P = 0.0000	**Control = 0.3141**	Date – D.F. = 15, F = 89.03, P = 0.0000
	Treatment = 0.2464	**Site – D.F. = 1, F = 55.25, P = 0.0000, N = 94**	**Treatment = 0.2689**	**Site – D.F. = 1, F = 5.04, P = 0.0276, N = 94**
LOG NO_2 (mg/L)	**Control = 0.00697**	Date – D.F. = 15, F = 58.97, P = 0.0000	**Control = 0.1447**	Date – D.F. = 15, F = 39.39, P = 0.0000
	Treatment = 0.00828	**Site – D.F. = 1, F = 12.56, P = 0.0007, N = 94**	**Treatment = 0.1028**	**Site – D.F. = 1, F = 5.60, P = 0.0204, N = 94**
NO_3 (mg/L)	**Control = 0.6601**	Date – D.F. = 15, F = 348.03, P = 0.0000	**Control = 0.1965**	Date - D.F. = 15, F = 28.07, P = 0.0000,
	Treatment = 0.4935	**Site – D.F. = 1, F = 112.75, P = 0.0000, N = 94**	**Treatment = 0.1439**	**Site – D.F. = 1, F = 13.24, P = 0.005, N = 94**
NO_x (mg/L)	**Control = 0.676**	Date – D.F. = 15, F = 358.90, P = 0.0000	**Control = 0.1291**	Date – D.F. = 15, F = 40.42, P = 0.0000
	Treatment = 0.5129	**Site – D.F. = 1, F = 107.35, P = 0.0000, N = 94**	**Treatment = 0.1006**	**Site – D.F. = 1, F = 4.60, P = 0.0350, N = 94**
ISS	**Control = 5.7199**	Date – D.F. = 15, F = 15.96, P = 0.0000	**Control = 5.1838**	Date – D.F. = 17, F = 14.04, P = 0.0000
	Treatment = 4.2848,	**Site – D.F. = 1, F = 9.33, P = 0.0031, N = 94**	**Treatment = 7.7685**	**Site – D.F. = 1, F = 42.40, P = 0.0000, N = 104**
TSS	Control = 9.9055	Date - D.F. = 15, F=19.07, P=0.0000	**Control = 12.852**	Date- D.F.=17, F=20.36, P=0.0000
	Treatment = 8.8427	Site- D.F. = 1, F = 2.69, P = 0.1048, N = 94	**Treatment = 18.649**	**Site - D.F. = 1, F = 46.51, P = 0.0000, N = 104**
TVS	Control = 4.1833	Date – D.F. = 15, F = 42.23, P = 0.0000	**Control = 7.6647**	Date – D.F. = 17, F = 17.25, P = 0.0000
	Treatment = 4.5579	Site – D.F. = 1, F = 2.22, P = 0.1403, N = 94	**Treatment = 10.869**	**Site – D.F. = 1, F = 23.03, P = 0.0000, N = 104**

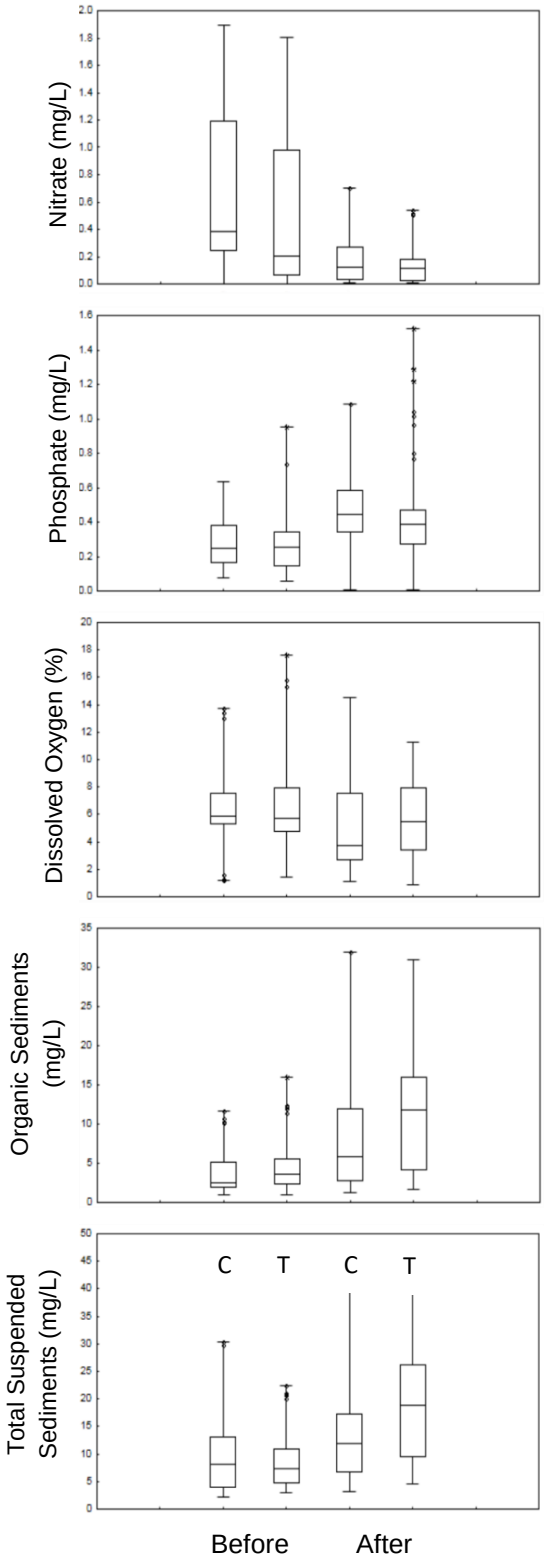


Figure 2.4. Summary of monthly environmental parameters from control and experimental ponds at the Hamilton Zoo. Means and quartiles are presented for the ‘before’ and ‘after’ periods. Whiskers represent the range of values encountered. C represents the Control pond, and T represents the Treatment pond.

Using BIOENV, a BEST analysis was used to determine the parameters that best describe the zooplankton variation. Of the 32 parameters, 7 explained 50.3% of the variation; water temperature, total suspended sediments (TSS), organic suspended sediments (TVS), inorganic suspended sediments (ISS), particle sizes 7.8 µm – 15.6 µm, NH_4, and PO_4. The DistLM analysis (Figure 2.6) showed a clear separation between the 'before' and 'after' periods, but did not indicate a difference between the ponds, before or after treatment. Axis one variables (56.2% of total variation) included TSS, TVS, ISS concentrations, and the 7.8 µm – 15.6 µm particle sizes. The model indicates that the 'after' period had higher concentrations of sediments and more particles in the water column, in both the control and treatment pond. Axis two variables (11.3% of total variation) included water temperature, PO_4 and NH_4. The model suggests that as water temperature increases, the nutrient concentrations in the water column decrease. The marginal tests indicated that TSS ($p = 0.001$), TVS ($p = 0.001$), ISS ($p = 0.007$), and the particle size ($p = 0.012$) were significant factors in shaping the zooplankton community. Water temperature, PO_4, and NH_4 were not significantly different between ponds ($p > 0.05$).

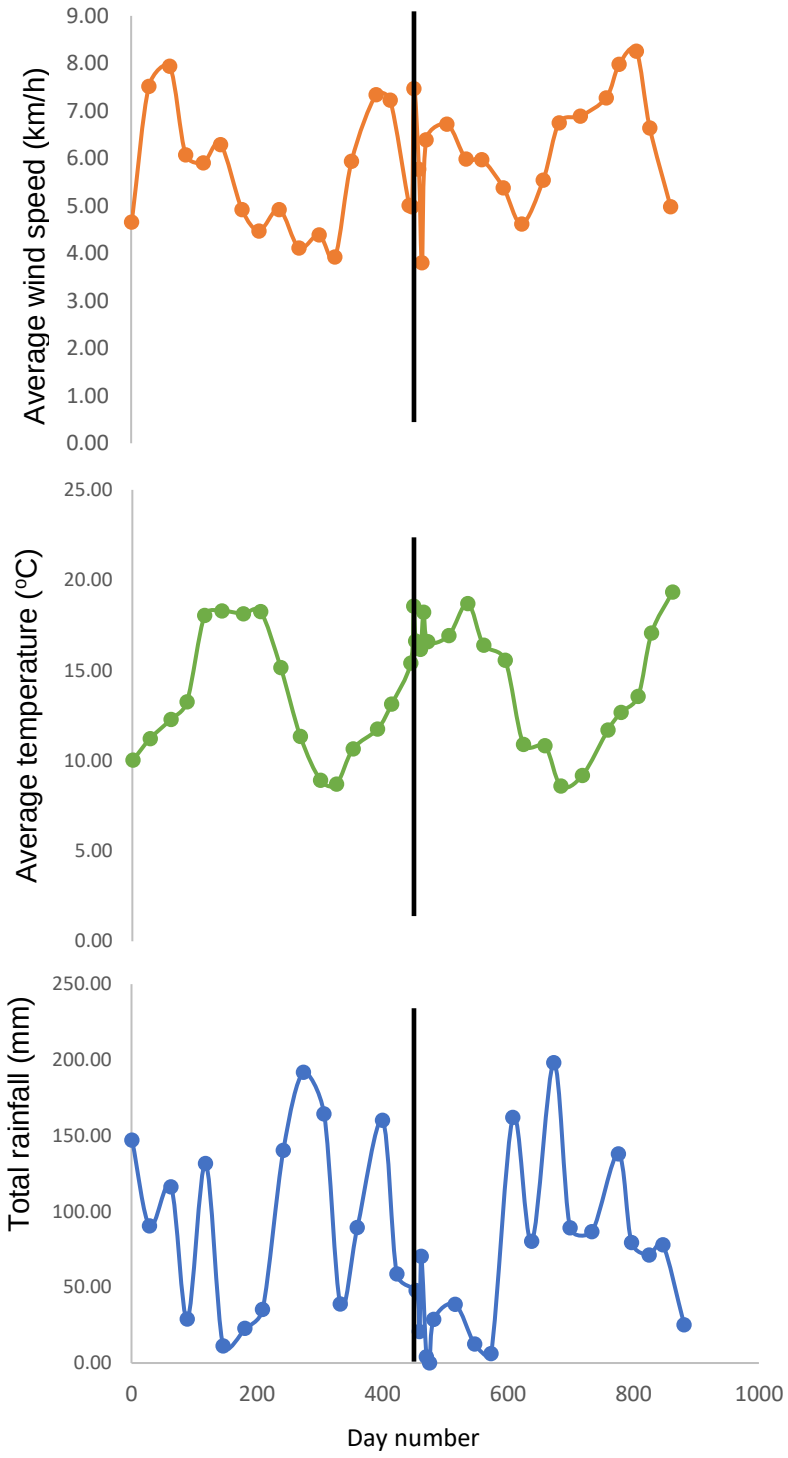


Figure 2.5. Seasonal differences in average wind speed (orange), average temperature (green) and total rainfall (blue). Solid line indicates when treatment occurred.

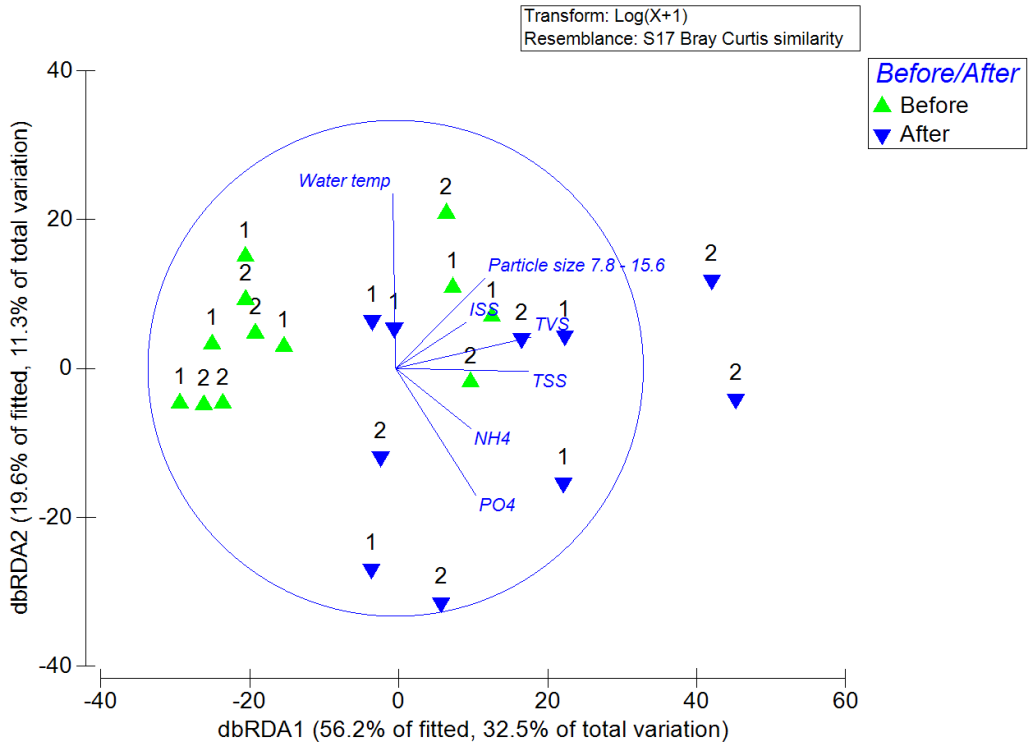


Figure 2.6. DistLM of zooplankton communities in relation to environmental parameters. A BEST analysis was used to determine the variables driving the zooplankton community change. Number 1 represents the control pond, while number 2 represents the treatment pond.

Discussion

Using a “Before-After Control-Impact” (BACI) design, I found evidence that carp increased the inorganic suspended sediments, nitrogen concentrations, as well as increased densities of rotifer and cyclopoid copepod, and decreased densities of cladocerans in the treatment pond ecosystem. However, my results also suggested that, overall, the differences in environmental variables likely had little consequence on the health of the ecosystem, and zooplankton community structure was relatively unchanged following carp addition. The introduced carp biomass of 400 kg ha^{-1} was double the threshold (~ 200 kg ha^{-1}) at which the effects are predicted to occur (Vilizzi et al., 2015). The final biomass (986 kg ha^{-1}) was well above this threshold, yet still within the range found for aquatic habitats elsewhere (e.g. King et al., 1997; Meijer et al., 1990).

The zooplankton taxa commonly found in the control and treatment ponds included the rotifers *Brachionus angularis* and *B. calyciflorus*, cladoceran *Bosmina* spp., and cyclopoid copepods, and have all been previously reported in similarly eutrophic habitats (e.g. Attayde and Bozelli, 1998; Stemberger and Lazorchak, 1994; Angeler et al., 2007; Pinto-Coelho et al., 2005; Sousa et al., 2008). In contrast, taxa associated with more oligotrophic habitats, such as calanoid copepods (Pace, 1986; Pinto-Coelho et al., 2005), were not recorded from my experimental ponds.

While the overall PERMANOVA analysis suggested a significant difference in the zooplankton communities between the ponds, this is likely the result of the power of the statistic, as the post-hoc results found no

significant difference at any sampling period, before or after fish addition. When each individual sampling event are examined there are no significant differences during each of the 34 sampling periods, the significant difference only becomes apparent when all the datasets are combined, suggesting the ecological difference between the ponds is minimal. This is similar to findings of Lougheed et al. (1998), who found no change in the zooplankton community composition in enclosures containing high densities of carp.

The zooplankton communities in both the control and treatment ponds were dominated by rotifers, cyclopoid copepods, and small cladocerans (*Bosmina meridionalis*). This is similar to previous studies that showed that in potentially degraded environments, and in the presence of carp, large cladocerans were typically absent (Angeler et al., 2007; Havens, 1993; Khan et al., 2003; Nieoczym and Kloskowski, 2014). Furthermore, when cladocerans were present in previous studies, they were typically smaller taxa (e.g. Havens, 1993; Tátrai et al., 2005), similar to my findings. Rotifers are not known to be displaced or out-competed by smaller cladocerans such as *Bosmina* spp. (MacIsaac and Gilbert, 1989).

Low densities of *Daphnia* were found throughout the study and were likely the result of unfavourable environmental conditions. Other studies have suggested that *Daphnia* populations can be limited by carp indirectly (e.g. increases in inorganic suspended sediments; Arruda et al., 1983; Kirk, 1991a; 1992; Rellstab and Spaak, 2007). My results suggest that the slight increases in suspended sediments caused by carp addition,

when sediment concentrations are already high, were unlikely to have limited *Daphnia* populations.

Although inorganic sediment loads were slightly, but significantly, higher in the control pond prior to treatment, they were comparatively (and significantly) higher in the treatment pond following carp addition. Organic sediment loads were also significantly higher in the treatment pond following the manipulation. This was expected given that carp can increase the suspended sediment loading in shallow lakes due to their feeding habits disturbing and resuspending benthic sediment (e.g. Akhurst et al., 2012; Angeler et al., 2007; Driver et al., 2005; Nieoczym and Kloskowski, 2014).

TN was significantly higher in the treatment pond after fish addition. Carp are known to increase TN and TP concentrations through their excretions and bioturbations (e.g. Akhurst et al., 2012; Chumchal and Drenner, 2004; Matsuzaki et al., 2007; Wahl et al., 2011). Concentrations decrease when carp are removed from the system (Meijer et al., 1990). Although TP concentrations did increase in the treatment pond following carp addition, the differences were not significant. This was somewhat unexpected as the sediment in the ponds had a high organic content. However, previous studies have also found no relationship between carp presence and TP concentrations (Matsuzaki et al., 2007; Richardson et al., 1990; Roberts et al., 1993).

Algal biomass can increase in the presence of carp, due to increases of nutrients through excretions and release of sediment bound

nutrients (Nieoczym and Kloskowski, 2014; Tátrai et al., 2005). However, I found no evidence to suggest an increase in the algal biomass in the treatment pond relative to the control pond. Indeed, algal biomass was slightly higher in the control pond relative to the treatment pond. I suggest that the ambient nutrient contributions to the ponds were already at such a high level in the ponds that the additional nutrients from the carp did not translate into detectable differences for phytoplankton. Increases in suspended sediments can inhibit phytoplankton production through decreased light penetration (Henley et al., 2000). However, in the ponds Secchi disk readings were similar in the before and after periods, and readings often reached the substrate in both ponds, so this too was unlikely.

In eutrophic systems, one of the predictors of zooplankton community change is the presence or absence of planktivorous fish (Jeppesen et al. 1997). There was no significant difference in the zooplankton community between the control and treatment pond following carp addition, which suggests that the presence of planktivorous carp had little impact on the zooplankton community composition.

The experimental ponds were already devoid of macrophytes prior to commencement of my study and were dominated by rotifer genera, tolerant of highly eutrophic conditions (e.g. *Brachionus* and Keratella spp; Duggan et al., 2001), before and after treatment. The lack of large changes in zooplankton densities and community composition in the treatment pond was perhaps not surprising. As the ponds were already in

a eutrophic state, the introduction of carp may have had minimal influence on overall community structure, despite showing changes in densities of individual species.

Zambrano et al. (2001) and Vilizzi et al. (2015) predicted that there is a biomass threshold at which carp elicit a catastrophic change in the ecosystem. At high densities, carp can initiate a shift from a clear state to a turbid state (e.g. Zambrano and Hinojosa, 1999; Cahn, 1929). If a lake is in the already degraded state, drastic shifts in population dynamics of zooplankton may be difficult to assess. For example, several previous studies have also observed minimal changes in zooplankton community composition in the presence of carp (e.g. Akhurst et al., 2012; Lougheed et al., 1998; Miller and Crowl, 2006; Wahl et al., 2011). These systems may also have already been in an advanced state of degradation. It is possible that major changes in zooplankton communities are only apparent in systems that were previously only ‘moderately’ damaged, or those that are still in the macrophyte-dominated state. The algal dominated state of the ponds suggests that they were already in an advanced stage of degradation. Accordingly, any remediation work involving the removal of carp should first focus on the protection and remediation of terrestrial catchment as well (Weber and Brown, 2009). This may be especially relevant given that shifting a lake from the degraded state back to a macrophyte dominated state requires a considerable effort (Scheffer et al., 2001), particularly when nutrient levels remain high (Tátrai et al., 2005).

Acknowledgments

I am extremely grateful to Stephen Standley, Adrian Peterson and staff at the Hamilton Zoo for allowing access to the zoo ponds and for help with field logistics. I also thank Dan Bowater, Warrick Powrie, Grant Tempero, Adam Daniel, Gwyneth Verkirk, Susie Wood, Barry O'Brien, Blake Hirschi, Diana Huffman, Gljs Riel, Vanessa Cotterill, Stacey Meyer, and Jasmine Whanga for help in the field and/or laboratory.

Literature Cited

Akhurst, D.J., Jones, G.B., Clark, M. and A. Reichelt-Brushett. 2012. Effects of carp, gambusia, and Australian bass on water quality in a subtropical freshwater reservoir. Lake and Reservoir Management, 28(3), 212-223.

Anas, M.U.M, Scott, K.A., Cooper, R.N. and B. Wissel. 2014. Zooplankton communities are good indicators of potential impacts of Athabasca oil sands operations on downwind boreal lakes. Can. J. Fish. Aquat. Sci., 71, 719-732.

Angeler, D.G., Sánchez-Carrillo, S., Rodrigo, M.A., Alvarez-Cobelas, M. and C. Rojo. 2007. Does seston size structure reflect fish-mediated effects on water quality in a degraded semiarid wetland? Environ. Monit. Assess., 125, 9-17.

Arruda, J.A., Marzolf, R. and R.T. Faulk. 1983. The role of suspended sediments in the nutrition of zooplankton in turbid reservoirs. Ecology, 64(5), 1225-1235.

Attayde, J.L. and R. L. Bozelli. 1998. Assessing the indicator properties of zooplankton assemblages to disturbance gradients by canonical correspondence analysis. Can. J. Fish. Aquati. Sci., 55, 1789-1797.

Attayde, J.L. and L.A. Hansson. 2001. The relative importance of fish predation and excretion effects on planktonic communities. Limnology and Oceanography, 46(5), 1001-1012.

Bajer, P.G., Sillivan, G. and P.W. Sorensen. 2009. Effects of a rapidly increasing population of common carp on vegetative cover and waterfowl in a recently restored Midwestern shallow lake. Hydrobiologia, 632, 235-245.

Cahn, A.R. 1929. The effect of carp on a small lake: the carp as a dominant. Ecology, 10(3), 271-274.

Carpenter, S.R. 1996. Microcosm experiments have limited relevance for community and ecosystem ecology. Ecology, 77(3), 677-680.

Carpenter, S.R., Kitchell, J.F., Cottingham, K.L., Schindler, D.E., Christense, D.L., Post, D.M. and N. Voichick. 1996. Chlorophyll variability, nutrient input, and grazing: Evidence from whole-lake experiments. Ecology, 77(3), 725-735.

Chumchal, M.M. and R.W. Drenner. 2004. Interrelationships between phosphorus loading and common carp in the regulation of phytoplankton biomass. Arch. Hydrobiol., 161(2), 147-158.

Clarke, KR, Gorley, RN, 2006. PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth

Crivelli, A.J. 1981. The biology of the common carp, *Cprinus carpio* L. in the Camargue, southern France. Journal of Fish Biology, 18(3), 271-290.

Crivelli, A.J. 1983. The destruction of aquatic vegetation by carp. Hydrobiologia, 106, 37-41.

Driver, P.D., Closs, G.P. and T. Koen. 2005. The effects of size and density of carp (*Cyprinus carpio* L.) on water quality in an experimental pond. Arch. Hydrobiol., 163(1), 117-131.

Duggan, I.C., Green, J.D. and R.J. Shiel. 2001. Distribution of rotifers in North Island, New Zealand, and their potential use as bioindicators of lake trophic state. Hydrobiologia, 446/447, 155-164.

Fowler, E.C. 2006. Spatio-temporal distribution and diversity of zooplankton in zoo ponds relative to water quality parameters. Masters Thesis, University of Waikato, New Zealand. 64 pp.

Gannon, J.E. and R.S. Stemberger. 1978. Zooplankton (especially crustaceans and rotifers) as indicators of water quality. Transactions of the American Microscopical Society, 97(1), 16-35.

Gilbert, J.J. 1990. Differential effects of *Anabaena affinis* on cladocerans and rotifers: mechanisms and implications. Ecology, 71(5), 1727-1740.

Haberman, J. and M. Haldna. 2014. Indices of zooplankton community as valuable tools in assessing the trophic state and water quality of eutrophic lakes: long term study of Lake Võrtsjärv. J. Limnol., 73(2), 263-273.

Hansson, L.A., Bergman, E. and G. Cronberg. 1998. Size structure and succession in phytoplankton communities: The impact of interactions between herbivory and predation. Oikos, 81(2), 337-345.

Havens, K.E. 1993. Responses to experimental fish manipulations in a shallow, hypereutrophic lake: the relative importance of benthic nutrient recycling and trophic cascade. Hydrobiologia, 254, 73-80.

Henley, W.F., Patterson, M.A., Neves, R.J. and A.D. Lemly. 2000. Effects of sedimentation and turbidity on lotic food webs: A concise review for natural resource manages. Reviews in Fisheries Science, 8(2), 125-139.

Hicks, B.J., Ling, N., Osborne, M.W., Bell, D.G. and C.A. Ring. 2005. Boat electrofishing survey of the lower Waikato River and its tributaries. CBER Contract Report 39. Centre for Biodiversity and Ecology Research, University of Waikato, New Zealand, 17pp.

Jeppesen, E., Lauridesen, T., Mitchell, S.F. and C.W. Burns. 1997. Do planktivorous fish structure the zooplankton communities in New Zealand lakes? New Zealand Journal of Marine and Freshwater Research, 31, 163-173.

Jeppesen, E., Nõges, P., Davidson, T.A., Haberman, J., Nõges, T., Blank, K., Lauridsen, T.L., Søndergaard, M., Sayer, C., Laugaste, R., Johansson, L.S., Bjerring, R. and S.L. Amsinck. 2011. Zooplankton as indicators in lakes: a scientific-based plea for including zooplankton in the ecological quality assessment of lakes according to the European Water Framework Directive (WFD). Hydrobiologia, 676, 279-297.

Khan, T.A., Wilson, M.E. and M.T. Khan. 2003. Evidence for invasive carp mediated trophic cascade in shallow lakes of western Victoria Australia. Hydrobiologia, 509-509, 465-472.

King, A.J., Robertson, A.L. and M.R. Healey. 1997. Experimental manipulations of the biomass of introduced carp (*Cyprinus carpio*) in billabongs. 1. Impacts on water-column properties. Mar. Freshwater Res., 48, 435-443.

Kirk, K.L. and J.J. Gilbert. 1990. Suspended clay and the population dynamics of planktonic rotifers and cladocerans. Ecology, 71(5), 1741-1755.

Kirk, K.L. 1991a. Suspended clay reduces *Daphnia* feeding rate: behavioural mechanisms. Freshwater Biology, 25, 357-365.

Kirk, K.L. 1991b. Inorganic particles alter competition in grazing plankton: the role of selective feeding. Ecology, 72(3), 915-923.

Kirk, K.L. 1992. Effects of suspended clay on *Daphnia* body growth and fitness. Freshwater Biology, 28, 103-109.

Lougheed, V.L. and P. Chow-Fraser. 1998. Factors that regulate the zooplankton community structure of a turbid, hypereutrophic Great Lakes wetland. Can. J. Fish. Aquat. Sci., 55, 150-161.

Lougheed, V.L., Crosbie, B. and P. Chow-Fraser. 1998. Predictions on the effect of common carp (*Cyprinus carpio*) exclusion on water quality,

zooplankton, and submergent macrophytesin a Great Lakes wetland. Can. J. Fish. Aquat. Sci., 55, 1189-1197.

Lowe, S., Browne, M., Boudjelas, S. and M. De Poorter. 2000. 100 of the world's worst invasive alien species. A selection from the Global Invasive Species Database. The Invasive Species Specialist Group, 12 pp.

MacIsaac, H.J. and J.J. Gilbert. 1989. Competition between rotifers and cladocerans of different body sizes. Oecologia, 81(3), 295-301.

Matsuzaki, S.S., Usio, N., Takamura, N. and I. Washitani. 2007. Effects of common carp on nutrient dynamics and littoral community composition: roles of excretion and bioturbation. Archiv für Hydrobiology, 168(1), 27-38.

McArdle, B.H. and M.J. Anderson. 2001. Fitting multivariate models to community data: a comment on distance-based redundancy analysis. Ecology, 82, 290-297.

Meijer, M.-L., de Haan, M.W., Breukelaar, A.W. and H. Buiteveld. 1990. Is reduction of the benthivorous fish an important cause of high transparency following biomanipulation in shallow lakes? Hydrobiologia, 200/201, 303-315.

Miller, S.A. and T.A. Crowl. 2006. Effects of common carp (*Cyprinus carpio*) on macrophytes and invertebrate communities in a shallow lake. Freshwater Biology, 51, 85-94.

Nieoczym, M. and J. Kloskowski. 2014. The role of body size in the impact of common carp *Cyprinus carpio* on water quality, zooplankton, and macrobenthos in ponds. International Review of Hydrobiology, 99, 212-221.

Pace, M.L. 1986. An empirical analysis of zooplankton community size structure across lake trophic gradients. Limnol. Oceanogr., 31(1), 43-55.

Pace, M.L., Cole, J.J. and S.R. Carpenter. 1998. Trophic cascades and compensation: Differential responses of microzooplankton in whole-lake experiments. Ecology, 79(1), 138-152.

Parkos, J.J., Santucci, V.J. and D.H. Wahl. 2003. Effects of adult common carp (*Cyprinus carpio*) on multiple trophic levels in shallow mesocosms. Can. J. Fish. Aquat. Sci., 60, 182-192.

Pinto-Coelho, R., Pinel-Alloul, B., Méthot, G. and K.E. Havens. 2005. Crustacean zooplankton in lakes and reservoirs of temperate and tropical regions: variation with trophic status. Can. J. Fish. Aquat. Sci., 62, 348-361.

Rellstab, C. and P. Spaak. 2007. Starving with a full gut? Effect of suspended particles on the fitness of *Daphnia hyaline*. Hydrobiologia, 594, 131-139.

Richardson, W.B., Wickham, S.A. and S.T. Threlkeld. 1990. Foodweb response to the experimental manipulation of a benthivore

(*Cyprinus carpio*), zooplanktivore (*Menidia beryllina*) and benthic insects. Arch. Hydrobiol., 119(2), 143-165.

Roberts, J., Chick, A., Oswald, L. and P. Thompson. 1993. Effect of carp, *Cyprinus carpio* L., an exotic benthivorous fish, on aquatic plants and water quality in experimental ponds. Mar. Freshwater Res., 46, 1171-1180.

Scheffer, M., Hosper, S.H, Meijer, M.L. and B. Moss. 1993. Alternative equilibria in shallow lakes. Trends Ecol. Evol. 8, 275-279.

Scheffer, M., Carpenter, S., Foley, J.A., Folkes, C. and B. Walker. 2001. Catastrophic shifts in ecosystems. Nature, 413, 591-596.

Schindler, D.W. 1998. Whole-ecosystem experiments: Replication versus realism: The need for ecosystem-scale experiments. Ecosystems, 1(4), 323-334.

Scott, W.B. and E.J. Crossman. 1973. Freshwater fishes of Canada. Bulletin 184. Fisheries Research Board of Canada, 966 pp.

Shapiro, J., V. Lamarra, and M. Lynch. 1975. Biomanipulation: an ecosystem approach to lake restoration. Pages 85-96 in P. L. Brezonik and J. L. Fox, editors. Proceedings of a Symposium on Water Quality Management Through Biological Control. University of Florida, Gainesville, Florida, USA.

Shapiro, J. and D.I. Wright. 1984. Lake restoration by biomanipulation: Round Lake, Minnesota, the first two years. Freshwater Biology. 14(4), 371-383.

Shiel, R.J. 1995. A guide to identification of rotifers, cladocerans and copepods from Australian inland waters. Cooperative Research Centre for Freshwater Ecology identification guide 3. 144 pp.

Sousa, W., Attayde, J.L., Da Silva Rocha, E. and E.M. Eskinazi-Sant'anna. 2008. The response of zooplankton assemblages to variations in the water quality of four man-made lakes in semi-arid northeastern Brazil. Journal of Plankton Research, 30(6), 699-708.

Stemberger, R.S. and J.M. Lazorchak. 1994. Zooplankton assemblage responses to disturbance gradients. Can. J. Fish. Aquat. Sci., 51, 2435-2447.

Stewart-Oaten, A., Murdoch, W.W. and K.R. Parker. 1986. Environmental impact assessment: "Pseudoreplication" in time? Ecology, 67(4), 929-940.

Swee, U.B. and H.R. McCrommon. 1966. Reproductive biology of the Carp, *Cyprinus carpio* L., in Lake St. Lawrence, Ontario. Transactions of the American Fisheries Society, 95(4), 372-380.

Tátrai, I., Mátyás, K., Korponai, J., Szabó, G., Pomogyi, P. and J. Héri. 2005. Response of nutrients, plankton communities and macrophytes to fish manipulation in a small eutrophic wetland lake. Internat. Rev. Hydrobiol., 90(5-6), 511-522.

Tempero, G.W., Ling, N., Hicks, B.J. and M.W. Osborne. 2006. Age composition, growth, and reproduction of koi carp (*Cyprinus carpio*) in the lower Waikato region, New Zealand. New Zealand Journal of Marine and Freshwater Research, 40(4), 571-583.

Vilizzi, L., Tarkan, A.S. and G.H. Copp. 2015. Experimental evidence from causal criteria analysis for the effects of common carp *Cyprinus carpio* on freshwater ecosystems: a global perspective. Reviews in Fisheries Science and Aquaculture, 23, 253-290.

Wahl, D.H., Wolfe, M.D., Santucci Jr., V.J. and J.A. Freedman. 2011. Invasive carp and prey community composition disrupt trophic cascades in eutrophic ponds. Hydrobiologia, 678, 49-63.

Weber, M.J. and M.L. Brown. 2009. Effects of common carp on aquatic ecosystems 80 years after “Carp as a dominant’: Ecological insights for fisheries management. Reviews in Fisheries Science, 17(4), 524-537.

Zambrano, L. and D. Hinojosa. 1999. Direct and indirect effects of carp (*Cyprinus carpio* L.) on macrophyte and benthic communities in experimental shallow ponds in central Mexico. Hydrobiologia, 408/409, 131-138.

Supplementary Information

Supplementary Table 2.1. Mean density (individuals/L) of identified zooplankton from monthly sampling in both the control and treatment ponds from 2013 to 2015. ** Represents species that occurred in low numbers (<3 individuals) and removed from analysis.

	Control Before	Treatment Before	Control After	Treatment After
Rotifera				
Anuraeopsis sp.	0.55	1.59	6.04	12.45
Asplanchna brightwellii (Gosse, 1850)	5.52	20.59	17.92	38.42
Bdelloid sp.	5.85	11.77	35.49	90.68
Brachionus angularis (Gosse, 1851)	24.75	57.80	27.00	65.22
Brachionus budapestinensis (Daday, 1885)	0.00	0.06	1.51	6.40
Brachionus calyciflorus (Pallas, 1766)	37.70	28.19	142.57	206.91
Brachionus urceolaris (Müller, 1773)	3.96	3.58	0.14	1.16
Calamoecia lucasi (Brady, 1906)	0.00	0.15	0.00	0.07
Cephalodella catellina (Müller, 1786)	0.00	0.02	0.00	0.00
Cephalodella forficula (Ehrenberg, 1832)	0.26	0.16	0.29	0.23
Cephalodella gibba (Ehrenberg, 1830)	0.09	0.12	0.00	0.22
Cephalodella ventripes (Dixon-Nuttall, 1901)	1.67	2.77	0.24	1.03
Colurella uncinata (Müller, 1773)	0.02	0.02	0.00	0.00
Dicranophoroides caudatus (Ehrenberg, 1834)	0.07	0.03	0.02	2.07
Epiphanes macrourus (Barrois & Daday, 1894)	0.00	0.00	3.37	5.22
Euchlanis incisa (Carlin, 1939)	**	**	**	**
Euchlanis pyriformis (Gosse, 1851)	**	**	**	**
Euclanis deflexa (Gosse, 1851)	0.06	0.22	0.00	0.00
Filinia longiseta (Ehrenberg, 1834)	42.03	16.68	415.12	708.29
Filinia terminalis (Plate, 1886)	**	**	**	**
Gastropus hyptopus (Ehrenberg, 1838)	83.57	62.51	69.09	69.31
Itura myersi (Wulfert, 1935)	**	**	**	**
Keratella cochlearis (Gosse, 1851)	**	**	**	**
Keratella slacki (Berzins, 1963)	219.01	426.49	353.35	581.03
Keratella tecta (Gosse, 1851)	**	**	**	**
Keratella tropica (Apstein, 1907)	**	**	**	**
Lecane bulla (Gosse, 1851)	1.49	0.21	0.75	0.96
Lecane closterocerca (Schmarda, 1859)	0.54	0.59	0.85	0.50
Lecane flexilis (Gosse, 1886)	0.41	0.11	0.08	0.00
Lecane furcata (Murray, 1913)	0.08	0.04	0.00	0.00
Lecane inermis (Bryce, 1892)	**	**	**	**
Lecane luna (Müller, 1776)	0.30	0.27	0.06	0.11
Lecane lunaris (Ehrenberg, 1832)	0.18	0.20	0.10	0.00
Lepadella ovalis (Müller, 1786)	0.45	0.42	0.55	0.36
Lophocharis salpina (Ehrenberg, 1838)	0.02	0.01	0.00	0.00
Monommata sp.	0.06	0.00	0.00	0.00
Mytilina bisulcata (Lucks, 1912)	0.03	0.01	0.00	0.03
Platyias quadricornis (Ehrenberg, 1832)	0.17	0.09	0.28	0.29
Pleurotrocha petromyzon (Ehrenberg, 1830)	0.91	1.57	0.09	0.14

Polyarthra dolicoptera (Idelson, 1925)	25.35	27.68	587.47	901.79
Proales sp.	0.17	0.16	0.10	0.00
Squatinella mutica (Ehrenberg, 1832)	0.19	0.02	0.02	0.00
Synchaeta oblonga (Ehrenberg, 1832)	2.04	1.06	2.39	1.19
Synchaeta pectinata (Ehrenberg, 1832)	16.87	4.25	4.97	1.91
Synchaeta stylata (Wierzejski, 1893)	0.01	0.08	0.00	0.00
Testudinella patina (Hermann, 1783)	0.07	0.04	0.01	0.00
Trichocerca pusilla (Jennings, 1903)	**	**	**	**
Trichocerca similis (Wierzejski, 1893)	31.80	36.41	17.09	27.53
Trichocerca stylata (Gosse, 1851)	0.81	3.36	4.05	13.86
Trichotria tetractis (Ehrenberg, 1830)	0.09	0.21	0.00	0.00
Cladocera				
Alona c.f. *affinis* (Leydig, 1860)	0.40	0.34	0.17	0.22
Bosmina meridionalis (Sars, 1904)	0.21	0.16	464.83	195.92
Chydorus sp.	0.34	0.33	0.00	0.00
Daphnia galeata (Sars, 1863)	36.84	55.31	40.58	47.00
Copepoda				
Mesocyclops sp.	11.95	27.80	22.72	46.38
Copepod nauplii	135.88	167.49	126.04	194.44
Harpacticoid nauplii	0.29	0.74	0.00	0.08
Ostracods				
Ostracod sp.	0.26	0.63	0.88	0.58

CHAPTER 3: TESTING THE POTENTIAL OF SHORT 28S RIBOSOMAL DNA SEQUENCES FOR ROUTINE IDENTIFICATION OF ZOOPLANKTON SPECIES

*to be published under the same title as: Woods, S., Hogg, I.D., Duggan, I.C., Banks, J.C.

Abstract

Molecular barcoding is a promising tool to aid in the identification of zooplankton taxa, in combination with trained taxonomists. However, obtaining sequences for zooplankton using standard barcodes (*COI*) is difficult, often requiring multiple primers to successfully amplify and sequence all taxa. I tested the use of a slower evolving, nuclear DNA region (28S) to determine the suitability of this region for use in a metabarcoding study. Zooplankton were collected from a small pond in the Waikato region of New Zealand, morphologically identified and sequenced. Sequence success was high (79%), and support was generally high at the species level (Maximum Likelihood bootstrap >0.70) for rotifer, cladoceran, and copepod taxa. Taxa with low support typically had only one specimen represented. Results from the copepod sequences revealed a population undetected in the morphological counts, supporting the usefulness of a DNA based approach to identify morphological similar taxa (e.g. copepod nauplii). Of the three typical approaches to delineate OTU's (ABGD, PTP, GMYC), ABGD was most accurate when compared with the morphological identifications. I conclude that the 28S region is a suitable marker to use in a metabarcoding study aimed to identify full communities of zooplankton.

Introduction

Zooplankton are key components of freshwater ecosystems providing a trophic link between phytoplankton and the higher trophic levels which feed on them (Carpenter et al., 1985). Zooplankton can also provide a useful indicator of water quality owing to the variable tolerances of taxa to a range of environmental conditions (Jeppesen et al., 2011). Unfortunately, identification of zooplankton can be problematic and often relies on a high level of taxonomic expertise, especially when similar and/or cryptic species are involved (McManus and Katz, 2009). There is also a shortage of well-trained experts able to undertake accurate identifications of zooplankton taxa (Boero, 2001).

The shortage of taxonomists has been at least partially addressed through use of molecular methods to assist with the routine identification of taxa (e.g. Hebert and Beaton, 1989). The more recent development of DNA barcodes (*sensu* Hebert et al., 2003) has shown much promise for accurately identifying a range of zooplankton taxa. For example, short fragments (658 bp) of the cytochrome c oxidase subunit 1 (*COI*) gene have been useful in identifying taxa and revealing cryptic species diversity in several groups including the Rotifera (Fontaneto, 2014), Cladocera (Costa et al., 2006; Elías-Gutiérrez et al., 2008; Petrusek et al., 2004), and Copepoda (Elías-Gutiérrez et al., 2008). However, obtaining sequences for *COI* from a single set of primers can be difficult because as divergence among the study taxa increases, there is increased probability that

substitutions have occurred in the primer binding regions resulting in PCR failure.

Failed PCR amplification can be particularly problematic for so-called “metabarcoding” approaches which seek to identify the full range of taxa within a given sample or habitat using their DNA barcodes alone. Zooplankton are a relatively diverse; thus, the successful amplification of the *COI* barcode region can be difficult for zooplankton, necessitating the use of multiple primers and or combinations of primers to obtain sequences for all taxa (Bucklin et al., 2010). Universal primers to produce *COI* barcodes for metabarcoding of divergent taxa such as zooplankton are not yet available (Taberlet et al., 2012). Until primer failure is resolved, an option is to use a conserved genetic marker where primer sites are less likely to contain substitutions leading to primer failure, and thus provide a higher sequencing success rate. This approach could also be used in conjunction with additional genetic markers, where available, to improve accuracy.

One possible solution to reduce the risk of failing to detect taxa from primer failure, and thus wrongly estimating abundance, is to amplify ribosomal DNA (rDNA) which often contains conserved regions suitable for the design of universal primers, while still containing enough diversity to distinguish species. However, these variable sections need to be tested to determine if there is enough variability to discriminate among taxa. Previous studies have trialed gene regions including 18S and 28S rDNA. The 18S gene has been used with some success in zooplankton (Abad et

al., 2016; de Vargas et al., 2015; Pearman and Irigoien, 2015). However, multiple primer pairs have been required to cover the full range of taxa (Pearman et al., 2014), and the level of variation needed to discriminate Operational Taxonomic Units (OTU's) may vary among different taxonomic groups (Brown et al., 2015). Although universal primers exist for both 18S and 28S that may be suitable for metabarcoding approaches (Machida and Knowlton, 2012), these have been criticized as they may underestimate the true diversity of taxa particularly when dealing with cryptic species due to a lack of genetic differences among closely related species (Machida and Tsuda, 2010; Tang et al., 2012).

A promising alternative is the large subunit (LSU) of the ribosomal 28S gene, specifically the D1-D3 regions. These regions have been found to have enough sequence diversity to discriminate between closely related taxa (De Ley et al., 1999; Hirai et al., 2013; Sonnenberg et al., 2007), including detecting cryptic species (De Ley et al., 2005). Here, I test the use of the D1 region of the LSU to discriminate taxa of copepods, cladocerans, and rotifers, and to assess species-level identifications. My study focused on obtaining sequence data for all species found in a small lentic (pond) habitat to build a reference library for potential use in metabarcoding studies. These data were further compared to morphological data to determine if the obtained sequences would accurately reflect the composition of taxa within the targeted habitat.

Methods

Zooplankton samples were collected monthly from ponds at the Hamilton Zoo, Hamilton, New Zealand (37^{o} 46', 27"S, 175^{o} 12' 51"E) between August 2012 and November 2014. Samples were collected using a PVC pipe, (100mm X 720mm) which was filled with pond water and the contents filtered through a 37 µm mesh. To capture other zooplankton species, that may have been present in the vicinity of the ponds, samples were also collected from nearby ponds within the Waikato region of New Zealand. For these additional locations, a 37 µm plankton net was deployed by hand from the shore and towed at a rate of 1m s^{-1}, just below the water surface. All samples were stored in 70% ethanol for later sorting and processing in the laboratory. Zooplankton individuals were picked, identified, separated, photographed, and stored in 70% ethanol at 4^{o}C prior to processing for DNA analyses. I aimed to collect at least three individuals of each morphological species (taken from different samples/sites) to determine any intraspecific variability as well as to minimize any issues with contamination of specimens.

Each specimen was photographed, and DNA was extracted from individual specimens using the Red Extract n Amp (Sigma-Aldrich, St. Louis, MO) kit by placing the whole specimen into 10 µL of the DNA extraction solution, and 2.5 µL tissue preparation solution. Following three hours of incubation at room temperature, 10 µL of the provided neutralising solution was added to stop the extraction. The primers 300R (CAACTTTCCCTCACGGTACTTG) and F63.2

(ACCCGCTGAAYTTAAGCATAT) were used to target the 28S, D1 loop region of our specimens. PCR reactions were carried out with 10 µL PCR mastermix solution (i-Taq™ containing DNA polymerase, dNTPs, PCR reaction buffer and gel loading buffer), 0.5 µL each primer (10 µMol/L), 2 µL of DNA extract and 7 µL deionised H_2O. The thermal cycling profile consisted of three minutes denaturation at 94ºC, followed by 40 cycles of 94ºC for 30 seconds, 52ºC for 30 seconds, and 72ºC for 30 seconds, with a final extension step of 72ºC for five minutes. PCR products (5 µL) were purified using 1 µL EXO I enzyme, 1 µL shrimp alkaline phosphatase (SAP) (Exo-SAP-IT, Affymetrix, Santa Clara, CA), as per the manufacturer's guidelines. Sequencing was performed on an ABI 3130XL DNA sequencer (Applied Biosystems Inc., Foster City, CA). Sequences were manually edited using Geneious (ver. 4.8.4, Biomatters, Auckland, New Zealand; Kearse et al., 2012) and verified as being zooplankton using the nBLAST algorithm (Altschul et al., 1990) in GenBank.

All unique sequences (identical sequences removed) were used in pair-wise distance analyses and chi-square tests for homogeneity. PAUP* (4.0b10; Swofford, 2003) was used to determine the homogeneity of the nucleotides for comparison with previous studies. A corrected pair-wise distance matrix was constructed using the Kimura 2-paramater distance model (K2P; Kimura, 1980) in MEGA v5.05 to determine the levels of intra- and inter-specific distances. Pairwise distances were not analysed for copepods or cladocerans due to the low number of species of these taxa (<10 species in both cases).

Sequence data were separated into their respective taxa (copepod, cladoceran, rotifer) and aligned using the MUSCLE alignment tool in Geneious (Edgar, 2004). A maximum likelihood (ML) analysis was conducted for each of the three zooplankton groups. JModelTest v.2.1.1 (Darriba et al., 2012; Guindon and Gascuel, 2003) was used to determine the best model, based on the Akaike Information Criterion (AICc), corrected for finite sample sizes. Trees were generated with 1000 bootstrap replicates in MEGA v5.05 (Tamura et al., 2011). Copepods used a General Time Reversible with invariant sites (GTR + I) models. Rotifers and cladocerans were analysed using a GTR model with Gamma distributed data.

From the sequence data, OTU's were determined using three different methods. The Automatic Barcode Gap Discovery Analysis (ABGD; Puillandre et al., 2012) used pairwise distances, grouping organisms when the intraspecific divergence values are less than the interspecific divergence values. I used a relative gap distance of 0.5, and an imported corrected pair-wise distance matrix based on a Jukes-Cantor model (JC69; Jukes and Cantor, 1969). The second model, the Poisson Tree Process (PTP; Zhang et al., 2013) used the number of substitutions in the sequence to determine OTU’s. A rooted, maximum likelihood tree with the outgroup *Rotaria neptunia* was used. Finally, the Generalized Mixed Yule Coalescent (GMYC) used a Yule model, assuming a constant speciation rate and no extinction (Pons et al., 2006), and distinguished OTU’s based on branch lengths. An ultrametric Bayesian tree was created in BEAUti and BEAST (Drummond et al., 2012) with a GTR model and

Gamma distributed sites. A relaxed clock was used with Yule process speciation, and a burn in rate of 10%. Analysis was conducted in R, using the Splits package (Ezard et al., 2009) and a single threshold model to reduce the risk of over-splitting species as recommended by Fujisawa and Barraclough (2013). Sequences and associated metadata were uploaded to the BOLD database (http://v3.boldsystems.org/).

Results

336 zooplankton individuals were sequenced from pond habitats at the Hamilton Zoo and vicinity, including 60 morphologically identified species from 19 families of rotifers; nine identified cladoceran taxa from four families; and seven identified copepod taxa covering calanoids, cyclopoids, and harpactacoids. Of these, I obtained 264 successful sequences (78.6% success rate), for all the common rotifer, copepod, and cladoceran species found in our ponds. A pilot study was also conducted on 20 species of rotifers comparing results between *COI* and 28S. The sequence success rate for 28S was 90% and the success rate for *COI* was 60%.

Rotifers

A 356 bp portion of the D1 region of the 28S rDNA gene was sequenced. 103 haplotypes were found that differed by at least one nucleotide in the 60 species examined. The nucleotide composition of the region showed an A-G bias (A = 27.9%, C = 20.7%, G = 26.1%, T = 25.3%). Base frequencies were not significantly different across all sites (χ^2 = 106.89, P = 1.00), and across the informative sites (χ^2 = 174.81, P = 1.00). The mean between-family divergence across all rotifer taxa was 3.4% (Table 3.1). Sequence divergences among species within families ranged from 0 - 15.8% (mean = 3.6%) and sequence divergences within-species ranged from 0 - 2.6% (mean = 0.8%).

The maximum likelihood analysis delineated two rotifer orders identified from the morphological analysis (Figure 3.1); Ploima (13

families), and Flosculariaeceae (4 families), with bootstrap values >0.95. Bootstrap support at the family level, was generally lower, with only Lecanidae, Hexarthridae, Trochosphaeridae, Asplanchnidae, Collothecidae, Conochilidae, Gastropodidae, and Epiphanidae (>0.70) and all others <0.70. At the species level, bootstrap support was generally high with values >0.70 for 43 of the 60 morphologically identified species. Of those species with bootstrap support <0.70, nine were represented by a single sequence each (Fig. 3.2). Despite the low bootstrap support values, all morphologically identified species were clearly delineated on the ML tree (Fig. 3.2).

Based on the Automatic Barcode Gap Discovery (ABDG) OTU analysis, there were 75 groups (p=<0.01), covering the 60 morphologically identified species. Two OTU's were found for each of *Brachionus angularis*, *Brachionus quadridentatus*, *Cephalodella forficula*, *Cephalodella gibba*, *Cephalodella ventripes*, *Dicranophorus caudatus*, *Euchlanis meneta*, *Filinia longiseta*, *Hexarthra mira*, *Lecane flexilis*, *Lepadella ovalis*, *Polyarthra dolicoptera*, and *Testudinella patina.* In contrast, the Poisson Tree Processes (PTP) analysis produced only 37 OTU's. These OTU's were mostly grouped at the family level. For example, group 1 included *Brachionus calyciflorus*, *B. quadridentatus*, *B. angularis*, *B. urceolaris, Plationus patulus, Platyais quadricornis* and *Epiphanes macrourus*, members of the Brachionidae and Epiphanidae families. The GMYC model revealed 71 OTU's using the single threshold method (p > 0.05). Multiple OTUs were found for five of the 60 morphologically identified species (*Brachionus angularis*, *Cephalodella ventripes*, *Cephalodella gibba*,

Dicranophorus caudatus, and *Euchlanis meneta*). However, grouping of taxa was also found. For example, *Trichocerca tigris* and *Trichocerca longiseta*, *Lecane flexilis* and *Lecane decepiens*, and *Keratella slacki* and *Keratella tropica* were grouped together to produce single OTUs.

Table 3.1. Minimum, maximum and mean between species corrected pairwise distances in families of rotifers and cladocerans sequenced, using unique sequences. N/C represents families with too few individuals for the comparisons.

Family	Minimum	Maximum	Mean	Number of sequences
Rotifers				
Asplanchnidae	0.00%	4.83%	2.41%	6
Atrochidae	N/C	N/C	N/C	1
Brachionidae	0.00%	10.25%	5.89%	49
Collothecidae	N/C	N/C	N/C	1
Conochilidae	0.00%	7.5%	3.77%	7
Dicranophoridae	0.32%	9.40%	5.14%	6
Epiphanidae	0.00%	0.31%	0.16%	4
Euchlanidae	0.00%	4.85%	2.75%	11
Gastropodidae	0.00%	0.00%	0.00%	3
Hexarthridae	0.00%	2.53%	1.26%	6
Lecanidae	0.00%	6.98%	4.15%	19
Lepadellidae	0.00%	14.01%	6.69%	10
Notommatidae	0.00%	15.81%	7.93%	15
Philodinidae	0.62%	4.46%	3.18%	5
Synchaetidae	0.00%	8.98%	4.53%	11
Testudinellidae	0.00%	6.16%	3.68%	7
Trichocercidae	0.00%	5.99%	3.60%	19
Trichotriidae	0.00%	0.31%	0.16%	3
Trochosphaeridae	0.00%	4.02%	2.18%	8
Cladocerans				
Bosminidae	0.03%	7.67%	6.12%	10
Sididae	N/C	N/C	N/C	1
Chydoridae	0.00%	8.04%	7.49%	12
Daphnidae	0.00%	6.63%	3.05%	17

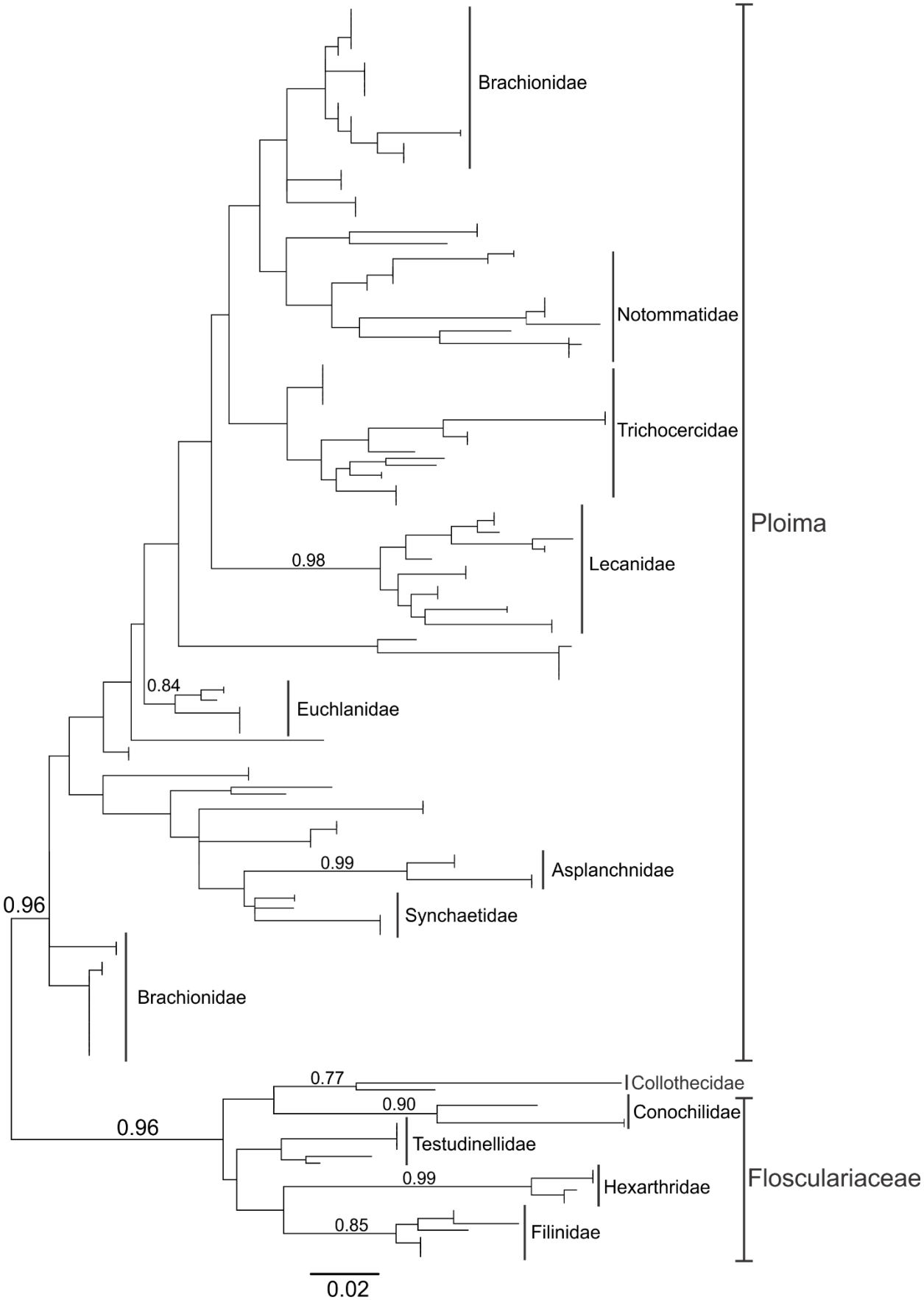


Figure 3.1. A phylogeny for rotifers estimated using maximum likelihood. The solid bars in the tree identify the families, and the solid bars on the right identify the orders. Samples were collected between 2012 and 2014 at the Hamilton Zoo and surrounding area. Numbers represent the bootstrap values.

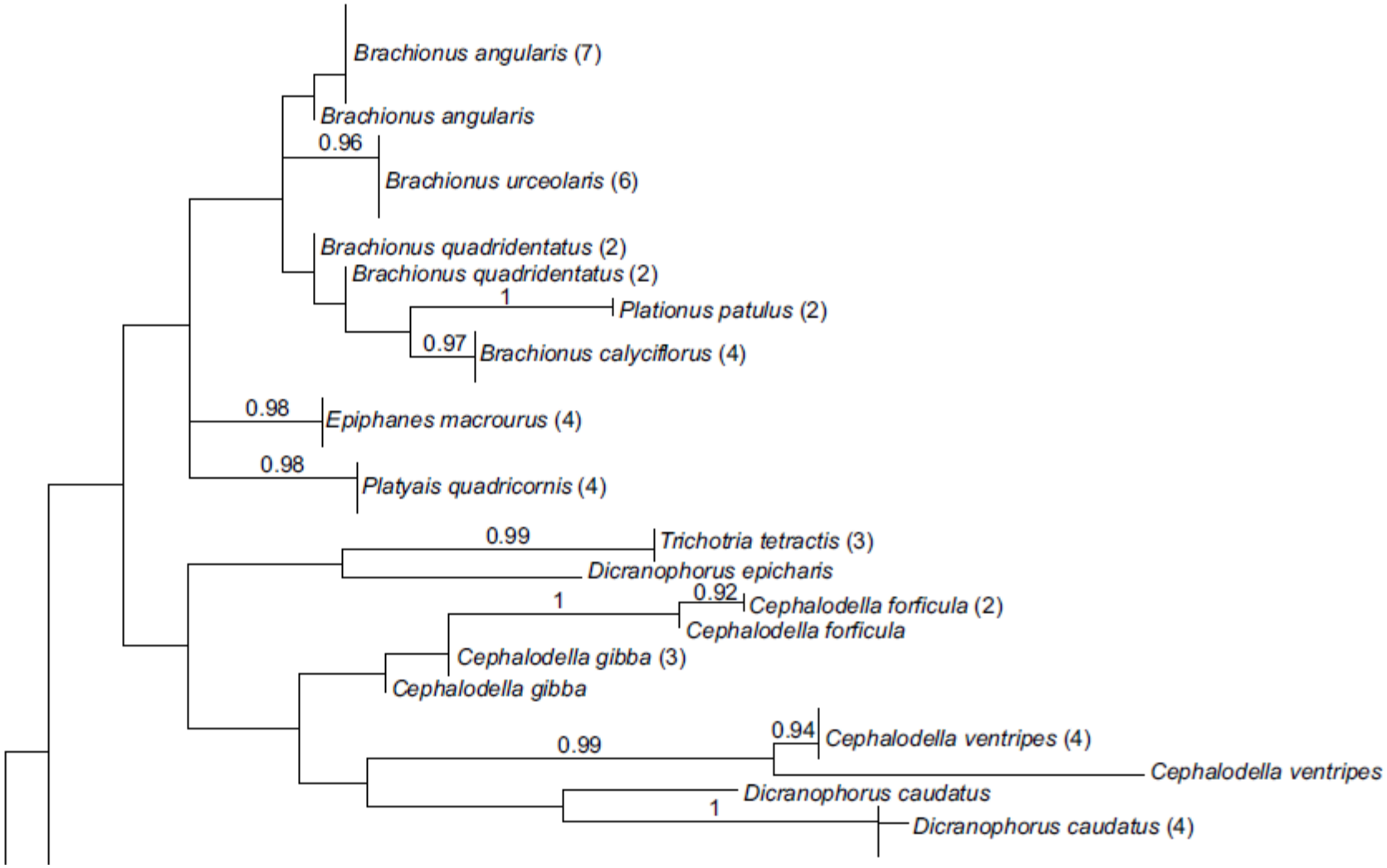


Figure 3.2. Enlarged view of the phylogeny for rotifers collected from the Hamilton Zoo and surrounding area from 2012-2014 estimated using Maximum likelihood analysis of 356 nucleotides of the D1 region of the 28S rDNA gene using the GTR+G model. Numbers on tree represent the bootstrap values, and numbers in brackets represent the number of individuals sequenced.

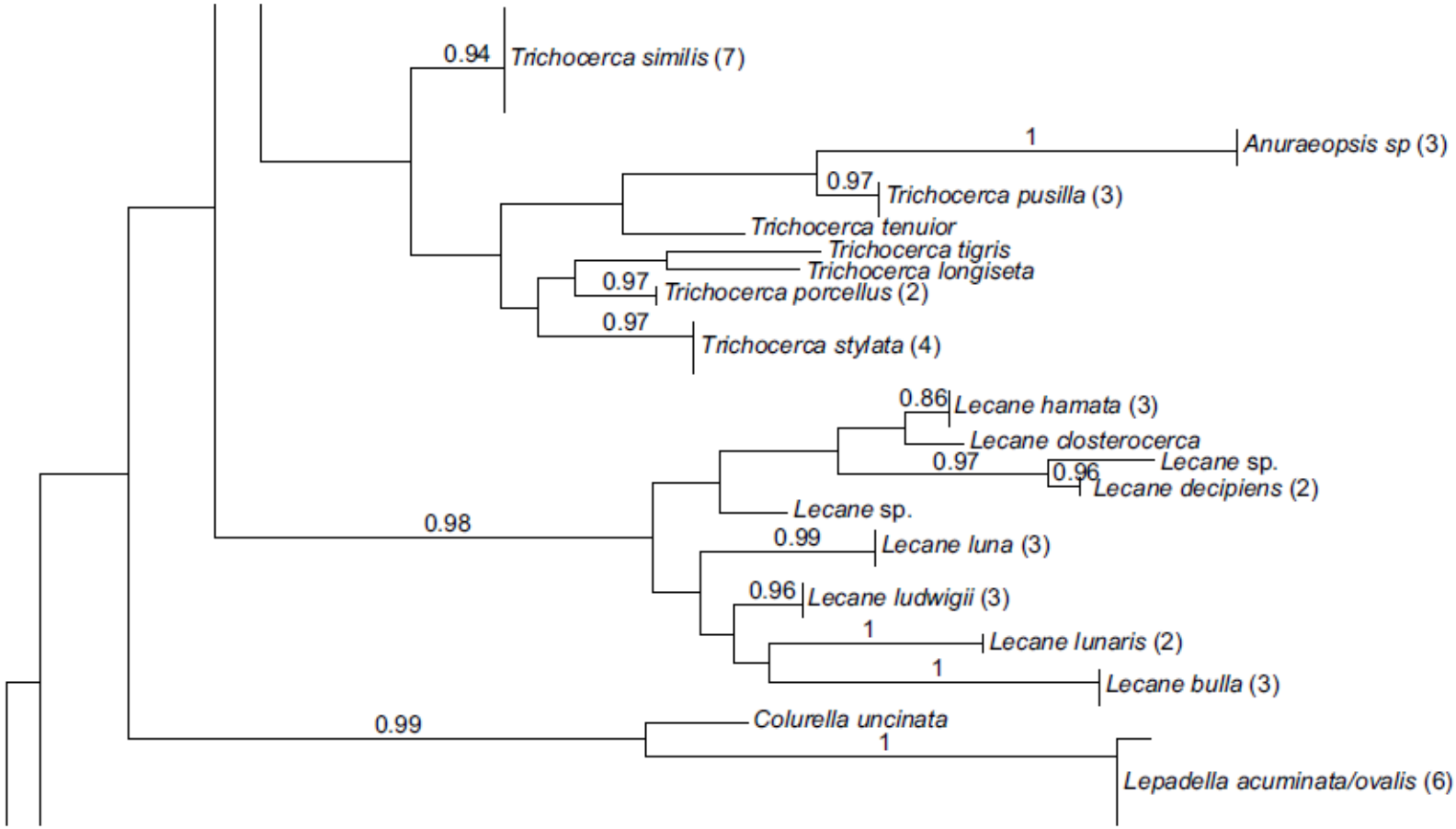


Figure 3.2 continued. Enlarged view of the phylogeny for rotifers collected from the Hamilton Zoo and surrounding area from 2012-2014 estimated using Maximum likelihood analysis of 356 nucleotides of the D1 region of the 28S rDNA gene using the GTR+G model. Numbers on tree represent the bootstrap values, and numbers in brackets represent the number of individuals sequenced.

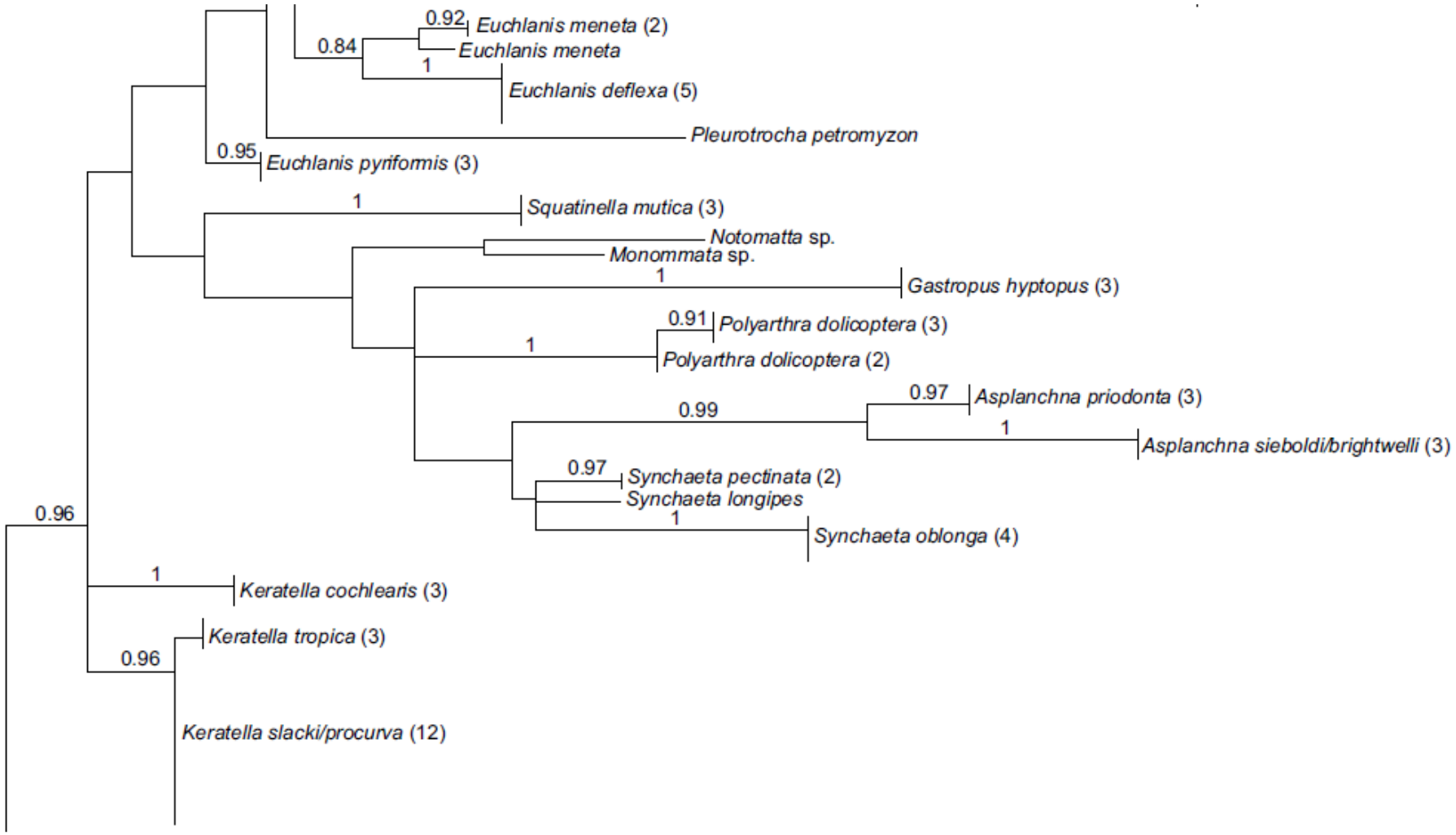


Figure 3.2 continued. Enlarged view of the phylogeny for rotifers collected from the Hamilton Zoo and surrounding area from 2012-2014 estimated using Maximum likelihood analysis of 356 nucleotides of the D1 region of the 28S rDNA gene using the GTR+G model. Numbers on tree represent the bootstrap values, and numbers in brackets represent the number of individuals sequenced.

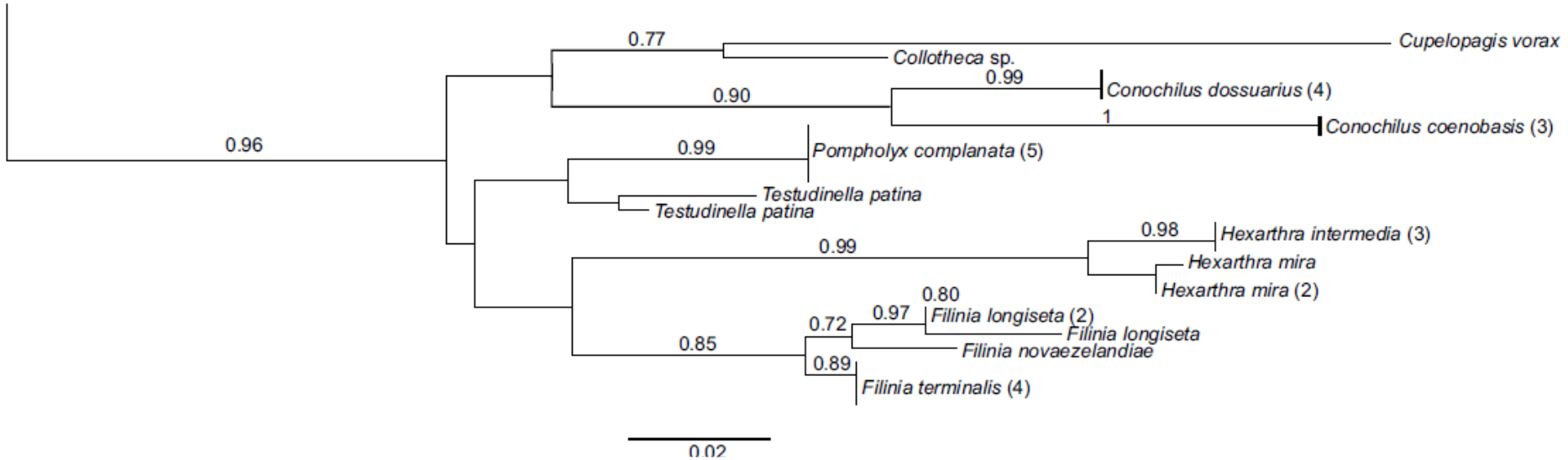


Figure 3.2 continued. Enlarged view of the phylogeny for rotifers collected from the Hamilton Zoo and surrounding area from 2012-2014 estimated using Maximum likelihood analysis of 356 nucleotides of the D1 region of the 28S rDNA gene using the GTR+G model. Numbers on tree represent the bootstrap values, and numbers in brackets represent the number of individuals sequenced.

Cladocerans

Nucleotide composition was A-G biased (A = 26.7%, C = 24.0%, G = 29.0%, T = 20.4%). Base frequencies were not significantly different across all sites ($\chi^2 = 19.50$, P = 0.996). Conspecific sequence divergence ranged from 0-6.6% (mean = 2.9%). Mean within-family sequence divergence was 7.85%.

Nine morphologically identified species of Cladocera were recovered: *Daphnia* sp., *Daphnia galeata, Ceriodaphnia dubia*, *Ceriodaphnia* c.f. *pulchella*, *Penilia avirostirs*, *Chydorus* sp., *Alona* c.f. *affinis, Bosmina* sp., and *Bosmina meridionalis*, from four families (Daphniidae, Sididae, Chydoridae, and Bosminidae). In the ML tree (Figure 3.3), six of the nine taxon groups were well supported (bootstrap value >0.70). Only three groups were not supported (bootstrap support <0.70), with two of those having only one specimen. *Ceriodaphnia dubia* sequences were split into two groups with one well supported group (2 sequences), and an unsupported group (3 sequences).

The ABGD analysis revealed 12 distinct OTU's (p = 0.001). Splitting occurred within *Ceriodaphnia dubia, Chydorus* sp. and *Daphnia galeata*. The AGBD results were supported by the PTP analysis, which identified 12 cladoceran OTU's. *Daphnia* spp., and *Ceriodaphnia dubia* were split into multiple OTU's. In contrast to these results, the GMYC analysis showed only 4 OTU's, with no significance from the likelihood ratio test (p > 0.05). As an example, OTU 1 included *Ceriodaphnia* c.f. *pulchella, Ceriodaphnia dubia,* and *Daphnia galeata.* OTU 3 included *Chydorus* sp.,

Alona c.f. *affinis*, *Bosmina sp*., and *Bosmina meridionalis.* Plotting the bootstrap values on the groups revealed low support for all morpho-species analyzed (min 0.02, max 0.45).

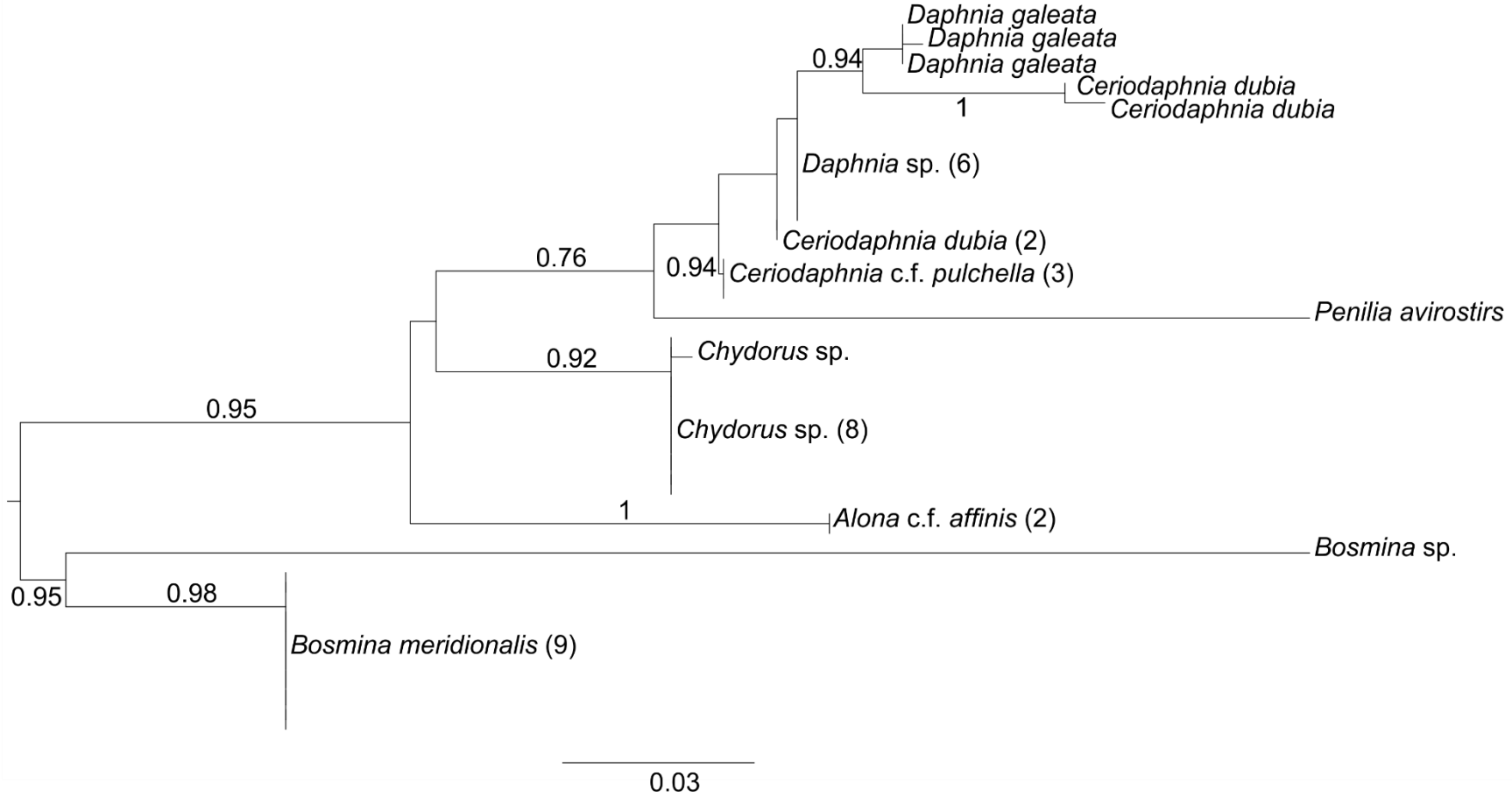


Figure 3.3. Phylogeny for cladocerans collected from the Hamilton Zoo and surrounding area from 2012-2014 estimated using Maximum likelihood analysis of 353 nucleotides of the D1 region of the 28S rDNA gene using the GTR+G model. Numbers on tree represent the bootstrap values, and numbers in brackets represent the number of individuals sequenced.

Copepods

I obtained sequences for 28 individual copepods including 10 cyclopoids (4 species) and 18 calanoids (3 species). Nucleotide composition was A-G biased (A = 26.6%, C = 24.9%, G = 30.4%, T = 18.1%). Base frequencies were not significantly different across all sites (χ^2 = 5.239, P = 1.000), and across the informative sites (χ^2 = 34.32, P = 0.793).

The ML tree identified multiple haplotypes for *Boeckella propinqua*, albeit with low support (bootstrap value < 0.70). *Gladioferens pectinatus* and *Calamoecia gibbossi* were well supported (bootstrap value 1). Unidentified nauplii made up the majority of the cyclopoids collected, with a maximum sequence divergence of 5.4%, suggesting a mixture of species. There were distinct groups formed within the nauplii with supported values (bootstrap support >0.70). However, these sequences did not group with the adult populations. The adult population of Cyclopidae formed a distinct, highly supported group (bootstrap support = 1), with *Acanthocyclops robustus* (Figure 3.4).

Sequences for six morpho-species were analysed for the copepod taxa. The ABGD analysis revealed 7 OTU's (p = 0.005). This analysis separated *Gladioferens pectinatus*, *Calamoecia gibbossi*, *Boeckella propinqua*, *Eucyclops serrulatus*, and *Cyclopoid* sp. into their own OTU's. Splitting occurred with the unidentified nauplii, which was consistent with the maximum likelihood analysis. The Multiple Poisson Tree Processes results suggested only three OTU's were present. OTU 1 included

Gladioferens pectinatus, *Calamoecia gibbossi*, and *Boeckella propinqua*. OTU 2 included only *Boeckella propinqua*. OTU 3 grouped together the Cyclopidae members; unidentified nauplii, *Eucyclops serrulatus*, *Acanthocyclops robustus*, and *Mesocyclops* sp. The GMYC analysis revealed seven OTU's ($p > 0.05$). Splitting occurred in the unidentified nauplii. However, in this analysis, some of the unidentified nauplii were combined into one OTU with the adult populations. Clumping also occurred with *Calamoecia gibbossi* and *Gladioferens pectinatus*.

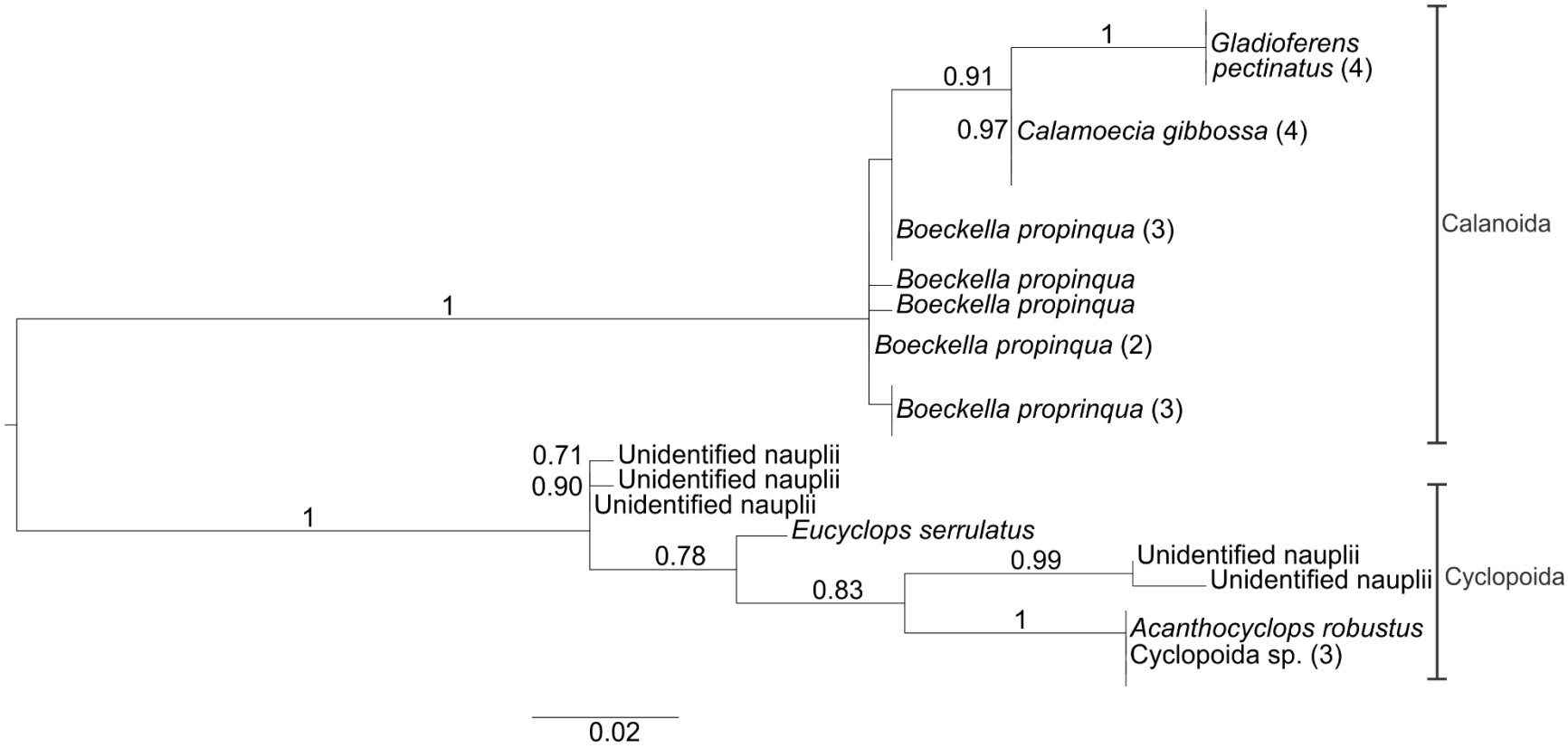


Figure 3.4. Phylogeny for copepods collected from the Hamilton Zoo and surrounding area from 2012-2014 estimated using Maximum likelihood analysis of 326 nucleotides of the D1 region of the 28S rDNA gene using the GTR+I model. Numbers on tree represent the bootstrap values, and numbers in brackets represent the number of individuals sequenced.

Discussion

I used the 28S rDNA gene region to successfully discriminate the zooplankton taxa from small pond habitats. I analyzed 60 morphologically identified species of rotifers, nine species of cladocerans, and seven species of copepods. I obtained a 78.6% sequencing success rate, a level similar to other studies (Hirai et al., 2013).

Within-species sequence divergence in rotifer taxa was low, with most having <1%. Interspecific variation ranged from 0.4% - 19.0% (mean 8.2%) and generally exceeded intraspecific values. In contrast, mitochondrial DNA (*COI*) conspecific mean divergence values are generally higher and provide more resolution at the species-level for distinguishing between taxa (Elías-Gutiérrez et al., 2008; Garciá-Morales and Elías-Gutiérrez, 2013; Gilbert and Walsh, 2005; Xiang et al., 2011). However, even with the low divergence values, 28S was able to identify specimens to a species level.

Generally, 28S also has a higher sequencing success rate than *COI*. For example, Hirai et al., (2013) in a study of hymenopterans had a 95% success rate for 28S versus 31.6% for *COI*. A pilot study conducted for this research had a 90% success rate for 28S, and a 60% success rate for *COI*. My study had high bootstrap support values for most taxa and captured within-species variation. A visually, clear barcode gap (*sensu* Hebert et al., 2003) was not obvious for the three groups of zooplankton studied, thus I used three different methods to delineate taxa in the study.

The ABGD method was the most consistent of the three, delineating the closest number of OTU's as the morphological identifications.

The limitations of the GMYC and the PTP analyses are likely the result of the lower divergence rate for the *28S* region compared with *COI* (e.g. Machida and Tsuda, 2010). The GMYC and PTP processes group together taxa that have only recently diverged (Fontaneto et al., 2015). These two methodologies use a phylogenetic based approach which requires monophyly. As the slow-diverging nuclear 28S region likely did not demonstrate monophyly at the tips, the small divergences between the taxa resulted in grouping at the genus or family level.

The rotifers *Cephalodella ventripes*, *Dicranophorus caudatus*, and *Testudinella patina* were split in the GMYC model and the ABGD method. These are of interest as their intra-specific sequence divergence values are >2% suggesting the presence of cryptic species. Cryptic species and species complexes are known to occur within many rotifer genera (see Fontaneto, 2014 for review), with morphologically similar species differing in their genetic sequences. For example, *Testudinella patina* is known to have cryptic species (Garciá-Morales and Elías-Gutiérrez, 2013), and the difference of 2.5% in my study was enough to separate out two taxa in the ABGD analysis. It is possible that the "split" taxa are part of cryptic species complexes (Fontaneto et al., 2007).

Despite the low species richness found for the cladocerans, I still found high variation in *Ceriodaphnia dubia*, with two distinct groups suggesting possible cryptic species similar to that found by Elías-Gutiérrez

et al., (2008) and Petrusek et al., (2004). For the calanoid and cyclopoid copepods, I had expected that the unidentified nauplii would group with the identified adult species. However, the unidentified nauplii formed their own separate groups suggesting that these were different species. It is possible that there is a low density of the adult population, or seasonal variation in species composition not captured in the individuals collected.

One of the issues with a slowly evolving marker such as 28S is that it may not detect more recently diverged species (e.g. Guerra et al., 2016). However, my data suggest that 28S can detect at least some cryptic species among the rotifers. However, it may be less important to distinguish closely related species when these species are being used as bioindicators of environmental change or ecosystem health. Previous studies have shown that species may respond differently to environmental factors (e.g. salinity; Ciros-Péréz et al., 2001; Montero-Pau et al., 2011). As closely related species are likely to share similar environmental preferences, failure to distinguish cryptic species may not impact upon the assessment of environmental quality (Montero-Pau et al., 2011; Ortells et al., 2003; Walsh et al., 2009),

Metabarcoding communities to identify the species present in a community requires “universal” PCR primers to ensure that as many as possible of the species present are identified. Accurate community characterization is particularly important when the communities are being used as bioindicators to assess ecosystem health. I found that 28S can routinely distinguish between morphologically identified species already

used as indicators of water quality (Duggan et al., 2001; Gannon and Stemberger, 1978; Haberman and Haldna, 2014). Based on this data, I conclude that 28S is an effective marker to use in the identification zooplankton. However, further research will benefit from studies linking environmental data to sequence data (e.g. Leasi et al., 2013; Ortells et al., 2003; Walsh et al., 2009) to test whether the environmental variables affect the overall community structure of zooplankton, and if this can be detected using 28S sequence data.

Acknowledgments

I would like to thank the staff and students of the PBRL laboratory for providing assistance and guidance throughout this project; Stacey Meyer, Matt Knox, Gemma Collins, Chrissen Gemmill, Kristi Holland, Clare Beet, and Phil Ross.

Literature Cited

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and D.J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403-410.

Boero, F. 2001. Light after dark: the partnership for enhancing expertise in taxonomy. TRENDS in Ecology & Evolution, 15(5), 1pp.

Brown, E.A., Chain, F.J.J., Crease, T.J., MacIsaac, H.J. and M.E. Cristescu. 2015. Divergence thresholds and divergent biodiversity estimates: can metabarcoding reliably describe zooplankton communities? Ecology and Evolution, 5(11), 2234-2251.

Bucklin, A., Ortman, B.D., Jennings, R.M., Nigro, L.M., Sweetman, C.J., Copley, N.J., Sutton, T. and P.H. Wiebe. 2010. A “Rosetta Stone” for metazoan zooplankton: DNA barcode analysis of species diversity of the Sargasso Sea (Northwest Atlantic Ocean). Deep-Sea Research II, 57, 2234-2247.

Carpenter, S.R., Kitchell, J.F. and J.R. Hodgson. 1985. Cascading tropic interactions and lake productivity. Bioscience, 35(10), 634-639.

Ciros-Péréz, J., Gómez, A. and M. Serra. 2001. On the taxonomy of three sympatric sibling species of the *Brachionus plicatilis* (Rotifera) complex from Spain, with the description of *B. ibericus* n. sp. Journal of Plankton Research, 23(12), 1311-1328.

Costa, F.O., deWaard, J.R., Boutillier, J., Ratnasingham, S., Dooh, R.T., Hajibabaei, M. and P.D.N. Hebert. 2006. Biological identifications

through DNA barcodes: the case of the Crustacea. Can. J. Fish. Aquat. Sci., 64, 272-295.

Darriba, D., Taboada, G.L., Doallo, R. and D. Posada. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nature Methods, 9(8), 772.

De Ley, P., De Ley, I.T., Morris, K., Abebe, E., Mundo-Ocamp, M., Yoder, M., Heras, J., Waumann, D., Rocha-Olivares, A., Burr, A.H.J., Baldwin, J.G and W.K. Thomas. 2005. An intergrated approach to fast and informative morphological vouchering of nematodes for applications in molecular barcoding. Phil. Trans. R. Soc. B., 360, 1945-1958.

De Ley, P., Félix, M-A., Frisse, L.M., Nadler, S.A., Sternberg, P.W. and W.K. Thomas. 1999. Molecular and morphological characterisation of two reproductively isolated species with mirror-image anatomy (Nematoda: Cephalobidae). Nematology, 1(6), 591-612.

de Vargas, C., Audic, S., Henry, N., Decelle, J., Mahé, F., Logares, R., et al. 2015. Eukaryotic plankton diversity in the sunlit ocean. Science, 348, 1261605-1/11.

Drummond, A.J., Suchard, M.A., Xie, D. and A. Rambaut. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Molecular Biology and Evolution, 29, 1969-1973.

Duggan, I.C., Green, J.D. and R.J. Shiel. 2001. Distribution of rotifers in North Island, New Zealand, and their potential use as bioindicators of lake trophic state. Hydrobiologia, 446/447, 155-164.

Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nuclei Acids Res., 32, 1792-1797.

Elías-Gutiérrez, M., Jerónimo, F.M., Ivanova, N.V., Valdez-Moreno, M. and P.D.N. Hebert. 2008. DNA barcodes for Cladocera and Copepoda from Mexico and Guatemala, highlights and new discoveries. Zootaxa, 1839, 1-42.

Ezard, T., Fujisawa, T. and T. Barraclough. 2009. Splits: Species' limits by threshold statistics. R package version 1.0. URL: http://R-Forge.R-project.org/projects/splits/

Fontaneto, D. 2014. Molecular phylogenies as a tool to understand diversity in rotifers. International Review of Hydrobiology, 99, 178-187.

Fontaneto, D., Herniou, E.A., Boschetti, C., Caprioli, M., Melone, G., Ricci, C. and T.G. Barraclough. 2007. Independently evolving species in asexual bdelloid rotifers. PLos Biol., 5(4), 914-921.

Fontaneto, D., Flot, J.F. and C.Q. Tang. 2015. Guidelines for DNA taxonomy, with a focus on the meiofauna. Marine Biodiversity, 45, 433-451.

Fujisawa, T. and T.G. Barraclough. 2013. Delimiting species using single-locus data and the generalized mixed yule coalescent approach: a revised method and evaluation on simulated data sets. Syst. Biol. 62, 707-724.

Gannon, J.E. and R.S. Stemberger. 1978. Zooplankton (especially crustaceans and rotifers) as indicators of water quality. Transactions of the American Microscopical Society, 92(1), 16-35.

Garciá-Morales, A.E. and M. Elías-Gutiérrez. 2013. DNA barcoding of freshwater Rotifera in Mexico: Evidence of cryptic speciation in common rotifers. Molecular Ecology Resources, 13, 1097-1107.

Gilbert, J.J. and E.J. Walsh. 2005. *Brachionus calyciflorus* is a species complex: Mating behavior and genetic differentiation among four geographically isolated strains. Hydrobiologia, 546, 257-265.

Guindon, S. and O. Gascuel. 2003. A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. Systematic Biology, 52, 696-704.

Haberman, J. and M. Haldna. 2014. Indices of zooplankton community as valuable tools in assessing the trophic state and water quality of eutrophic lakes: long term study of lake Võrtsjärv. J. Limnol., 73(2), 263-275.

Hebert, P.D.N. and M.J. Beaton. 1989. Methodologies for allozyme analysis using cellulose acetate electrophoresis: A practical handbook. Helena Laboratories.

Hebert, P.D.N., Cywinska, A., Ball, S.L. and J.R. deWaard. 2003. Biological identifications through DNA barcodes. Proc. R. Soc. Lond., 270, 313-321.

Hirai, J., Shimode, S. and A. Tsuda. 2013. Evaluation of ITS2-28S as a molecular marker for identification of calanoid copepods in the subtropical western North Pacific. J. Plankton Res., 35(3), 644-656.

Jeppesen, E., Nõges, Davidson, T.A., Haberman, J., Nõges, T., Blank, K., Lauridsen, T.L., Søndergaard, M., Sayer, C., Laugaste, R., Johansson, L.S., Bjerring, R. and S.L. Amsinck. 2011. Zooplankton as indicators in lakes: a scientific-based plea for including zooplankton in the ecological quality assessment of lakes according to the European Water Framework Directive (WFD). Hydrobiologia, 676, 279-297.

Jukes, T.H. and C.R. Cantor. 1969. Evolution of protein molecules. *In* Munro, H.N., editor, Mammalian Protein Metabolism, pp 21-132, Academic Press, New York.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P. and A. Drummond. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics, 28(12), 1647-1649.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution, 16, 111-120.

Leasi, F., Tang, C.Q., De Smet, W.H. and D. Fontaneto. 2013. Cryptic diversity with wide salinity tolerance in the putative euryhaline *Testudinella clypeata* (Rotifera, Monogononta). Zoological Journal of the Linnean Society, 168, 17-28.

Leray, M., Yang, J.Y., Meyer, C.P., Mills, S.C., Agudelo, N., Ranwez, V., Boehm, J.T. and R.J. Machida. 2013. A new versatile primer set targeting a short fragment of the mitochondrial *COI* region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. Frontiers in Zoology, 10(34), 14pp.

Machida, R.J. and A. Tsuda. 2010. Dissimilarity of species and forms of planktonic *Neocalanus* copepods using mitochondrial *COI*, 12S, nuclear ITS, and 28S gene sequences. PLoS ONE, 5(4), 6pp.

Machida., R.J. and N. Knowlton. 2012. PCR primers for metazoan nuclear 18S and 28S ribosomal DNA sequences. PLoS ONE, 7(9), 11pp.

McManus, G.B. and L.A. Katz. 2009. Molecular and morphological methods for identifying plankton: what makes a successful marriage? Journal of Plankton Research, 31(10, 1119-1129.

Montero-Pau, J., Ramos-Rodríguez, E., Serra, M. and A. Gómez. 2011. Long-term coexistence of rotifer cryptic species. PLoS ONE, 6(6), 9pp.

Ortells, R., Gómez, A. and M. Serra. 2003. Coexistence of cryptic rotifer species: ecological and genetic characterization of *Brachionus plicatilis*. Freshwater Biology, 48, 2194-2202.

Pearman, J.K., El-Sherbiny, M.M., Lanzén, A., Al-Aidaroos, A.M. and X. Irigoien. 2014. Zooplankton diversity across three Red Sea reefs using pyrosequencing. Front. Mater. Sci., 1(27), 11 pp.

Pearman, J.K. and X. Irigoien. 2015. Assessment of zooplankton community composition along a depth profile in the Central Red Sea. PLoS ONE, 10(7), 14pp.

Petrusek, A., Černý, M. and E. Audenaert. 2004. Large intercontinental differentiation of *Moina micrura* (Crustacea: Anomopoda): one less cosmopolitan cladoceran? Hydrobiologia, 526, 73-81.

Pons, J., Barraclough, T.G., Gomez-Zurita, J., Cardoso, A., Duran, D.P., Hazell, S., Kamoun, S., Sumlin, W.D., Vogler, A.P. and M. Hedin. 2006. Sequence-based species delimitation for the DNA taxonomy of undescribed insects. Syst. Biol., 55(4), 595-609.

Puillandre, N., Lambert. A., Brouillet S. and G. Achaz. 2012. ABGD, Automatic Barcode Gap Discovery for primary species delimitation. Molecular Ecology, 21(8), 1864-1877.

Swofford, D.L. 2003. PAUP* ver 4.0. b10. Phylogenetic analysis using parsimony and other methods, version. Sunderland, Massachusetts, Sinauer Associates, Sunderland.

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C. and E. WIllerslev. 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. Molecular Ecology, 21, 2045-2050.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and S. Kumar. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Bio. Evol., 28, 2731-2739.

Tang, C.Q., Leasi, F., Obertegger, U., Kieneke, A., Barraclough, T.G. and D. Fontaneto. 2012. The widely used small subunit 18S rDNA molecule greatly underestimates true diversity in biodiversity surveys of the meiofauna. PNAS, 109(40), 16208-16212.

Walsh, E.J., Schröder, T., Wallace, R.L. and R. Rico-Martinez. 2009. Cryptic speciation in *Lecane bulla* (Monogononta: Rotifera) in Chihuahuan Desert waters. Verh. Internat. Verein. Limnol., 30(7), 1046-1050.

Xiang, X., Xi, Y., Wen, X., Zhang, G., Wang, J. and K. Hu. 2011. Genetic differentiation and phylogeographical structure of the *Brachionus calyciflorus* complex in eastern China. Molecular Ecology, 20, 3027-3044.

Yang, J., Zhang, X., Xie, Y., Song, C., Zhang, Y., Yu, H. and G.A. Burton. 2017. Zooplankton community profiling in a eutrophic freshwater ecosystem-Lake Tai basin by DNA metabarcoding. Scientific Reports, 7(1773), 11pp.

Zhang, J., Kapli, P., Pavlidis, P. and A. Stamatakis. 2013. A general species delimitation method with applications to phylogenetic placements. Bioinformatics, 29(22), 2869-2876.

CHAPTER 4: ASSESSING THE ZOOPLANKTON COMMUNITIES OF FRESHWATER PONDS USING DNA METABARCODING.

*to be published under the same title as: Woods, S., Hogg, I.D., Duggan, I.C., Banks, J.C.

Abstract

I evaluated the suitability of DNA metabarcoding for use in ecological studies determining the species composition and abundance in zooplankton communities. Ten ‘natural pond’ samples containing zooplankton (>300 individuals) were collected from a small pond in the Waikato region of New Zealand, and 20 constructed communities from the same pond community containing varying amounts of Cyclopidae spp., and *Bosmina meridionalis* were created and sequenced on the Illumina MiSeq platform. Ten constructed community samples, and three natural pond community samples were eliminated from analysis due to the low number of sequences generated. All highly abundant (>50 individuals/sample) and moderately abundant (10-50 individuals/sample) were successfully identified with the sequencing data. Less abundant taxa (<10 individuals/sample) showed primer bias, and sequencing results failed to identify some taxa. Sequences showed a bias towards larger individuals and did not correspond to the abundance of individuals in the samples. I conclude that metabarcoding of zooplankton communities would be useful in ecological studies determining the trophic state of lotic environments, as less abundant taxa would have little effect on the designation, and the number of sequences generated would likely be increased with more individuals in the sample.

Introduction

Environmental changes such as global warming, land use shifts, and eutrophication are having profound effects on Earth's freshwater ecosystems (Pimm et al., 2014; Sala et al., 2000). These changes will influence the spatial distributions of aquatic taxa, and result in changes in species' composition and distributions as systems are progressively affected (Pimm et al., 2014; Sala et al., 2000). Accurate assessments of biodiversity will be required to monitor and, potentially, manage these changes. In order to adequately monitor biotic responses, there is an urgent need to quickly and efficiently assess community composition across a range of habitats.

Biodiversity assessments of aquatic habitats have traditionally used morphologically-based surveys such as quantitative and qualitative surveys of fish, benthic invertebrates, zooplankton and algae (Hynes 1970). However, these approaches can be time consuming and also require taxonomic expertise. As an alternative, DNA-based approaches have been proposed for routine surveys and biomonitoring, and would minimize the taxonomic expertise required (Hebert et al. 2003). Further, DNA-based approaches can assign species level identifications to juvenile stages such as copepod nauplii, and morphologically similar species (Fontaneto, 2014) that are often omitted or grouped together at the family or order level in morphological assessments (Wright et al. 1984). A further benefit of DNA-based approaches is the ability to standardize and simplify biodiversity assessments across a range of habitats and geographic

locations (Hebert et al., 2003). In particular, once a DNA sequence has been associated with a particular species or location, subsequent collections can then be linked.

To facilitate analyses of entire communities, DNA “metabarcoding” approaches have been increasingly used (Taberlet et al. 2012). Communities of interest are sampled and sequenced for short (<400 bp) sequence fragments using next generation sequencing platforms. The sequencing can combine samples from multiple, individually-tagged samples or habitats and potentially generate millions of sequences. Accordingly, metabarcoding can also be used to capture temporal and spatial variability among communities (Brannock et al., 2016; Chain et al., 2016; Pearman and Irigoien, 2015). A bioinformatics approach is then used to target desired taxa and to match these with a reference database to obtain identification of individuals within each of the samples. Previous metabarcoding studies of communities have included analyses of gut contents (De Barba et al., 2013), freshwater invertebrates (Dowle et al., 2016; Hajibabaei et al., 2011), arthropods (Gibson et al., 2014), and zooplankton (e.g. Abad et al., 2016; Brown et al., 2015; de Vargas et al., 2015; Machida and Knowlton, 2012; Pearman and Irigoien, 2015).

Similar to single sequence DNA barcoding and identification, community-based analyses rely on comprehensive databases of appropriate sequences for the different taxa under study. The most comprehensive library of COI sequences is found in the Barcode of Life Datasystems (BOLD) database (Ratnasingham et al. 2014). Other

databases can also provide useful references including Silva (Quast et al., 2013) for the small subunit of the 18S region, GenBank (www.ncbi.nlm.nih.gov/genbank/) and BOLD for the large subunit of the 28S region. However, even with these global databases, there is often a lack of available sequences for comparison or reference (Carugati et al., 2015; Dowle et al., 2016). A further challenge is finding universal primers that provide sequences of appropriate length (~400 bp) for use with current next generation sequencing platforms. At present, the 658 nucleotide fragment of the COI gene provided by the Folmer et al. (1994) primers currently exceeds this length (van Dijk et al., 2014). To date, the lack of universal primers that target shorter COI fragments has limited the use of COI for diverse taxonomic groups such as zooplankton which comprise multiple taxonomic classes (Deagle et al., 2014; Hirai et al., 2013; Taberlet et al., 2012).

Here, using a purpose-built 28S reference library (Woods et al. in prep; Chapter 3), I tested the use of a metabarcoding approach to evaluate zooplankton community composition in pond habitats using the 28S rDNA gene. Morphological assessments of zooplankton communities were conducted and compared to data obtained from the metabarcoding approach. Based on these data, I discuss the possible benefits of 28S metabarcoding and its use in ecological assessments.

Methods

As part of a study to determine the effects of common carp of pond habitats (Chapter 2; Woods et al. in prep), zooplankton samples were collected monthly from ponds at the Hamilton Zoo (37° 46' 27"S, 175° 12' 51"E) between August 2012- January 2014 to assess the accuracy of 28S for barcoding zooplankton communities. Complete details of the study sites and sampling of zooplankton are provided in Woods et al. (in prep; Chapter 2). Briefly, a PVC tube (100 mm X 720 mm) was placed into the water column at a 45° angle to avoid disturbing the sediment and 5.68 L of water collected. The water samples collected from the Hamilton zoo ponds were filtered through a 37 µm plankton net, and stored in 70% ethanol. Two types of zooplankton communities were sent for metabarcoding; whole pond samples containing natural communities, and constructed communities containing two abundant species found at the study site. For the whole pond samples, 5 mL aliquots were taken from collected samples, and zooplankton counted using a stereo microscope until 300 individuals were counted. If the sample did not contain more than 300 specimens, the entire sample was processed. The aliquots were then transferred to 15 mL conical centrifuge tubes and stored in 95% ethanol at -20°C for DNA extraction. Ten samples were taken from different months and study ponds to cover both spatial and temporal variability that is known to occur within these habitats.

Twenty communities were constructed using two abundant zooplankton found consistently in the two ponds sampled (*Bosmina*

meridionalis and Cyclopoida). Constructed communities consisted of 50 individuals; comm 1 (50 *B. meridionalis*), comm 2 (35 *B. meridionalis* and 15 Cyclopoida), comm 3 (25 *B. meridionalis*, 25 Cyclopoida), comm 4 (15 *B. meridionalis*, 35 Cyclopoida), comm 5 (50 Cyclopoida). Communities were assembled in MQ water and individuals were brushed to remove phytoplankton, preventing contamination from the pond water. Four replicates of each of the five constructed communities were sent for metabarcoding.

To extract the DNA of the communities, the zooplankton aliquots were filtered through a 0.45 µm, sterile filter pad using a Venturi vacuum system. The filter pads were folded, and placed in a 2 mL tube for extraction. The PowerSoil® DNA Isolation Kit (MO BIO, Carlsbad, CA) was used for the DNA extractions, following the manufacturer's instructions. Following DNA extraction, PCR amplifying 28S used 10 µL of a PCR mastermix solution (i-Taq™ containing DNA polymerase, dNTP's, PCR reaction buffer and gel loading buffer), 0.5 µL 300R (CAACTTTCCCTCACGGTACTTG) and F63.2 (ACCCGCTGAAYTTAAGCATAT) primers, 2 µL DNA template and 7 µL distilled, deionised water. The thermo cycling conditions consisted of an initial denaturation at 94 ^{o}C for 3 min, followed by 40 cycles of 94 ^{o}C for 30 s, 52 ^{o}C for 30 s, and 72 ^{o}C for 30 s, followed by a final extension step of 72 ^{o}C for 5 min. Samples were cleaned twice using Agencourt AMPure XP beads (Beckman Coulter, Indianopolis, IN), following the manufacturer's guidelines. To ensure sufficient amplicons were in the samples, DNA was quantified using a Qubit™ fluorometric quantitation assay (Thermo Fisher

Scientific, Waltham, MA). Samples (n=30, including 20 constructed communities) were sent to New Zealand Genomics Limited (NZGL) at the University of Auckland for sequencing on the Illumina MiSeq platform (Illumina Inc, San Diego, CA).

A reference library for the 28S rDNA gene was constructed from identified specimens of 76 species of rotifers, calanoid, cyclopoid and harpacticoid copepods, and Diplostraca taken from the BOLD database (Woods et al. unpubl. data; Chapter 3). These data were supplemented with a further 51 sequences for 28S from Genbank to provide a comprehensive coverage of taxa likely to be observed in the study ponds. Sequence length for the reference library was 314 nucleotides.

Mothur (Schloss et al., 2009) was used to align the sequence data to the reference library and designate the sequences to their corresponding taxonomic classifications. Samples with few useable sequences (<100) were excluded from further analyses. Sequences were 'denoised' by removing ambiguities, contaminants, chimeras (UCHIME; Edgar et al., 2011) and duplicate sequences. Sequences were pre-clustered according to the accepted parameters of one difference per 100 nucleotides (<3 nucleotide differences in total; MiSeq SOP, https://www.mothur.org/wiki/MiSeq_SOP, Accessed 21-11-2017; Kozich et al., 2013), and thus assumed to be the same species. Sequences were then assigned to their putative taxa based on sequences obtained from the morphologically identified specimens in the reference library. Sequences not identified as animal or plant taxa were removed from the analysis.

Constructed zooplankton community assemblages were analyzed using a Bray-Curtis dissimilarity matrix on log(x+1) transformed data Using Primer v6 (Clarke and Gorley, 2006), and visualized using multidimensional scaling (MDS).

Results

I obtained an acceptable number of sequences from 10 of the constructed communities. The mean length of the sequence fragments was 281 bp, with a minimum length of 259 bp and a max length of 300 bp. Chimeras were observed in low frequency (0.01%). The results for 10 of the 20 constructed communities were excluded due to low numbers of sequences (<70). For the natural pond habitat samples, three samples were excluded as they contained fewer than 1000 animal sequences. There were 246,191 sequences used in the analysis from the communities that remained.

Constructed communities

A total of 54,795 sequences were generated from the 10 constructed community samples used in the analysis. The species composition of the constructed communities assessed using sequence data was comparable to that of the morphological assessments in most cases (Table 4.1, Figure 4.1). Communities consisting of either *Bosmina meridionalis* or Cyclopidae matched well with the morphological community composition. For example, communities consisting of only *B. meridionalis* (RC69, RC70) generated 98.22% (mean) cladoceran sequences. Similarly, communities consisting of only Cyclopidae (RC83 - RC86) generated 99.57% (mean) copepod sequences. However, in communities RC75 and RC76 that had equal parts *B. meridionalis* and Cyclopidae (25 individuals each) there was a bias towards Cyclopidae genera, as the sequences were dominated by Cyclopidae (mean 91.42%).

False positives indicating a presence of rotifer and plant DNA in the samples were present in low quantities, making up an average of 0.74% of the sequences.

Communities containing only *B*. *meridionalis* (RC69, RC70), and communities containing only Cyclopidae (RC83-86) were clearly distinguishable from the mixed communities using the 28S reads to characterise each community (Figure 4.1). There were fewer differences among the communities that contained a mixture of cladocerans and copepods. Considerably more copepod sequences than cladoceran sequences were generated in the mixed communities; 5037 cladoceran sequences compared with 49,469 copepod sequences.

Table 4.1. Number of sequences generated from each constructed community. RC69, RC70 contained 50 B. meridionalis (Cladocera), RC75, RC76 contained 25 B. meridionalis, 25 copepods. RC79, RC80 contained 15 B. meridionalis and 35 copepods. RC83, RC84, RC85, RC86 contained 50 copepods.

Taxon	RC69	RC70	RC75	RC76	RC79	RC80	RC83	RC84	RC85	RC86
Cladocerans	873	800	1180	745	742	697				
Copepods										
Mesocyclops			12443	635	11124	10052				5290
Acanthocyclops robustus			71	42	52	57	105	153	71	49
Eucyclops serratulus			5	1	8	3	4	3		4
Harpacticoida			405	7890	411	358				233
Rotifera	10	18	15	25	38	17	0	0	0	3
Plantae	1	1	16	37	43	45	0	0	1	16

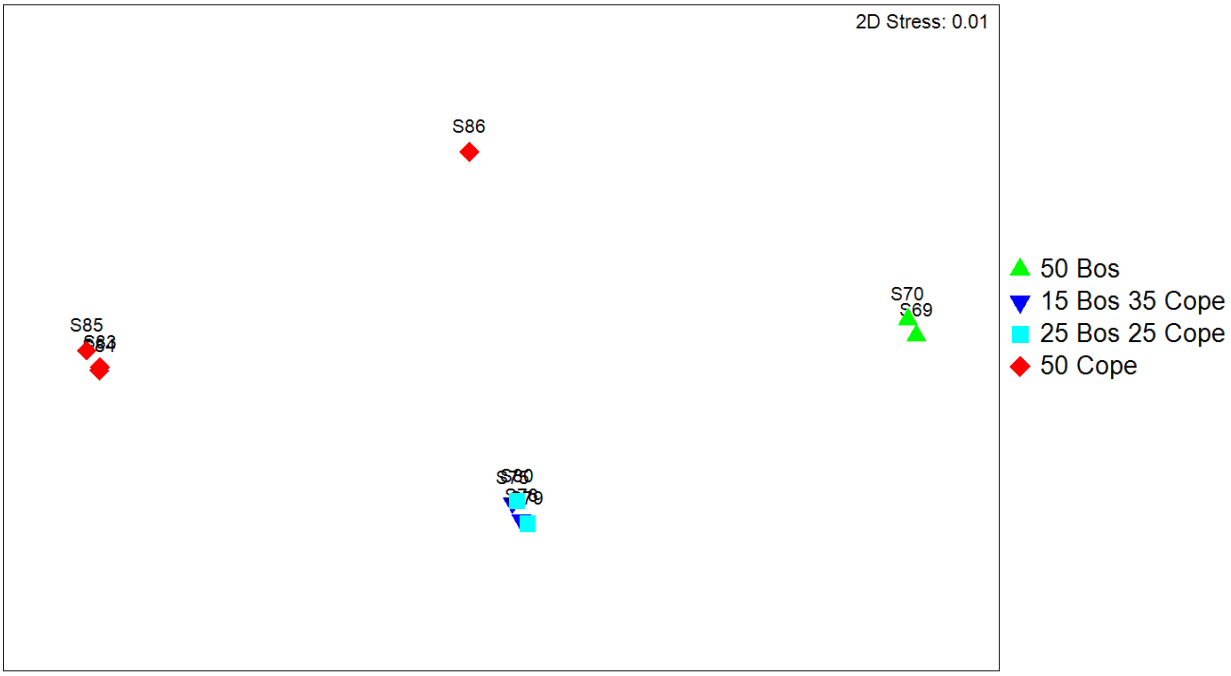
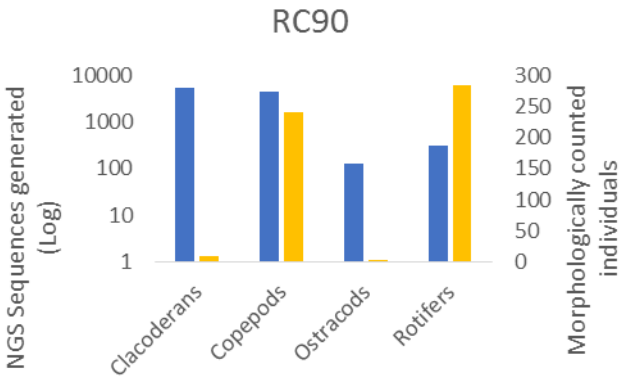


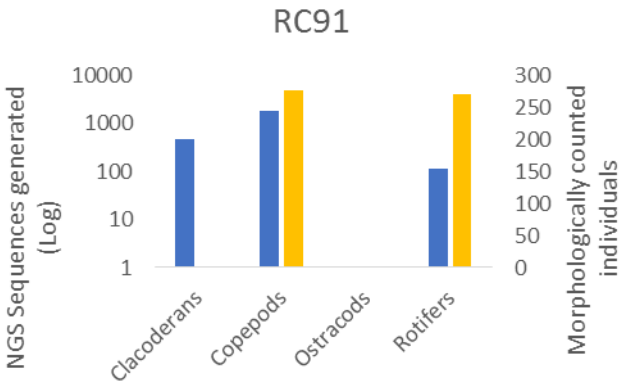
Figure 4.1. nMDS plot of 10 constructed communities containing Bosmina meridionalis, and/or Cyclopoid copepods. RC69, RC70 contained 50 *B. meridionalis* (Cladocera), RC75, RC76 contained 25 *B. meridionalis*, 25 copepods. RC79, RC80 contained 15 *B. meridionalis* and 35 copepods. RC83, RC84, RC85, RC86 contained 50 copepods.

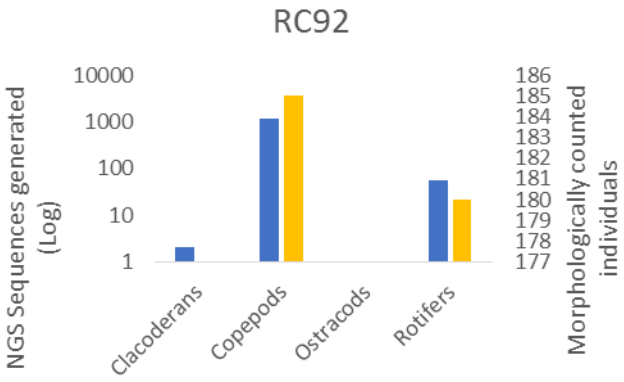
Natural pond community characterisation

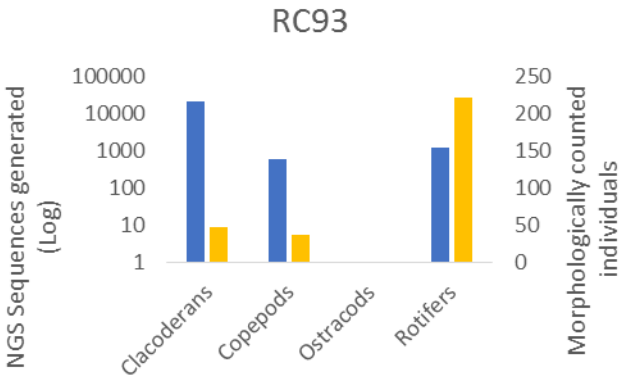
I obtained 191,396 sequences from the natural pond samples that ranged between 247 bp and 305 bp length (mean 277 bp), corresponding to the targeted region. Chimeras were observed for 0.04% of the post-quality-control reads. Sequence data were dominated by plants, making up 55% of the generated sequence reads. The cladocerans *Alona* c.f. *affinis*, *B. meridionalis*, and *Daphnia galeata*, generated 70,564 sequences. Copepods, comprised of predominantly unidentified cyclopoid nauplii, generated 8,319 sequences. Rotifers were the most diverse and abundant taxa (30 morphologically identified species) but only generated 6,693 sequences of the total number of reads.

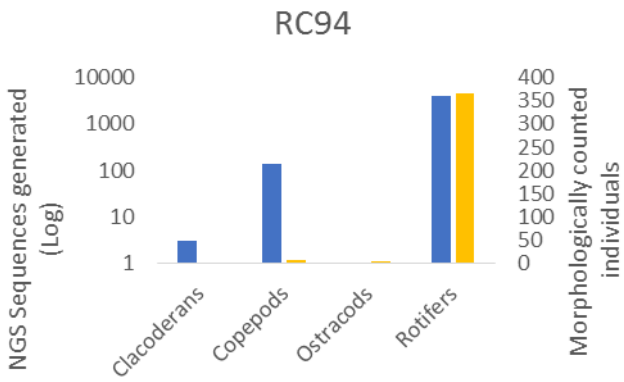
There was no evidence of sequence abundance correlating with morphological species abundance (Figure 4.2). Unlike the constructed communities, the natural pond communities generated more sequences for cladocerans than copepods. To highlight the bias towards cladoceran taxa, in sample RC90, eight cladoceran (*Daphnia galeata*) individuals were counted in the morphological assessments and generated 5,563 sequences. In contrast, the rotifers *Polyarthra dolichoptera* (n=134 individuals) and *Trichocerca similis* (n=126), generated 70 and 143 sequences, respectively. In sample RC93, Branchiopoda (*Bosmina meridionalis*) had 47 individuals producing 20,083 sequences. *Filinia longiseta* was the dominant taxa with 140 individuals, although produced only 776 sequences.

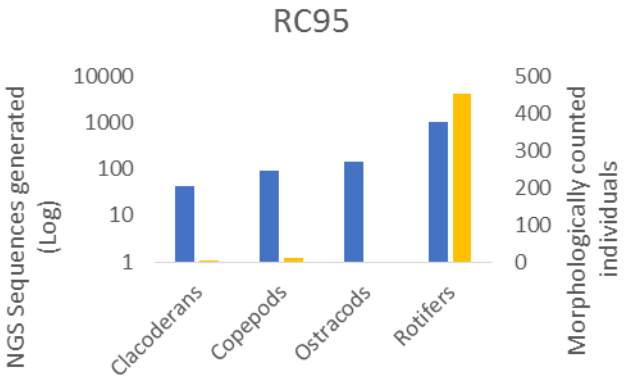












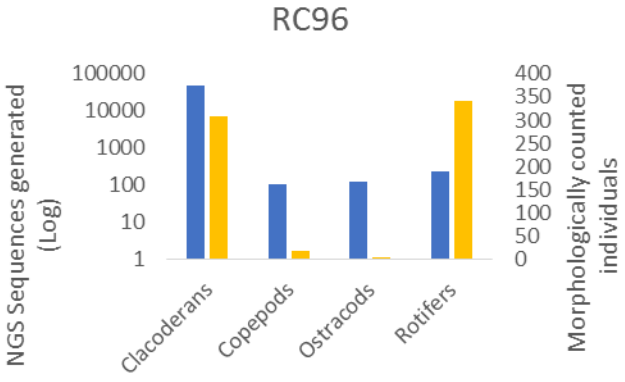


Figure 4.2. Comparison of sequences generated (blue bars) and number of individuals counted (orange bars) for the four groups of zooplankton seen in the whole pond community samples.

In RC96, *B. meridionalis* was counted 307 times and produced 44,410 sequences. *Filinia longiseta* had 252 individuals counted and generated 120 sequences. There were also biases in the rotifer taxa when Branchiopoda were absent. In sample RC92, Cyclopoid copepods were the dominant taxa with 185 individuals, generating 1108 sequences. *P. dolichoptera*, the only other abundant species in the morphological counts (n=123) produced only seven sequences. *F. longiseta* and *Keratella slacki* were both abundant (121 and 112 individuals) in RC94, but produced 498 and 2,957 sequences, respectively. False positives, where sequences were produced when no individuals were counted in the morphological samples, were present in low quantities in samples RC90 (0.33% of sequences), RC91 (2.22%), RC92 (2.92%), RC93 (0.52%), RC94 (0.96%), and RC96 (0.11%). However, in RC95, there was a large number of false positives, making up 15.47% of the sequences generated.

Characterisation of natural communities using 28S detected every species observed in a sample when species were abundant (> 50 individuals counted, Table 4.2). There were nine identified taxa, represented in the seven samples. This was also true when considering the moderately abundant taxa (20-50 individuals counted; Table 4.3). For the less abundant taxa(1-10 individuals; Table 4.4) in the samples, 17 of the 31 morphologically counted taxa had at least one detection failure from the NGS approach. Of the undetected taxa, 13 of the 33 had only one individual present in the morphological counts.

Table 4.2. Zooplankton taxa with >50 individuals observed and detected in lake samples. O represents taxa that were observed in the morphological samples, and detected in the MiSeq data.

	RC90	RC91	RC92	RC93	RC94	RC95	RC96
Cladocerans							
Bosmina meridionalis							O
Copepods							
Cyclopidae spp.	O	O	O				
Rotifers							
Bdelloid spp.					O		
Filinia longiseta				O	O	O	O
Gastropus hyptopus						O	
Keratella slacki					O	O	
Polyarthra dolichoptera	O	O	O			O	
Trichocerca similis	O	O					

Table 4.3. Zooplankton taxa with 10-50 individuals observed and detected in lake samples. O represents taxa that were observed in the samples, and detected in the MiSeq data.

	RC90	RC91	RC92	RC93	RC94	RC95	RC96
Cladocerans							
Bosmina meridionalis				O			
Copepods							
Cyclopidae spp.				O		O	O
Rotifers							
Anuraeopsis sp.							O
Asplanchna brightwelli				O			
Bdelloid spp.	O			O			
Brachionus angularis							O
Cephalodella ventripes					O		
Keratella slacki				O			
Polyarthra dolichoptera				O			O
Trichocerca similis			O				

Table 4.4. Taxa with 1-10 individuals observed in lake samples. O represents taxa that were observed in the morphological samples, and detected in the MiSeq data. X represents taxa that were observed in the morphological samples, but not detected in the MiSeq data.

	RC90	RC91	RC92	RC93	RC94	RC95	RC96
Cladocerans							
Alona c.f. *affinis*						O	
Daphnia galeata	O	O					
Copepods							
Cyclopidae spp.					O		
Rotifers							
Anuraeopsis sp.				O	X	O	
Asplanchna brightwelli						O	O
Bdelloid spp.		O	O			X	O
Brachionus angularis				O		O	
Brachionus calyciflorus		X			X	O	O
Brachionus urceolaris		X					
Cephalodella catellina			X				
Cephalodella forficula						O	
Cephalodella ventripes	X		X				
Colurella uncinata			X				
Dicranophoroides caudatus					X		
Euclanis deflexa					X		
Gastropus hyptopus				X			
Keratella slacki	O	O					O
Lecane bulla			X				
Lecane closterocerca		X	X		X		
Lecane furcata		X	X				
Lepadella acuminata		X	O		O		
Lepadella ovalis	X	X	X				
Pleurotrocha petromyzon					O		
Squatinella mutica	O		O				
Synchaeta oblonga							O
Synchaeta pectinata			X		X		X
Synchaeta stylata	X	X	X				
Trichocerca similis					O		
Trichocerca stylata				X			X
Trichotria tetractis					O		
Ostracods							
Ostracod spp.	O				O		O

Discussion

Metabarcoding is a potentially powerful tool that can provide valuable data on community composition with less effort and time than traditional taxonomic methods. Here, I have demonstrated using metabarcoding to monitor the community composition of zooplankton in freshwater ponds. A similar approach could be extended to zooplankton in other aquatic habitats including marine (e.g. Harvey et al., 2017).

A critical component of metabarcoding studies is access to reference sequences (Carugati et al., 2015, Dowle et al., 2016) held in databases such as BOLD and GenBank that have been generated from morphologically identified specimens. The small scale of the experiment allowed for the development of a comprehensive reference list of morpho-species found at the study site. An exception was the copepods as only one adult species was seen and morphologically identified (*Mesocyclops* spp.) in the study ponds through the sample collection period. Although copepod nauplii were abundant in the morphological counts, they are unidentifiable using morphology. Analysis of the reference library sequences showed that the nauplii were genetically distinct from the adult population (Woods et al. unpubl. data, Chapter 3). The samples sent for metabarcoding were dominated by morphologically unidentifiable nauplii, and generated sequence data predominantly from *Mesocyclops* spp., but also other cyclopoid taxa not identified through the morphological identifications. Difficulties in assigning sequences to their appropriate taxonomic classification is common in metabarcoding studies and

highlights the need for adequate reference libraries (Leray and Knowlton, 2015; Lindeque et al., 2013). By contrast, I had a very high success rate in identifying rotifers to species-level using the generated sequences, potentially due to the wide diversity of taxa collected and sequenced at the study sites, thus compiling a more comprehensive reference set.

There were also difficulties in identifying cladoceran species from the metabarcoding generated sequences. *Alona*, *Bosmina*, and *Daphnia* species were morphologically identified in the pond community samples, but were only identified only to class in the metabarcoding data. Brown et al., (2015) warn about using the same preclustering thresholds (i.e., 1 bp difference per 100 nucleotides) for zooplankton, suggesting that the high diversity warrants each taxonomic group (rotifers, cladocerans, copepods) to be treated independently. My data indicates that an adjustment in the preclustering step may be required with the cladoceran genera and will need to be further validated in the future.

The sequencing success rate (i.e. samples generating >70 zooplankton sequences) for the constructed communities was 50%, possibly as a consequence of the low number of individuals in the samples, or a problem with the extraction techniques used. By contrast, the pond samples had a higher success rate (70%), which could potentially be increased further if samples containing 1000’s of individual zooplankton were used, versus the sub-samples of approximately 300 individuals that were used.

There was some evidence of primer bias in detecting taxa within the samples. This was particularly true for the less abundant taxa, as some morpho species occurring in more than one counted sample were not successfully detected using the sequence data (e.g. *Synchaeta stylata*, *Cephalodella ventripes*, *Lecane closterocerca*, *Lecane furcata*, *Lepadella ovalis*), while others were detected (e.g. *Asplanchna brightwellii*, *Brachionus angularis*, *Daphnia galeata*, *Keratella slacki*, *Lepodella acuminate*, *Ostracod* spp. *Squatinella mutica*). The inability to recover sequences from less abundant species has also been documented in other studies (Dowle et al., 2016; Hajibabaei, et al., 2011). However, for the purposes of assessing ecosystem health, less abundant taxa, such as those making up less than 0.5% abundance, are often of little relevance, and can be removed from analyses to reduce the effect of species sampled by chance. The ability to detect the highly abundant and moderately abundant taxa within the samples was therefore encouraging and it seems likely metabarcoding can provide insight on the trophic status of the ecosystem (e.g. Duggan et al., 2001), as well as provide species richness, used in diversity indices. There were cases of DNA being sequenced when no individuals were counted in the morphological samples (false positives). This is not entirely suprising, as there would be undigested material in the stomachs of individuals which could produce sequences, or dead individuals not counted as part of the morphological sample. Aside from RC95, which had a large number of false positives, all samples contained low amounts that are unlikely to bias the results. Most of the error in RC95 came from one taxon (Ostracod; 11.37 of the 15.47%

error), which was most likely a missed or dead individual in the morphological sample.

There was also a PCR bias for physically larger taxa to be over-represented in the sequences. Other studies have shown a correlation between biomass and the number of sequence reads recovered (Dowle et al., 2016; Elbrecht and Leese, 2015; Hirai et al., 2015; Lindeque et al., 2013). In my study, the larger cyclopoid and cladoceran taxa generated considerably more sequences than the smaller rotifers in in the samples.

Estimates of taxon abundance through next-generation sequencing have been proposed (e.g. Deagle et al., 2013; Porazinska et al., 2010; Saitoh et al., 2016). However, my results provided no evidence for a link between taxon abundance and the number of sequences generated. In order to eliminate potential biomass bias, samples could be sorted or sieved into various size classes (Elbrecht et al., 2017) which could possibly reduce the bias in number of sequences generated due to differences in taxa size. However, my data suggested there may still be problems with assessing abundance even if similar sized taxa are used to characterise communities. For example, in one sample *P*. *dolichoptera* (approximately 125 µm in length) comprised 25% of the morphological counts but generated 70 sequences. The other abundant species, *T*. *similis* (approximately 200 µm in length; 24% of the morphological counts), generated 143 sequences. Accordingly, further study of taxon size relative to number of sequences generated will be required to advance the use of NGS approaches to assess abundance.

In summary, the D1 region of the LSU 28S gene can adequately determine species composition of zooplankton from natural pond samples. PCR amplification and number of sequences generated was linked to taxon size, rather than abundance, similar to other studies (Dowle et al., 2016; Hirai et al., 2015). The results also highlight the success of classifying sequence data to species-level for the rotifer taxa, which would be helpful to determine trophic levels of aquatic ecosystems (Duggan et al., 2001). However, in the context of using this methodology for use in ecological studies, the limitatons presented here far exceed the benefits compared to a morphological assessment. Until the limitations and biases are addressed it would be more beneficial to use morphological techniques to gain insight on the zooplankton community changes through time.

Acknowledgements

I thank technicians Matt Knox and Stacey Meyer in the Pacific Biosystematics Research Laboratory for their support in the lab. Thanks to Andrew Gilman for creating the taxonomy file and for helpful advice with the data analyses. Staff at New Zealand Genomics Limited provided support and advice with the next generation sequencing component.

Literature Cited

Abad, D., Albaina, A., Aguirre, M., Laza-Martínez, A., Uriarte, I., Iriarte, A., Villate, F. and A. Estonba. 2016. Is metabarcoding suitable for estuarine plankton monitoring? A comparative study with microscopy. Mar. Biol., 163(149), 13pp.

Brannock, P.M., Ortmann, A.C., Moss, A.G. and K.M. Halanych. 2016. Metabarcoding reveals environmental factors influencing spatio-temporal variation in pelagic micro-eukaryotes. Molecular Ecology, 25, 3593-3604.

Brown, E.A., Chain, F.J.J., Crease, T.J., MacIsaac, H.J. and M.E. Cristescu. 2015. Divergence thresholds and divergent biodiversity estimates: can metabarcoding reliably describe zooplankton communities? Ecology and Evolution, 5(11), 2234-2251.

Carugati, L., Corinaldesi, C., Dell'Anno, A. and R. Danovaro. 2015. Metagenetic tools for the census of marine meiofaunal biodiversity: an overview. Marine Genomics, 24, 11-20.

Chain, F.J.J., Brown, E.A., MacIsaac, H.J. and M.E. Cristescu. 2016. Metabarcoding reveals strong spatial structure and temporal turnover of zooplankton communities among marine and freshwater ports.

Clarke, K.R. and R.N. Gorley. 2006. PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth, UK, 192pp.

Deagle, B.E., Thomas, A.C., Shaffer, A.K., Trites, A.W. and S.N. Jarman. 2013. Quantifying sequence proportions in a DNA-based diet study using Ion Torrent amplicon sequencing: which counts count? Molecular Ecology Resources, 13, 620-633.

Deagle, B.E., Jarman, S.N., Coissac, E., Pompanon, F. and P. Taberlet. 2014. DNA metabarcoding and the cytochrome *c* oxidase subunit I marker: not a perfect match. Biology letters, 10, 4pp.

De Barba, M., Boyer, C.M.F., Mercier, C., Rioux, D., Coissac, E. and P. Taberlet. 2013. DNA metabarcoding multiplexing and validation of data accuracy for diet assessment: application to omnivorous diet. Molecular Ecology Resources, 18pp.

de Vargas, C., Audic, S., Henry, N., Decelle, J., Mahé, F., Logares, R., et al. 2015. Eukaryotic plankton diversity in the sunlit ocean. Science, 348, 1261605-1/11.

Dowle, E.J., Pochon, X., Banks, J.C., Shearer, K. and S.A. Wood. 2016. Targeted gene enrichment and high-throughput sequencing for environmental biomonitoring: a case study using freshwater macroinvertebrates. Molecular Ecology Resources, 16, 1240-1254.

Duggan, I.C., Green, J.D. and R.J. Shiel. 2001. Distribution of rotifers in North Island, New Zealand, and their potential use as bioindicators of lake trophic state. Hydrobiologia, 446/447, 155-164.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C. and R. Knight. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics, 27(16), 2194-2200.

Elbrecht, V., Taberlet, P., Dejean, T., Valentini, A., Usseglio-Polatera, U., Beisel, J., Coissac, E., Boyer, F. and F. Leese. 2016. Testing the potential of a ribosomal 16S marker for DNA metabarcoding of insects. PeerJ. 12pp.

Elbrecht, V., Peinert, B. and F.Leese. 2017. Sorting things out-assessing effects of unequal specimen biomass on DNA metabarcoding. Peer J Preprints.

Folmer, O., Black, M., Hoeh, W., Lutz, R. and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology, 3(5), 294-299.

Fontaneto, D. 2014. Molecular phylogenies as a tool to understand diversity in rotifers. International Review of Hydrobiology, 99, 178-187.

Gibson, J., Shokralla, S., Porter, T.M., King, I., van Konynenburg, S., Janzen, D.H., Hallwachs, W. and M. Hajibabaei. 2014. Simultaneous assessment of the microbiome and microbiome in a bulk sample of tropical arthropods through DNA metasystematics. PNAS, 111(22), 8007-8012.

Hajibabaei, M., Shokralla, S., Zhou, X., Singer, G.A.C. and D.J. Baird. 2011. Environmental barcoding: a next-generation sequencing approach for biomonitoring applications using river benthos. PLos ONE, 6(4), 7pp.

Harvey, J.B.J., Johnson, S.B., Fisher, J.L., Peterson, W.T. and R.C. Vrijenhoek. 2017. Comparison of morphological and next generation DNA sequencing methods for assessing zooplankton assemblages. Journal of Experimental Marine Biology and Ecology, 487, 113-126.

Hebert, P.D.N., Cywinska, A., Ball, S.L. and J.R. deWaard. 2003. Biological identifications through DNA barcodes. Proc. R. Soc. Lond., 270, 313-321.

Hirai, J., Shimode, S. and A. Tsuda. 2013. Evaluation of ITS2-28S as a molecular marker for identification of calanoid copepods in the subtropical western North Pacific. J. Plankton Res., 35(3), 644-656.

Hirai, J., Kuriyama, M., Ichikawa, T., Hidaka, K. and A. Tsuda. 2015. A metagenetic approach for revealing community structure of marine planktonic copepods. Molecular Ecology Resources, 15, 68-80.

Hynes, H.B.N. 1970. The ecology of running waters. Liverpool University Press, Liverpool. 555pp.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K. and P.D. Schloss. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the

MiSeq Illumina sequencing platform. Applied and Environmental Microbiology, 79(17), 5112-5120.

Leray, M. and N. Knowlton. 2015. DNA barcoding and metabarcoding of standardized samples reveal patterns of marine benthic diversity. PNAS, 112(7), 2076-2081.

Lindeque, P.K., Parry, H.E., Harmer, R.A., Somerfield, P.J. and A. Atkinson. 2013. Next generation sequencing reveals the hidden diversity of zooplankton assemblages. PLoS ONE, 8(11), 14pp.

Machida, R.J. and N. Knowlton. 2012. PCR primers for metazoan nuclear 18S and 28S ribosomal DNA sequences. PLoS ONE, 7(9), 11pp.

Pearman, J.K. and X. Irigoien. 2015. Assessment of zooplankton community composition along a depth profile in the Central Red Sea. PLoS ONE, 10(7), 14pp.

Pimm, S.L., Jenkins, C.N., Abell, R., Brooks, T.M., Gittleman, J.L., Joppa, L.N., Raven, P.H., Roberts, C.M. and J.O. Sexton. 2014. The biodiversity of species and their rates of extinction, distribution, and protection. Science, 344 (6187), 1246752-1-1246752-10.

Porazinska, D.L., Sung, W., Giblin-Davis, R.M. and W.K. Thomas. 2010. Reproducibility of read numbers in high-throughput sequencing analysis of nematode community composition and structure. Molecular Ecology Resources, 10, 666-676.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and F.O. Glöckner. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research, 41, D590-D596.

Ratnasingham, S. and P.D.N. Hebert. 2007. BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). Molecular Ecology Notes, 7, 355-364.

Saitoh, S., Aoyama, H., Fujii, S., Sunagawa, H., Nagahama, H., Akutsu, M., Shinzato, Kaneko, N. and T. Nakamori. 2016. A quantitative protocol for DNA metabarcoding of springtails (Collembola). Genome, 59, 705-723.

Sala, O.E., Chapin, F.S., Armesto, J.J., Berlow, E., Bloomfield, J., Dirzo, R., Huber-Sanwald, E., Huenneke, L.F., Jackson, R.B., Kinzig, A. et al. 2000. Global biodiversity scenarios for the year 2100. Science, 287, 1770-1774.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., et al. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol., 75(23), 7537-7541.

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C. and E. WIllerslev. 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. Molecular Ecology, 21, 2045-2050.

van Dijk, E.L., Auger, H., Jaszczyszyn, Y. and C. Thermes. 2014. Ten years of next-generation sequencing technology. Trends in Genetics, 30(9), 418-426.

Wright, J. F., Moss, D., Armitage, P. D., & Furse, M. T. (1984). A preliminary classification of running-water sites in Great Britain based on macro-invertebrate species and the prediction of community type using environmental data. Freshwater Biology, 14, 221-256

Supplementary Information

Supplementary Table 4.1. Comparison of species counted in morphological assessment (morpho) and the number of sequences generated (NGS) for the natural pond samples.

	RC90		RC91		RC92		RC93		RC94		RC95		RC96	
	NGS	**Morpho**	**NGS**	**Morpho**	**NGS**	**Morpho**	**NGS**	**Morpho**	**NGS**	**Morpho**	**NGS**	**Morpho**	**NGS**	**Morpho**
Cladocerans														
Branchiopoda	5563		459		2		20083		3		44		44410	
Alona c.f. affinis												1		
Bosmina meridionalis								47						307
Daphnia galeata		8		2										
Copepods														
Copepod nauplii		222		258		170		33		4		9		14
Boeckella minuta											1		3	
Boeckella montana													1	
Skistodiaptomus pallidus											1		1	
Acanthocyclops robustus	28		22		3		3				1		2	
Eucyclops serratulus	7		5		3						2		2	
Mesocyclops spp.	4288	18	1733	18	1102	15	556	3	124	2	89	2	11	4
Harpacticoida	132		60		32		10		14	1	1		82	
Ostracods														
Ostracoda	126	1							1	1	147		123	1
Rotifers														
Anuraeopsis spp.							6	3		8	14	5	6	17
Anuraeopsis fissa							2							
Asplanchna brightwelli							198	14			11	6	15	3
Asplanchna sieboldi			1										1	
Bdelloid spp.		14		9		4		23		65		1		2
Brachionus angularis							14	5			5	1	5	12

Supplementary Table 4.1 (continued). Comparison of species counted in morphological assessment (morpho) and the number of sequences generated (NGS) for the natural pond samples.

	RC90		RC91		RC92		RC93		RC94		RC95		RC96	
	NGS	**Morpho**	**NGS**	**Morpho**	**NGS**	**Morpho**	**NGS**	**Morpho**	**NGS**	**Morpho**	**NGS**	**Morpho**	**NGS**	**Morpho**
Brachionus calyciflorus				1			96			1	28	1	2	3
Brachionus urceolaris				1										
Cephalodella catellina						2								
Cephalodella forficula											1	1		
Cephalodella gibba			4						14				2	
Cephalodella ventripes		2				1			70	40			1	
Collothecidae spp.									2		16		7	
Colurella uncinata	3					2					1			
Conochilus unicornis			2											
Cupelopagis vorax	4													
Dicranophoroides caudatus									1			1		
Epiphanes macrourus					1						14			
Euclanis deflexa										4				
Filinia longiseta			1		1		776	140	498	121	358	100	120	252
Filinia novaezelandia	1						1							
Filinia terminalis							7				5		1	
Gastropus hyptopus	1							2			35	160		
Hexarthra mira											1			
Keratella cochlearis					1				1					
Keratella slacki	10	4	8	5	1		17	15	2957	112	501	113	8	2
Keratella tecta	9		18		22		1				7		1	
Lecane bulla						1			2					
Lecane closterocerca	1			2		2				1				
Lecane furcata				1		1								

Supplementary Table 4.1 (continued). Comparison of species counted in morphological assessment (morpho) and the number of sequences generated (NGS) for the natural pond samples.

	RC90		RC91		RC92		RC93		RC94		RC95		RC96	
	NGS	**Morpho**	**NGS**	**Morpho**	**NGS**	**Morpho**	**NGS**	**Morpho**	**NGS**	**Morpho**	**NGS**	**Morpho**	**NGS**	**Morpho**
Lepadella acuminata				1	3	1			6	1				
Lepadella ovalis		2		5		3					5			
Monommata spp.									1				1	
Pleurotrocha petromyzon	7				1				54	5	1		11	
Polyarthra dolichoptera	70	134	34	161	7	123	7	16			1	63	4	46
Rotaria neptunia	46		2		5		30		192				1	
Rotaria rotatoria									2					
Rotaria socialis	6													
Rotaria tardigrada											2			
Squatinella mutica	2	1	3		1	3					1		1	
Synchaeta longipes			2										2	
Synchaeta oblonga	6								14				17	1
Synchaeta pectinata	2		20			1				2				2
Synchaeta stylata		2		4		8								
Trichocerca porcellus													2	
Trichocerca pusilla	1						4							
Trichocerca similis	143	126	14	81	11	28			4	4				
Trichocerca stylata								3						1
Trichocerca tenuior					1									
Trichotria tetractis			2				3		1	1			20	

CHAPTER 5: THESIS CONCLUSIONS

Thesis summary

With the threat of increasing eutrophication in New Zealand, new national policy initiatives have been implemented to protect and improve aquatic ecosystems, which include measures to manage nutrient inputs, and maintain or improve overall water quality (Ministry for the Environment, 2017). Common carp can enhance eutrophication processes and shift ecosystems into a degraded state, resulting in more effort required for remediation. Thus, the presence of common carp, or a threat of a new introduction, should be considered in management plans. However, to date, there has been a lack of experimental based studies of sufficient scope and scale to determine the effects common carp have on the dynamics of aquatic ecosystems in New Zealand. Using zooplankton as indicating taxa can provide insight into water quality and can be used to track trophic shifts over time (e.g. Duggan et al., 2001). However, using zooplankton as indicators requires effort and expertise and would benefit from a more expedited approach. DNA-based approaches have a benefit of being able to sample upwards of 100 samples simultaneously. A switch to a DNA-based approach could save time and require less effort from trained taxonomists once a reference library is compiled. However, DNA-based approaches are not yet readily used in ecology, and most taxa remain untested for their suitability. The work presented in this thesis describes ecosystem dynamics after an introduction of carp and outlines

an approach to using DNA-based techniques to sample the zooplankton communities used in water quality monitoring.

Multivariate analyses revealed no significant difference in the zooplankton communities between the control and treatment pond following carp addition (Chapter 2). Zooplankton communities in both ponds changed through time and were associated with increased suspended sediments and smaller particle sizes in the water column. A significant increase in inorganic suspended sediments and nitrogen concentrations was found in the treatment pond after the addition of carp. However, sediment and nutrient concentrations were at high levels prior to the carp introduction, and the minimal increase after introduction was unlikely to have any effects on the overall ecosystem dynamics. Despite the high density of fish introduction, the lack of drastic changes indicates that ecosystems in a degraded state are quite resilient to change. In the Waikato region, approximately 50% of lakes are eutrophic (Verburg et al., 2010), and many contain carp. Remediation efforts in these degraded ecosystems may be more problematic because of the resilience to change, and efforts should not only focus on removing carp biomass, but a holistic, catchment-based approach to reduce sediment and nutrient loading into lakes.

The 28S rDNA gene proved to be a successful marker for identifying zooplankton (Chapter 3). 28S achieved a higher sequencing success rate than that of the routinely used COI marker, and was able to distinguish between species despite the lower divergence values. There

was little primer bias, with all targeted taxa successfully sequenced. For the purposes of assessing water quality based on zooplankton taxa, 28S is an acceptable marker. Further, there was an indication that detection of cryptic species may be possible by targeting this region. Interestingly, the juvenile copepods found in the ponds formed a distinct group, separate from the adult population, suggesting multiple species of cyclopoid copepods inhabited the ponds, and highlights the benefits of using a DNA-based identification approach to identify morphologically similar species. It was deemed appropriate that the sequence library compiled in this Chapter be used as a reference for a metabarcoding study conducted on the zooplankton communities in the experimental ponds.

The metabarcoding study (Chapter 4) was able to generate sequences for all abundant taxa found in the morphological samples. Of the medium abundant taxa, only one species was not detected in the sequences. However, primer biases were apparent in the low abundant taxa, with 64% of the species identified in the morphological samples not detected in the sequences. There were a low number of false positives in the samples, and these were most likely body parts, dead individuals, or possibly stomach contents of the counted individuals. Because the false positives were low, it is unlikely that they biased the samples. The rotifers were the most diverse group with 30 species identified in the morphological counts and could be identified to the species level from the sequences generated. Cladocerans and copepods were predominantly identified to class, and order, respectively. The difficulties in assigning sequences to species indicates a need for a comprehensive reference

library, and/or an adjustment of the pre-clustering step in the analysis for cladocerans and copepods.

The approaches used in the latter chapters were designed to test whether a DNA-based approach is comparable to the traditional morphological techniques. Without data on abundance, the data is not comparable, and does not appear to be useful in ecological studies. The results from the carp addition study show a change in the zooplankton community over time, which would not be picked up in the genetic analyses. Finding methods to limit the PCR bias correlated with biomass would greatly help the science moving forward. However, based on the success of identifying rotifers, the metabarcoding analysis shows promise for use in management; identifying rotifer taxa indicative of trophic state.

Future directions

The data in Chapter 2 supports previous studies showing increases in suspended sediments (e.g. Akhurst et al., 2012; Angeler et al., 2007) and nutrient concentrations (e.g. Chumchal and Drenner, 2004; Matsuzaki et al., 2007) following an introduction of carp. It also provides insights into how stable states of aquatic ecosystems influence physical, chemical, and biological variables, and explains why the variables may not respond as predicted after an introduction. Further research should be conducted on lakes with varying levels of degradation to understand the processes that influence resilience, as well as determine the varying levels of ecological disturbance following a carp introduction. Carp will likely continue to spread, and colonize new lakes in New Zealand, and will present an

opportunity to explore these research avenues. Especially important sites would have high densities of large filter feeding cladocerans like *Daphnia* spp. and an abundance of submerged macrophytes. Monitoring changes before and after new carp introductions in these lakes would provide valuable information to the potential destructive capabilities of carp in New Zealand.

Chapter 3 forms a foundation for future metabarcoding studies designed for use in biological monitoring. Continual work is required to build comprehensive reference libraries. This work should be broadened to include, at a minimum, the entire Waikato region, if not all of the North and South Islands of New Zealand. This will help determine phylogenetic relationships between other closely related species not found in the experimental ponds and give further supporting or refuting evidence of 28S as a suitable marker for metabarcoding studies. As some of the taxa had high intra-specific divergence values (e.g. *Testudinella patina*), it may be possible that cryptic species can be identified using 28S. Based on COI sequences, data suggests possible cryptic speciation in New Zealand rotifers (e.g. *Polyarthra dolichoptera*, *Trichocerca similis*, *Lecane bulla*; Collins, unpublished data; Gilman, unpublished data). Comparing divergence values between COI and 28S in these taxa would give an indication if 28S is indeed sensitive enough to identify cryptic speciation. However, a more important aspect of this is linking environmental data to cryptic diversity. Care should be taken to identify micro-habitats and specific physical and chemical variables found at collection sites. If cryptic species live in distinct environments, a DNA-based method of zooplankton

identification could be a more sensitive method in determining water quality, rather than morphological identifications that are unable to distinguish between taxa. However, if taxa live in sympatry, a region that has little primer bias (e.g. 28S) would be a better genetic marker to use over one that requires multiple primers for amplification (e.g. *COI*).

In metabarcoding analyses, more research is required to determine appropriate clustering protocols to properly assign generated sequences to their designated taxon. As suggested by Brown et al., (2015), copepods, cladocerans, and rotifers may need to be treated individually and assigned varying levels of clustering methods. Identifying taxa to species level is essential if metabarcoding is to be used as a tool for ecological studies. For example, research has shown that high carp biomass is negatively associated with large cladocerans (e.g. *Daphnia* spp.), with small cladocerans (e.g. *Bosmina* spp.) remaining unaffected. The data was generally unable to distinguish between either taxa, and would miss out on crucial data in aquatic systems affected by a new introduction of carp. Another major limitation to current metabarcoding techniques is the inability to obtain abundance estimates from generated sequences. My data was able to identify the abundant zooplankton species which gives an indication of the trophic status of lakes (Duggan et al., 2001). However, abundance estimates would be beneficial to track changes over time and capture the more subtle zooplankton community changes, especially if different stressors (e.g. carp presence) are introduced. Thus, different methodologies to estimate abundance should continue to be tested (e.g.

Dowle et al., 2016) to demonstrate that DNA-based approaches can be a useful, and feasible option for monitoring of aquatic ecosystems.

Literature Cited

Akhurst, D.J., Jones, G.B., Clark, M. and A. Reichelt-Brushett. 2012. Effects of carp, gambusia, and Australian bass on water quality in a subtropical freshwater reservoir. Lake and Reservoir Management, 28(3), 212-223.

Angeler, D.G., Sánchez-Carrillo, S., Rodrigo, M.A., Alvarez-Cobelas, M. and C. Rojo. 2007. Does seston size structure reflect fish-mediated effects on water quality in a degraded semiarid wetland? Environ. Monit. Assess., 125, 9-17.

Brown, E.A., Chain, F.J.J., Crease, T.J., MacIsaac, H.J. and M.E. Cristescu. 2015. Divergence thresholds and divergent biodiversity estimates: can metabarcoding reliably describe zooplankton communities? Ecology and Evolution, 5(11), 2234-2251.

Chumchal, M.M. and R.W. Drenner. 2004. Interrelationships between phosphorus loading and common carp in the regulation of phytoplankton biomass. Arch. Hydrobiol., 161(2), 147-158.

Dowle, E.J., Pochon, X., Banks, J.C., Shearer, K. and S.A. Wood. 2016. Targeted gene enrichment and high-throughput sequencing for environmental biomonitoring: a case study using freshwater macroinvertebrates. Molecular Ecology Resources, 16, 1240-1254.

Duggan, I.C., Green, J.D. and R.J. Shiel. 2001. Distribution of rotifers in North Island, New Zealand, and their potential use as bioindicators of lake trophic state. Hydrobiologia, 446/447, 155-164.

Matsuzaki, S.S., Usio, N., Takamura, N. and I. Washitani. 2007. Effects of common carp on nutrient dynamics and littoral community composition: roles of excretion and bioturbation. Archiv für Hydrobiology, 168(1), 27-38.

Ministry of the Environment. 2017. National Policy Statement for Freshwater Management 2014 (Amended 2017). Publication ME 1324, 47 pp.

Verburg, P., Hamill, K., Unwin, M. and J. Abell. 2010. Lake water quality in New Zealand 2010: Status and trends. NIWA Client Report: HAM2010-107, National Institute of Water and Atmospheric Research Ltd. Hamilton, NZ.