USING ZOOPLANKTON METABARCODING FOR THE ECOTOXICOLOGICAL ASSESSMENT OF REMEDIATION PRACTICES FOR A SIMULATED PETROLEUM SPILL IN A BOREAL LAKE

A Thesis Submitted to the College of
Graduate and Postdoctoral Studies
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
In the Department of Toxicology
University of Saskatchewan
Saskatoon

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ABSTRACT

After oil spills occur, regulators require adequate information to select best practices to minimize impacts on environments and to remediate target freshwater ecosystems. Zooplankton are valuable indicators of structure and function of aquatic ecosystems since they play pivotal roles in biochemical cycles while stabilizing food webs. Traditional identification of zooplankton can be costly and time-consuming, while also being difficult to standardize. Compared with classification of individuals by identification, based on visual inspection of morphology, metabarcoding of DNA and or RNA has promise for cost-effective high-throughput and benchmarkable biomonitoring of zooplankton communities. These identification methods were applied in the context of assessing responses of the zooplankton community exposed to simulated spills of diluted bitumen (dilbit), with concurrent exposure of experimental remediation practices of enhanced monitored natural recovery and shoreline cleaner application. The objective of this study was also to apply DNA and RNA metabarcoding of zooplankton for ecotoxicological assessment and compare it with traditional morphological identification in experimental shoreline enclosures in a boreal lake. Metabarcoding detected 77.4% of the morphologically identified boreal zooplankton taxa down to the genus level, with a total of 24 shared genera. Metabarcoding-based relative abundance of shared genus also served as an acceptable proxy for biomass inferred by morphological identification at the genera-level. Overall, both DNA and RNA metabarcoding determined significant differences between genera richness between the no treatment enclosure and shoreline cleaner application, while morphological identification determined no difference. DNA metabarcoding determined overall differences in community composition between no treatment and treatments, shoreline cleaner application and enhanced monitored natural recovery, while RNA metabarcoding and morphological identification determined differences between one or the other. Shoreline cleaner application overall seemed to have the greatest effect on zooplankton communities relative to enhanced monitored natural recovery, regardless of zooplankton identification method. Both metabarcoding and morphological identification were able to discern the differences between the two experimental remediation practices. Metabarcoding of zooplankton can

provide informative results for ecotoxicological assessment of remediation practices of dilbit, advancing our knowledge of best practices for remediating oil-impacted aquatic ecosystems while serving to accelerate the assessment of at-risk freshwater ecosystems.

ACKNOWLEDGMENTS

I would first like to thank my committee members, Dr. Markus Brinkmann, Dr. Markus Hecker, and Dr, Paul Jones, for the time and expertise they have contributed throughout my thesis as well as my external examiner, Dr. Margaret Docker, for the helpful comments and questions regarding this thesis. Thank you to my supervisor Dr. John P. Giesy, for his support and patience throughout my master's thesis. The long talks regarding science and life have been helpful and important in the formation of this thesis. Dr. Yuwei Xie, thank you for providing me with your guidance, teaching me the methods and tools, and allowing me to play with data to help build and develop my current skill set. Thank you, Dr. Abigail Debofsky, for teaching me the basics of molecular biology and providing me with the tools and direction to begin the analysis of data, and for the support during the research process.

I would like to thank the GWF for providing me the funding for my stipend during my thesis research. I would also like to thank the Toxicology Centre for providing additional funding via the Toxicology Devolved Scholarship. This project would also not have been possible without my family and friends, each of whom made their unique contributions. Thank you to my fellow lab mates, including Alana Weber, for her support, including casual discussions after running into errors either via command line or in RStudio. A general thank to all the graduate students at the Toxicology Centre for making the time in Saskatoon an enjoyable experience. Thank you to Dr. Jon Challis and Dr. Yufeng Gong for giving me chemistry experiences outside of my thesis, as learning new ideas is always imperative to scientific thought. A special thanks to Alper James Alcaraz, who allowed me to work with him on qPCR and library preparation of his samples while teaching me the methods, and for training me on the basics of command line and bioinformatics. I would finally like to thank Dr. Stephanie Graves and Emily Kennedy for their assistance in the collection of samples.

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LIST OF ABBREVIATIONS

µL L-1	Micro-Liter per Liter
µm	Micrometer
18s rRNA	18S ribosomal RNA
α	Alpha
BOLD	Barcode of Life Data Systems
bp	Base Pair
BTEX	Benzene, Toluene, Ethylbenzene, Xylene
β	Beta
cDNA	Complementary DNA
CEWAF	Chemically Enhanced Water Accommodated Fraction
CLB	Cold Lake Blend
cm	Centimeter
COI	Mitochondrial Cytochrome Oxidase 1 Gene Region
Dilbit	Diluted Bitumen
DNA	Deoxyribonucleic Acid
eDNA	Environmental DNA
ee	Expected Error
ELA	Experimental Lakes Area
eMNR	Enhanced Monitored Natural Recover
eRNA	Environmental RNA
g	Gram
g/L	Gram per Liter
h	Hour(s)
IC25	Inhibition Concentration 25%
IISD	International Institute of Sustainable Development
L	Liter

LC50	Median Lethal Dose
LCA	Lowest Common Ancestor
min	Minutes
mL	Milliliter
morph-taxa	Morphological Taxonomy
m	Meter
mg/L	Milligram per Liter
n	Sample Size
ng/µL	Nanogram per Microliter
ORF	Open Reading Frames
OTU	Operational Taxonomical Unit
PAHs	Polycyclic Aromatic Hydrocarbons
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
ppm	Parts Per Million
QC	Quality Control
REF	Reference
ρ	Rho
RNA	Ribonucleic Acid
SCA	Shoreline Cleaner Application
SWA	Shoreline Washing Agent
tPAH	Total Polycyclic Aromatic Hydrocarbons
WAF	Water Accommodated Fraction
ZOTU	Zero-radius Operational Taxonomic Unit

NOTE TO READERS

This thesis is organized and formatted to follow the University of Saskatchewan College of Graduate Studies and Research guidelines for a manuscript-style thesis. Therefore, there is some repetition of material presented in each chapter. Chapter 1 is a general introduction and literature review, including project goals and objectives. Chapter 2 is organized as a manuscript for publication in a peer-reviewed scientific journal and a description of author contributions is provided following the preface for this chapter. Chapter 3 contains a general discussion and overall conclusion. References cited in each chapter are combined and listed in the References section of the thesis. Supporting information associated with the research chapter are presented in the Supporting Information section at the end of this thesis.

CHAPTER 1

BACKGROUND AND REVIEW OF PERTINENT LITERATURE

1.1 Biodiversity

Biodiversity is substantial for the wellbeing of humans, as it provides invaluable functioning to ecosystems and various goods and services to society (Cardinale et al., 2012; Díaz et al., 2006). Biodiversity, or biological diversity, is the complex interactions and sum of all biotic variations of life on all scales, including genotypes, species, populations, and ecosystems (Díaz et al., 2006; Gaston, 2000; Purvis and Hector, 2000). Global biodiversity has been declining in recent decades due to human influence, which along with other marked changes in the ecosphere, including biogeochemistry, indicates entrance into a new geological epoch termed by some the Anthropocene (Crutzen, 2006; Dirzo et al., 2014; Sax and Gaines, 2003). These changes in biodiversity can be attributed, in part, to the continuing destruction of habitat, introductions of exotic/invasive species, anthropogenic pollution, and changes in climate (Sala et al., 2000; Sax and Gaines, 2003). Changes in biodiversity and the drivers of change are measured by using unique indicators, including the state of biodiversity, pressures on biodiversity, and responses of biodiversity (Birk et al., 2012; Butchart et al., 2010; Friberg et al., 2011; Zhou et al., 2008). Since decreases in biodiversity pose risks to functions of and services provided by ecosystems, inventorying biodiversity, and estimating rates of change are critical for the assessment of ecosystem health and integrity (Bourlat et al., 2013; Cardinale et al., 2012; Hooper et al., 2005; Lefcheck et al., 2015).

1.1.1 Ecosystem Function and Services

Research into the link between biodiversity and ecosystem function is important, to better understand how biodiversity impacts ecosystems surged during the 1990s (Cardinale et al., 2012; Paquette and Messier, 2011; Schulze and Mooney, 2012). Ecosystem functions refer to natural processes of ecosystems, which can include amounts of energy and material stocks, flows of energy and materials and variations in those stocks over time (Hébert et al., 2017). Niche complementarity, the ability of species to co-exist due to different forms of resources, has been shown to enhance ecosystem function (Cardinale, 2011; Maherali and Klironomos, 2007; Paquette and Messier, 2011). This property is due to how species'

individual functional characteristics can greatly influence ecosystem properties by shaping flows of energy and materials (Chapin et al., 1998; Hooper et al., 2005). Cycling of nutrients is critical for the viability of ecosystems (DeAngelis et al., 1989; Vanni, 2002).

A recent study investigated how phytoplankton species richness and class richness determined biotransformation of a mixture of 37 structurally diverse pollutants, with a positive effect of species richness and class richness on the number of transformed products observed (Stravs et al., 2019). This indicates that a more diverse species composition can lead to greater biotransformation of toxic pollutants. An increase in richness of algal species can also increase efficiencies with which algae can assimilate inorganic resources into standing biomass (Cardinale et al., 2011). Past studies have also shown that species diversity dictates the resistance of ecosystems to environmental change, including pollution from activities of humans (Chapin et al., 1998; Jung et al., 2016; Walker et al., 1999). Species-rich communities are also better able to perform multiple ecosystem functions (Lefcheck et al., 2015).

Species also provide other services, including the case of ecosystem engineers, which are defined as organisms directly or indirectly influencing availabilities of resources to other species by modifying current or creating new habitats (Jones et al., 1994, 1997; Wright et al., 2002). Examples of this include freshwater phytoplankton intercepting light leading to shallower mixing depths or bioturbation by freshwater protozoa to change properties, such as oxygenation, of lake sediment (Jones et al., 1994).

1.1.2 Assessment of Biodiversity

Spatial analyses of biodiversity typically include measurement of the number of species observed or estimated to occur in an area, which is termed species richness (Gaston, 2000; Gotelli and Colwell, 2001). Species richness is a fundamental measurement of spatial patterns, of community and regional diversity of the number of species in an area (Gotelli and Colwell, 2001; Whittaker et al., 2001). Species richness is an instinctive and natural index of structures of communities that can also provide baselines for restorations of impacted sites (Gotelli and Colwell, 2011). The richness of species in local communities can be quantified

by alpha (α) diversity, whereas differences in species between or among communities are quantified by beta (β) diversity (Whittaker et al., 2001).

Alpha diversity is a measure of average single-location or single-community diversity, and beta diversity is a measure of relative difference in species composition between or among communities, with alpha and beta being independent (Jost, 2007, 2010). Alpha diversity can be measured by species richness, species evenness, a measure of how close in relative abundance species are in a defined area, or a manipulated combination of both, which includes the Shannon and Simpson indexes of diversity. The Shannon index of diversity estimates the average uncertainty of predicting which species will occur in a random subsample from an environment of interest and theoretically can range from zero (0) to infinity (Nagendra, 2002; Shannon, 1948). Simpson index is the probability of two equal samples having a different species detected and ranges from 0 to 1 (Nagendra, 2002; Simpson, 1949).

Beta diversity focuses on the turnover of species between sampling units and community composition through space or time (Koleff et al., 2003). Beta diversity represents changes in composition of species between two or more local and regional assemblages (Koleff et al., 2003). Presence-absence or count data can be used to compute distances or differences between or among assemblages of interest. Count data is commonly transformed or normalized for community data, especially in the case of microbial community sequence data to satisfy the requirements of compositional and sparse data (Barwell et al., 2015; Gloor et al., 2017). The Jaccard distance, which uses presence-absence data, is a common metric used for computing beta diversity between locations (Jaccard, 1912). A novel approach that can take into account both the relative counts or presence-absence of the community composition uses phylogenetic information to compute beta diversity between locations or assemblages of interest (Lozupone et al., 2011). Overall, when selecting a metric or index for beta diversity analysis, care needs to be taken in selecting the proper method for the community data being analyzed, as the metric/index can influence the results inferred due to the nature of the data itself as well as the assumptions of the metric/index (Anderson et al.,

2011). These two measurements, namely alpha and beta diversity, have become commonplace for characterizing biological diversity.

1.2 Novel Biological Monitoring Techniques

Aquatic biomonitoring techniques currently include measurements of biodiversity and the use of bioindicators. Long-term and large comprehensive data sets are often required for describing the status and predicting trends in structures of aquatic ecosystems in a changing world (Dodds et al., 2012; Hampton et al., 2013; LaDeau et al., 2017). This form of conventional biological monitoring of ecosystems is costly and time-consuming, as discerning taxa can be difficult to conduct, and the collection of biological samples can be tedious (Kelly et al., 2014). Traditional monitoring can also be invasive to the ecosystem or species under study, as it requires active searching and collection that can disrupt the target ecosystem or harm the biological organism (e.g., fishnets) (Kelly et al., 2014; Thomsen and Willerslev, 2015). Finally, the number of taxonomists able to accurately and efficiently identify environmental species has been in decline in recent decades (Pearson et al., 2011; Wägele et al., 2011). Declining number of taxonomists is of concern, with many species remaining undescribed and the benchmarking of taxa identification via conventional morphological approaches difficult to standardize. With growing concerns of changes in species assemblages due to environmental stressors and declines in biodiversity, more efficient techniques to measure these changes are warranted (Barnosky et al., 2011).

Genomics tools, such as metabarcoding or metagenomics, offer a high-throughput method for assessing community dynamics within aquatic ecosystems. DNA Barcodes were initially the primary molecular method used for species identification (Hebert et al., 2003). Barcodes consist of larger fragments (e.g., 500 bp) and are commonly used by researchers to identify taxa to the species-level (Cristescu, 2014). This method, however, was limited to screening one organism at a time. Development of metabarcoding has allowed for the high-throughput identification of organisms within target communities (Ji et al., 2013). This new approach did come with its limitations, including PCR biases, database gaps, and data

processing validation, which is an active area of research (Cristescu, 2014). The two methods, namely barcoding and metabarcoding, should be treated as complementary methods, with barcoding being used to build high-confident public databases to be used with metabarcoding approaches (Cristescu, 2014).

Metabarcoding provides an efficient and data-intensive method of capturing local biodiversity through detection of aquatic organisms via next-generation sequencing (Taberlet et al., 2012b; Valentini et al., 2016). Metabarcoding is the use of short amplicon sequences to detect taxa present in a community (Ji et al., 2013). This is achieved by using specific primers for the target community of interest or using universal primers for detection of several taxa communities. These standardized primers are used to amplify targeted genes of an organism to differentiate taxa (Taberlet et al., 2012b). After short regions (typically 200-450 bp) are amplified by polymerase chain reaction (PCR), samples are analyzed by use of next-generation sequencing. Next-generation sequencing instruments are provided by companies and technologies such as Illumina, Oxford Nanopore Technologies, and Pacific Biosciences, among others (Goodwin et al., 2016). After samples are sequenced, bioinformatics and data processing, including taxonomic annotation, are conducted to elucidate the structure of the community from which the sequences were derived. Metabarcoding includes the use of community DNA from homogenized tissues or environmental DNA (eDNA) from environmental samples (Hajibabaei et al., 2011; Taberlet et al., 2018; Taberlet et al., 2012a).

1.2.1 Environmental DNA and DNA Metabarcoding

eDNA refers to the complex genetic material released into the environment that can be obtained directly from environmental samples without the obvious presence of organisms (Creer et al., 2016; Thomsen and Willerslev, 2015). eDNA consists of either exogenous DNA or endogenous DNA that can be extracted from environmental samples without direct isolation of any target organisms (Taberlet et al., 2012a). The first reference to environmental DNA was in 1987 on the idea of extracting microbial DNA from sediments (Ogram et al., 1987). Since then, the technique has grown in use and applications, especially in the last

decade with advances in DNA sequencing techniques (Kelly et al., 2014; Thomsen and Willerslev, 2015). Total eDNA refers to intracellular DNA and extracellular DNA that can be found in samples from the environment (Barnes and Turner, 2016; Pietramellara et al., 2009). Intracellular DNA refers to DNA that originated from living cells or organisms and is usually of good quality, whereas extracellular DNA refers to DNA released from cells after death and destruction of cell structure, typically being degraded or in small fragments (Creer et al., 2016). Both types of DNA can be collected from the environment and isolated for DNA metabarcoding for species composition analyses.

Noninvasive eDNA and DNA metabarcoding has been widely applied for biomonitoring of bacterial, protist, zooplankton, benthic macroinvertebrate, and fish in freshwater ecosystems (Elbrecht and Leese, 2017; Evans and Lamberti, 2018; Hering et al., 2018; Li et al., 2019; Xie et al., 2018; Yang et al., 2017a). eDNA and DNA metabarcoding and morphological identification can be closely related for species detection (Elbrecht and Leese, 2017; Evans et al., 2016; Hänfling et al., 2016; Shaw et al., 2016; Yamamoto et al., 2017; Yang et al., 2017c). Furthermore, results of a recent study showed that using presence/absence of morphological benthic invertebrate data sets for ecological monitoring came to the same ecological status class 76.6% of the time (Buchner et al., 2019). This evidence shows that even with bias in abundance estimates for DNA metabarcoding, its use will prove to be extremely valuable for ecosystem health monitoring (Elbrecht and Leese, 2015). Metabarcoding can also characterize species composition from bulk samples of eukaryotes (Ji et al., 2013).

1.2.2 Metabarcoding in Ecotoxicology

Due to the widely diverse organisms present in ecosystems, the ecological effects of toxicants on freshwater ecosystems can be complex to decipher. Anthropogenic disturbances, such as the introduction of hydrocarbon contamination, can negatively affect community structure and biodiversity (Jung et al., 2016). DNA metabarcoding has been used to elucidate changes in freshwater invertebrate OTUs (Operational Taxonomic Units) between sites

impacted by the organophosphate chlorpyrifos and control sites, with strong differences observed among the two sample locations (Andújar et al., 2018). Operational Taxonomic Units are typically a clustering of sequences within 97% similarity to serve as a replacement to the conventional 'species-level' identification (Nguyen et al., 2016). Using eDNA metabarcoding, researchers deciphered that changes in structures among communities were mainly due to nutrient enrichment (Li et al., 2018). Studies have also utilized metabarcoding to show differences in zooplankton communities between two different ecosystems for biomonitoring purposes (Yang et al., 2017c). These studies detail how metabarcoding can offer a novel and predictive method to evaluate effects of pollution on aquatic ecosystems. In recent years, the use of eDNA metabarcoding for the assessment of chemical effects on biological communities has been considered one of the most important advances in ecotoxicology (Zhang et al., 2018b).

Since it might better represent currently active constituents of a community, RNA metabarcoding has been suggested as a useful method for analyses of ecological communities (Baldrian et al., 2012). RNA is degraded within cells at a rate that balances energetic costs and adaptability to varying environmental conditions (Hui et al., 2014). RNA metabarcoding can measure responses of communities at the time of sampling, without the common issue of persistence of DNA in the environment, making it potentially advantageous for measuring changes in community structure due to exposures to stressors (Cristescu, 2019). RNA metabarcoding can detect more significant changes in taxa richness due to treatment relative to DNA metabarcoding (Laroche et al., 2017). It has been suggested to use coupled DNA and RNA metabarcoding when assessing ecosystems (Laroche et al., 2017; Pochon et al., 2017). Coupled DNA and RNA metabarcoding could help eliminate erroneous detections while also indicating the presence of active taxa. Paired DNA and RNA metabarcoding could serve as a stand-alone assessment of ecosystem status or can be used as a complementary method to morphology-based monitoring (Laroche et al., 2018). Morphology approaches provide relatively better indications of abundance and biomass, as metabarcoding is still limited in the ability to accurately detect the absolute biomass/abundance. Using both methods would give

the most insight into the response of the community, however, it has been shown that using presence/absence data can suffice for recording the community response, eliminating the overall necessity of having “absolute” biomass/abundance data (Buchner et al., 2019).

1.3 Ecological Importance of Zooplankton

Zooplankton are classified as animal plankters found floating or suspended in the water column. Freshwater zooplankton can be mainly classified into three major groups: rotifers, cladocerans, and copepods. Rotifers are in the Phylum Rotifera and mainly live among aquatic vegetation in the littoral zone of lakes (Sládeček, 1983). Rotifers have been considered as indicators of water quality, including toxicity from introduced compounds to aquatic systems (Sládeček, 1983). Cladocerans, in the subphylum Crustacea, are commonly referred to as “water fleas” and include the well-known genus *Daphnia*. Daphnids have been shown to suppress blooms of cyanobacteria, which was inferred from the zooplankters' ability to maintain greater N:P ratios (Andersen and Hessen, 1991). Due to their availability and taxonomic stability, as well as sensitivity to pollution, cladocerans are universally used for ecotoxicological assessments (Sarma and Nandini, 2006). Copepods, also in the subphylum Crustacea, are important aquatic organisms that are also sensitive indicators of the presence of environmental toxicants (Kulkarni et al., 2013). Zooplankton can recycle a large amount of nutrients in lake ecosystems, supporting a large fraction of phytoplankton while transferring nutrients from deeper waters to the euphotic zone or vice versa during daily vertical migrations (Vanni, 2002). Zooplankton communities occupy a central trophic position, therefore making them key mediators of energy and material fluxes in ecosystems, providing valuable ecosystem functions (Hébert et al., 2016). Community-level analyses provide a holistic approach to assessing response of freshwater ecosystems to chemicals perturbations (Clements and Newman, 2003). Zooplankton communities can serve as a sensitive indicator ecosystem health and the subsequent response to anthropogenic pollution (Xiong et al., 2017; Yang and Zhang, 2020; Yang et al., 2017b).

1.3.1 Metabarcoding of Zooplankton for Biomonitoring

Metabarcoding of multiple communities provides a comprehensive view of the status of aquatic ecosystems. Significant advances have been made in macroinvertebrate metabarcoding as these aquatic organisms are commonly used in biomonitoring of freshwater ecosystems (Buss et al., 2015; Curry et al., 2018; Elbrecht et al., 2017; Metcalfe, 1989). Macroinvertebrate metabarcoding has been used to assess watershed conditions in boreal regions, proving the value of metabarcoding in biomonitoring programs (Emilson et al., 2017; Hajibabaei et al., 2019).

Zooplankton are widely used for biomonitoring of freshwater ecosystems since they are ubiquitous, ecologically important and can be sensitive to stressors (Lougheed and Chow-Fraser, 2002; Marmorek and Korman, 1993; Sládeček, 1983). Zooplankton metabarcoding has been used to assess species sensitivity distribution of zooplankton community to ammonia (Yang et al., 2017b). Investigations of zooplankton community variation by water pollution have also been conducted utilizing metabarcoding (Xiong et al., 2019; Xiong et al., 2017; Yang and Zhang, 2020). Metabarcoding can reveal rare taxon presence in zooplankton tissue DNA samples (Lindeque et al., 2013). Metabarcoding, combined with network learning, could further provide a network approach to quantify stressor impacts to freshwater ecosystems (Bohan et al., 2017; Gray et al., 2014; Vacher et al., 2016).

1.4 Petroleum and Diluted Bitumen in the Aquatic Environment

1.4.1 Effects of Crude Oil Spills on Aquatic Ecosystems

Aquatic ecosystems are continuously threatened by the extraction and transport of petroleum products. Due to the limited number of studies conducted in freshwater ecosystems, a more thorough literature search was done involving marine ecosystems with the Deepwater Horizon Oil spill serving as a case study. Between April 20 and July 15, 2010, the Deepwater Horizon oil spill released an estimated 3.19-6.24 million barrels of Louisiana sweet crude oil into the Gulf of Mexico, while a total of 6.9 million liters of chemical dispersants was used to help break up the oil (Barron, 2012; Beyer et al., 2016). Toxicity of

the Louisiana sweet crude and COREXIT® EC9500A, an oil dispersant used in the Deepwater Horizon oil spill, was assessed on phytoplankton species with decreases in the number of sensitive species and an increase in resistant species being observed (Ozhan and Bargu, 2014). With the 25% CEWAF (Chemically enhanced water accommodated fraction), exposure using 63 ppm dispersant, and a total petroleum hydrocarbon concentration of 132.2 ppm, diatoms were found to have the greatest resistance, whereas dinoflagellates were the most sensitive. Nutrient enrichment helped phytoplankton cope with the stressor of introduced toxicants, but decreases in biomass of phytoplankton from the Louisiana sweet crude exposure still occurred at concentrations as low as 2.6 ppm total petroleum hydrocarbons (Ozhan and Bargu, 2014). On July 21st, 2016, a buried pipeline near Maidstone, Saskatchewan, spilled approximately 225,000 liters of crude oil into the North Saskatchewan River (Yang et al., 2020). Details of the effects of this spill on the river's ecosystem are currently being studied, with a recent study reporting potential impacts on the gut microbiome of fish (DeBofsky et al., 2020).

Using a short-term (2 days) microcosm study, researchers observed that with exposure to Deepwater Horizon spill oil, concentrations of 50 µL L^{-1} caused increases in chlorophytes (algae) and cyanobacteria, while the number of cryptophytes, a class of algae that have plastids, decreased (Gilde and Pinckney, 2012). An additional mesocosm study showed that both dispersants alone and dispersed oil caused reductions in biomass of dinoflagellates (algae) and diatoms, with these alterations potentially diverting carbon from higher trophic levels due to decreases in biomass of primary producers (Ortmann et al., 2012). Researchers have also shown that an acute 48 h exposure to COREXIT 9500A treated crude oil was toxic to microzooplankton, zooplankton typically <200 µm, at low concentrations (Almeda et al., 2014; Calbet, 2008). It was found that the EC_{50} ranged from 0.85–2.29 µL L^{-1} for oligotrophic ciliates and tintinnids (Order Tintinnida) and 5.69–13.40 µL L^{-1} for heterotrophic dinoflagellates, indicating that dispersed crude oil could cause impacts on zooplankton food webs (Almeda et al., 2014). Crude oil has also been shown to be toxic to various zooplankton species, with median lethal toxicity seen at 16 h post-exposure of a

concentration of 32.4 μL L^{-1} (Almeda et al., 2014). In a report assessing the impacts of the Deepwater Horizon oil spill, it was determined that taking an ecosystem service approach would prove to be the most useful method (Council, 2013). Multiple studies have used metagenomic and metabarcoding techniques to better understand how microbial communities changed because of the Deepwater Horizon oil spill (Beazley et al., 2012; Hazen et al., 2010; Kimes et al., 2013; Mason et al., 2014).

Metabarcoding holds promise for biomonitoring and assessment of oil-contaminated sites. Researchers utilized DNA metabarcoding of sediment samples to assess the impacts of offshore oil drilling sites on eukaryotic fauna using the 18s rRNA gene. Samples were taken adjacent to the Norwegian continental shelf oil-drilling platforms, and the downstream results showed that relative abundance of metazoan and non-metazoan taxa was significantly correlated to contamination (Lanzén et al., 2016). eDNA and eRNA metabarcoding outperformed the traditional morphological-based monitoring in a study to measure the biological impacts of offshore oil and gas drilling. This was due to eDNA and eRNA providing more information on biota normally excluded from conventional monitoring surveys (Laroche et al., 2018). This study also showed that identified indicator taxa were specific to site conditions, providing a potential method for identifying a site with known contamination using eDNA and eRNA (Laroche et al., 2018).

1.4.2 Diluted Bitumen in Canada

Canada is the second-largest country in the world with vast and abundant natural resources, including the oil-sands region. Oil sands are loose sand deposits that contain high molar mass viscous petroleum, typically called bitumen (Masliyah et al., 2004). Large deposits of oil-sands, which account for greater than 95% of the bitumen in North America, are in Alberta, Canada (Garven, 1989; Hein and Cotterill, 2006). These deposits are separated into three regions in Alberta: Peace River, Athabasca (Fort McMurray area), and Cold Lake, with the greatest production of bitumen coming from the Athabasca region (Hein and Cotterill, 2006). Due to the current requirement for energy resources reliant on fossil fuels,

bitumen may become more in demand, and the extraction of petroleum may increase (Shah et al., 2010). Extraction of bitumen from oil sands has increased in past years from an equivalence of $\sim 1 \times 10^5$ barrels of oil/day in 1980 to $\sim 1.6 \times 10^6$ barrels/day in 2011, with growth likely to slow in upcoming years (Korosi et al., 2016). Bitumen can be recovered from these shallow oil sand deposits through surface mining, hot water extraction, and froth treatment (Long et al., 2002). Bitumen itself is a dense mixture of semi-solid hydrocarbons of a dark brown to black color with trace level metals, heteroatoms (e.g., any atom that is not carbon or hydrogen), and organometallic compounds (Strausz et al., 2010). Because oil sands are unconsolidated deposits of heavy hydrocarbon bitumen, they require multiple stages of processing before being refined (Jiang et al., 2007).

Bitumen has a high viscosity when at room temperature and must be diluted to be transported to refineries to be further processed to produce gasoline, jet fuel, heating oil, or diesel fuel (Masliyah et al., 2004). The viscosity of bitumen produced from longer-chain hydrocarbons is not conducive to transportation via pipeline. Therefore, to allow transportation, the addition of a diluent, such as natural gas condensate, is common (Philibert et al., 2016). This diluted bitumen, or dilbit, is a complex petroleum mixture that consists of 30% diluent and 70% bitumen (Crosby et al., 2013). Dilbit, like crude oil, contains a mixture of constituents ranging from lesser molecular mass, more water-soluble, to larger, less soluble compounds (King et al., 2017). Dilbit also contains several semi-volatile organic compounds, with the most notable being benzene, toluene, ethylbenzene, and xylene (BTEX), with BTEX components ranging from 0.8-1.2% by volume of dilbit (Dew et al., 2015). Bitumen itself consists of 16-17% saturates, 37% resins, 18-21% asphaltenes, and 25-29% aromatics, with some bitumen samples containing up to 38.6% aromatics (Strausz et al., 2010).

There are multiple compounds in bitumen that have toxic potential. These include polycyclic aromatic hydrocarbons (PAHs), metals, and naphthenic acids (Dew et al., 2015) as well as the BTEX compounds themselves. In one aromatic fraction alone, nearly 6000 component molecules were found, ranging in molecular mass from 200 to approximately 800 Daltons (Strausz et al., 2010). Compounds found in dilbit of toxicological interest are

polycyclic aromatic hydrocarbons (PAHs) due to their known source of toxicity to aquatic organisms. Polycyclic aromatic hydrocarbons (PAHs) are widespread and prevalent environmental pollutants, typically produced by high-temperature reactions, including pyrolysis of fossil fuels. (Juhasz and Naidu, 2000; Nikolaou et al., 1984). PAHs are formed naturally, as in the case of thermal geologic production and burning of vegetation, but due to increased anthropogenic activities in the last century, such as industrial development, significant rises can be seen in the natural environment (Juhasz and Naidu, 2000; Maliszewska-Kordybach, 1999). PAHs are genotoxic, carcinogenic, and have the capability to bioaccumulate in many different freshwater organisms (Newsted and Giesy, 1987; Pelletier et al., 1997). The prevalence of these compounds in aquatic environments and their inherent toxicity, makes them a reoccurring and consistent problem in freshwater ecosystems and the underlying health.

1.4.3 Dilbit Spills in Freshwater Ecosystems and Associated Effects

The nature of dilbit spills is important to understand for choosing proper methods for remediation and recovery of freshwater ecosystems, while also understanding the potential effects it could have on the target environment. Due to its complex composition, dilbit can be unpredictable when it is released into the environment (Dew et al., 2015). Densities of dilbit components can range from greater or lesser than water, which makes predicting what will occur when it is released into the environment difficult (Crosby et al., 2013). In 2010, a 30-inch pipeline ruptured near Marshall, Michigan, releasing approximately 843,000 gallons that firstly went into Talmadge Creek with subsequent movement into the Kalamazoo River (EPA, 2013). Because the lower molecular mass compounds in dilbit are more volatile, the dilbit became denser after weathering leading to sinking to the sediment within the Kalamazoo River. For this reason, a fraction of the spilled dilbit remains unrecovered in sediment (Alderman et al., 2018). A simulated open tank experiment under natural environmental conditions (e.g., sunlight, wind, rain, seawater temperature, and salinity) was utilized to elucidate the behavior and fate of diluted bitumen spilled at sea. The authors found that the

products of the natural weathering of Access Western Blend (AWB) dilbit were able to form oil-balls that sank in brackish water (<20 practical salinity units) after 7 days (King et al., 2014). Because freshwater is less dense than brackish water, it will likely take less time for dilbit to sink in freshwater ecosystems with similar weathering processes. The fate of dilbit in a spill depends on the nature of the spill and location, as a spill into a calm lake or a fast-turbulent river will have different outcomes. The Kalamazoo River spill was a worst-case scenario, in that dilbit was first spilled onto land and then weathered, as it traveled overland to Talmadge Creek to become mixed with a river (Dew et al., 2015). With the increasing transport of dilbit in pipelines and spills occurring in North America, more information is needed on the behavior of this petroleum mixture in the environment.

Dilbit is acutely toxic to a variety of taxa within aquatic ecosystems, including fish (Alderman et al., 2017; Alderman et al., 2018; Alsaadi, 2018; Barron et al., 2018; Madison et al., 2015; Madison et al., 2017; Philibert et al., 2016), zooplankton (Barron et al., 2018; Cederwall et al., 2020; Robidoux et al., 2018), and phytoplankton (Cederwall et al., 2020), with limited studies involving the two latter taxonomic groups. With a continuing focus on zooplankton and macroinvertebrates, weathered-sediment bound dilbit has been shown to impair movement and respiration of *Hyalella azteca* due to the immediate adhering of dilbit to the body of the amphipods (order Amphipoda; subphylum Crustacea) (Everitt et al., 2020). *Ceriodaphnia dubia* (order Cladocera) was shown to be acutely sensitive to Cold Lake Blend Water Accommodated Fraction (CLB WAF), with an LC50 = 6.43 g/L; however, no mortality was observed with exposure to weathered CLB (Robidoux et al., 2018). LC50 is the concentration that will kill 50% of the test organisms with a single exposure. Effects on reproduction followed what was observed in lethality, with greater effects with CLB (Inhibition Concentration 25% (IC25) < 1.0 g/L) compared to the weathered CLB (IC25 = 3.99 g/L) for *Ceriodaphnia dubia* (Robidoux et al., 2018). Involving the same species, *Ceriodaphnia dubia*, a 7-day survival and reproduction assay determined an IC25 of 0.185 mg/L BTEX and 12.5 µg/L total PAH (tPAH) (Barron et al., 2018). Fresh CLB WAF was found to have a 48 LC50 of > 5.86 mg/L for measured BTEX and > 40.0 µg/L tPAH,

respectively. Currently, there is a lack of understanding of the overall impacts dilbit exposure can have on the entire freshwater ecosystem community. Recent studies have begun to unravel the potential effects dilbit exposure may have on freshwater communities (Black, 2019; Cederwall et al., 2020). Understanding the effects of oil spills on the entire aquatic community assemblages within ecosystems can give greater insight into the potential functions and services that could be impacted.

1.4.4 Remediation Practices for Oil-Impacted Aquatic Ecosystems

Oil spills can cause significant contamination of aquatic ecosystems that can lead to long-term negative consequences. Cleanup technology of oil spills is primarily composed of two approaches, protective and removal (Vandermeulen and Ross, 1995). Protective methods include the use of booms, skimmers, and sorbents to collect residual oil, whereas removal methods include sorbents, burning, chemical dispersion, and bioremediation (Vandermeulen and Ross, 1995). Remediation methods, including bioremediation, are used to aid in the recovery of contaminated ecosystems to restore them to a natural state. Methods of remediation can be classified as physical, chemical, thermal or biological (Dave and Ghaly, 2011). Increasing effectiveness and response time of remediation technologies is of primary focus to mitigate environmental damages due to oil spills (Prendergast and Gschwend, 2014). Several methods can be commonly employed when remediating petroleum-contaminated aquatic ecosystems. The methods can additionally be classified as active or passive methods, for example, shoreline cleaner application and enhanced monitored natural recovery, respectively.

1.4.5 Impacts of Shoreline Cleaner Application on Aquatic Ecosystems

Chemical methods of oil spill remediation help change the physical and chemical properties of oil (Dave and Ghaly, 2011). One method, shoreline cleaner application (SCA), is used to wash oil off impacted shorelines for collection by mechanical means, for example, through the use of absorptive pads. This approach uses surface-washing agents (SWA) to

enhance separation and removal of oil adhered to solid surfaces (Chen et al., 2019). SCA has been shown to have much less of a toxic effect relative to dispersant application; however, its use is for primarily different scenarios (Bhattacharyya et al., 2003). SCA helps remove oil from shorelines to be collected by use of traditional methods, whereas dispersants help to emulsify the oil and thus break up oil sheens to increase biodegradation by microbes (Pezeshki et al., 2000; Prince, 2015). SCA is an effective approach with the restoration of oiled shorelines and prevention of plant damage (Pezeshki et al., 1995; Teas et al., 1993).

SCA could be useful in low energy systems, such as isolated lakes, as it can assist with the collection of toxic petroleum components from contaminated shorelines. However, the effects of use in freshwater ecosystems are not well understood (Hansen et al., 2014; Stroski et al., 2019). More research is required to have a complete understanding of the ecological risks this remediation method could pose to freshwater ecosystems, with shoreline cleaner application currently not allowed in Canadian freshwater ecosystem due to limited information of these potential risks. COREXIT® EC9580A, an SWA approved for use in United States and marine oil spill response in Canada (Black et al., 2020), has been shown previously to be acutely toxic to the pelagic copepod species *Acartia tonsa* with a 48-h LC50 of 50.4 ± 4.47 mg/L, and a mysid (order Mysida; subphylum Crustacea), *Americamysis bahia*, with a 48-h LC50 of 32 mg/L (Bi et al., 2020; Fingas, 2013; Hansen et al., 2014). These results warrant further investigation of toxicity to additional aquatic taxa and communities.

1.4.6 Impacts of Enhanced Monitored Natural Recovery on Aquatic Ecosystems

Nutrient enrichment can be an effective method for remediating aquatic ecosystems of low activity. Oil bioremediation is the process of microorganisms degrading and metabolizing oil constituents to assist in restoring impacted ecosystems (Dave and Ghaly, 2011). Microbes require additional nutrients to stimulate the biodegradation of petroleum constituents, as the biodegradation of oil spills is commonly limited by the bioavailability of nutrients (Atlas and Hazen, 2011; Dave and Ghaly, 2011). Nutrient enrichment was utilized, along with washing of the shoreline, after the *Exxon Valdez* oil spill in Prince William Sound,

with more than 70 miles of shoreline having this treatment applied (Nauman, 1991). Previous research has suggested that degradation of petroleum by microbes can be increased by application of fertilizer (Prince et al., 1993). Fertilizers used on a large scale for the *Exxon Valdez* oil spill included Inipol EAP22, an oil adhering oleophilic liquid product, and Customblen™, a slow-release granular agricultural product (Prince and Bragg, 1997).

Nutrients are beneficial to oil-degrading microbes because petroleum components, more specifically hydrocarbons, provide little output of nitrogen or phosphorus when they mineralize (Mendelssohn et al., 2012). Nutrient enrichment, however, can be double-edged since increases in nutrient input can disrupt nutrient cycling leading to eutrophication and oxygen depletion as well as the formation of ammonia, which can be toxic to aquatic organisms (Mendelssohn et al., 2012). Bioremediation of oil-impacted freshwater ecosystems is less well understood, likely due to fewer high-magnitude oil-spills in these ecosystems. One study determined that there was an increase in biodegradation of oil constituents, particularly hydrocarbons, in a simulated freshwater wetland with the addition of nitrate and phosphorus (Purandare et al., 1999). Another study determined that while temperature played a major role in biodegradation of oil constituents, nitrogen and phosphorus nutrient levels can also limit oil biodegradation (Ward and Brock, 1976). There is evidence, however, that long-term nutrient enrichment of a freshwater boreal lake has been shown to have negative consequences on the biomass of planktonic zooplankton (Malley et al., 1988; Paterson et al., 2011).

1.7 Purpose of Research, Objectives, and Hypotheses

Zooplankton communities can serve as valuable indicators of ecosystem health and can model the potential impacts of stressors on aquatic communities. Identification of zooplankton by use of visual taxonomy based on structural morphology by traditional methods can be costly and time-consuming, which limits timely and cost-effective assessment of ecosystems. Meanwhile, taxonomists with the ability to identify zooplankton accurately and effectively by morphology have become fewer. Metabarcoding could serve as a complementary method to morphological identification for the rapid and high-throughput

identification of environmental zooplankton samples for the assessment of aquatic ecosystems. Zooplankton metabarcoding can also be benchmarkable, referring to the development and application of replicative and transferable protocols with methods that can be effectively used in the lab across the globe.

The purpose of this research was to benchmark zooplankton metabarcoding in a boreal lake using the mitochondrial cytochrome oxidase 1 gene region (COI) and compare it to traditional morphological identification. This research also assessed the ability of DNA and RNA zooplankton metabarcoding to assess the effects of two different remediation practices, enhanced monitored natural recovery (eMNR) and shoreline cleaner application (SCA), of experimentally spilled dilbit in boreal shoreline enclosures. The metabarcoding methods were compared to the ability of morphological identification to assess the response of the zooplankton community to remediation practices.

The specific objectives of this research were:

1. Compare the zooplankton (phylum Rotifera; orders Calanoida, Cyclopoida, and Cladocera) taxonomy profiles derived from the experimental shoreline enclosures as determined by DNA metabarcoding, RNA metabarcoding, and morphological identification. This includes assessing the presence and absence of family, genus, and species, and the relative abundances of the shared taxa within these ranks.
2. Determine the impact of the different remediation practices, eMNR, and SCA, coupled with the simulated dilbit spill on the zooplankton community using changes in alpha and beta diversity measurements and compatible statistical analysis.
3. Compare the ability of the three zooplankton identification methods, RNA and DNA metabarcoding and morphological identification, to determine the impacts of eMNR and SCA along with spilled dilbit on the zooplankton community in boreal shoreline enclosures.

The main hypotheses of this research were:

1. Zooplankton can be identified down to the family and genera level using metabarcoding. Previous research suggests that zooplankton require barcoding of species from the local region to identify down to the species level. The relative abundance of zooplankton inferred from metabarcoding will reflect the biomass inferred from morphological identification. There will, however, be some disconnect as PCR biases and variable organism size will skew the results.
2. SCA will likely have the greatest acute toxicity on the zooplankton community relative to eMNR. This can be inferred from the literature review, as previous studies suggest acute toxicity from the SWA, COREXIT® EC9580A (Nalco, Co., Illinois, USA).
3. RNA metabarcoding will be the most sensitive method for measuring species richness, or alpha diversity, response to treatments. This can be inferred from previous studies as RNA metabarcoding can potentially more closely reflect the changes in the active or alive community compared to DNA metabarcoding and morphological identification. For assessing changes in beta diversity to treatments, DNA metabarcoding will be the most sensitive method as shown by previous studies. DNA metabarcoding can potentially detect more taxa relative to RNA metabarcoding and morphological identification, as it can commonly detect residual or persistent DNA in the environment from a wider range of organisms, relative to the less stable RNA or the whole organism for morphological identification.

CHAPTER 2

USING ZOOPLANKTON METABARCODING TO ASSESS THE EFFICACY OF DIFFERENT OIL SPILL CLEAN-UP TECHNIQUES IN A BOREAL LAKE

PREFACE

The objective of chapter 2 is to evaluate the ability of DNA and RNA metabarcoding to determine the abundance and presence of the zooplankton community in boreal shoreline enclosures, while also assessing the impacts of different remediation practices of spilled diluted bitumen on the zooplankton community. Shoreline enclosures in a boreal lake were exposed to spilled diluted bitumen with select remediation practices being applied, enhanced monitored natural recovery and shoreline cleaner application, while zooplankton was collected and identified using morphology or metabarcoding for 3 days pre-exposure and 11 and 38 days after the simulated spill to meet this objective.

Author contributions:

Phillip Ankley (University of Saskatchewan) collected, generated, and analyzed the data, prepared all the figures, and drafted the manuscript.

Yuwei Xie (University of Saskatchewan) conceptualized the study, assisted with the methodology, and edited the manuscript.

Tyler A. Black (University of Guelph) provided resources and methodology, including zooplankton identification, and edited the manuscript.

Abigail DeBofsky (University of Saskatchewan) provided methodology.

McKenzie Perry (University of Manitoba) provided resources.

Michael J. Paterson (IISD-ELA) provided validation of zooplankton identification and resources and edited the manuscript.

Mark Hanson (University of Manitoba) provided resources.

Scott Higgins (IISD-ELA) provided resources.

John P. Giesy (University of Saskatchewan) provided funding, supervision, project conceptualization and administration during the project, and edited the manuscript.

Vince Palace (IISD-ELA) provided resources and edited the manuscript.

2.1 Abstract

Regulators require adequate information to select best practices with less ecosystem impacts for remediation of freshwater ecosystems after oil spills. Zooplankton are valuable indicators of aquatic ecosystem health as they play pivotal roles in biochemical cycles while stabilizing food webs. Compared with morphological identification, metabarcoding has promise for cost-effective high-throughput, and benchmarkable biomonitoring of zooplankton communities. The objective of this study was to apply DNA and RNA metabarcoding of zooplankton for ecotoxicological assessment and compare with traditional morphological identification in experimental shoreline enclosures in a boreal lake exposed to simulated spills of diluted bitumen (dilbit), with experimental remediation practices of enhanced monitored natural recovery and shoreline cleaner application. Metabarcoding detected boreal zooplankton taxa up to the genera level, with a total of 24 shared genera, and while metabarcoding-based relative abundance served as an acceptable proxy for biomass inferred by morphological identification. Morphological identification determined zooplankton community composition changes due to treatments at 11 days post-spill while metabarcoding methods indicated changes in zooplankton richness and communities at 38 days post-spill. Shoreline cleaner application overall seemed to have the largest impact on zooplankton communities relative to enhanced monitored natural recovery, regardless of zooplankton identification method. Both metabarcoding and morphological identification were able to discern the differences between the two experimental remediation practices. Metabarcoding of zooplankton can provide informative results for ecotoxicological assessment of remediation practices of dilbit, advancing our knowledge of best practices for remediating oil-impacted aquatic ecosystems while serving to accelerate the assessment of at-risk freshwater ecosystems.

2.2 Introduction

Aquatic ecosystems are continuously threatened by global activities of extraction and transport of oil, especially in cases of accidental oil spills (Atlas and Hazen, 2011; Beyer et al., 2016). Diluted bitumen (dilbit) is a complex petroleum mixture produced by the dilution of bitumen, a viscous heavy oil, by diluents to form a mixture that is transportable through pipeline and rail but toxic to aquatic organisms (Barron et al., 2018; Dew et al., 2015; Madison et al., 2015). Bitumen extracted from the Athabasca Oil Sands region in Alberta is diluted to form dilbit and transported across North America, leading to potential risks of spills occurring via pipeline or rail. North America has seen several large pipeline oil spills, including a 2010 spill of dilbit affecting the Kalamazoo River in Michigan (USA) and in 2016 where crude oil spilled into the North Saskatchewan River, Saskatchewan (Canada) (Dew et al., 2015; Yang et al., 2020). Several practices have been developed to help restore oil impacted marine aquatic ecosystems (Dave and Ghaly, 2011). These include active processes, such as shoreline cleaner application, and passive natural attenuation, such as nutrient enrichment application; however, the effects of these different oil remediation practices on boreal freshwater ecosystems of low energy needs to be better understood.

Shoreline cleaner application can be an effective strategy for shoreline remediation of oil spills in marine ecosystems. Cleaners wash oil from surfaces to be collected by traditional methods, whereas dispersants promote dispersion of petroleum components (Pezeshki et al., 2000; Prince, 2015). Cleaners have been shown to be an effective strategy for cleaning oiled shorelines, including prevention of plant damage (Pezeshki et al., 1995; Teas et al., 1993). Shoreline cleaner toxicity to various aquatic organisms, however, requires more information (Barron et al., 2020; Chen et al., 2019). Its potential use effects in freshwater ecosystems, let alone boreal ecosystems, is not well understood (Bhattacharyya et al., 2003; Hansen et al., 2014). A previous study has determined enhanced toxicity of oil exposure with the addition of shoreline cleaner, COREXIT® 9580, but it was to a lesser extent relative to the addition of a dispersant (Bhattacharyya et al., 2003). Shoreline cleaner application is currently not allowed

to be used in freshwater ecosystems in Canada, as more investigation would be required prior to approval.

Nutrient enrichment is used to stimulate hydrocarbon-degrading microorganisms to break down oil-residue (Atlas and Hazen, 2011; Prince, 1993). Nutrient enrichment is an effective approach (Bragg et al., 1994), but it may cause eutrophication of the aquatic system leading to harmful algae bloom formation and expanding water hypoxia (Pretty et al., 2003; Watson et al., 2016). Long-term nutrient enrichment in a Precambrian Shield lake has been shown to have negative consequences on the biomass of planktonic zooplankton (Malley et al., 1988; Paterson et al., 2011). The remediation practice that has the least effect on aquatic organisms has yet to be determined in-situ in a boreal freshwater ecosystem of low energy. Processes of wave energy to help break up oil into smaller droplets or remobilization of settled oil for optimal biodegradation is minimized in low energy environments, therefore efficacy and effects of these remediation practices needs to be better understood (Carls et al., 2001; Fitzpatrick et al., 2015).

Since zooplankton can respond quickly to altering environmental conditions and are sensitive to aquatic pollution, they are widely used as indicators of the status and trends of aquatic ecosystems (Parmar et al., 2016; Schindler, 1987). Zooplankton play pivotal roles in freshwater ecosystems by recycling nutrients (Steinberg et al., 2008) while also occupying central trophic positions, making them mediators of energy and material fluxes in ecosystems (Giering et al., 2019). Traditional, visual identification of zooplankton based on morphology can be costly and time-consuming (Pan et al., 2008; Wheeler et al., 2004). Furthermore, it is difficult to standardize and requires individuals with taxonomical expertise, a collective skill that has declined in recent decades (Hopkins and Freckleton, 2002; Thomsen et al., 2012). Application of the emerging technology of metabarcoding has been suggested for describing communities of zooplankton (Yang et al., 2017d). DNA metabarcoding can provide robust reproducible identification of taxa during ecological assessments (Valentini et al., 2016), but DNA based metabarcoding cannot distinguish whether organisms are dead or alive (Pochon et al., 2017), which should be of importance when tracking rapid changes of communities under

environmental stressors. RNA metabarcoding may serve as a useful measure in this regard, as it can reflect the active community upon sampling (Baldrian et al., 2012). RNA is broken down within individual organism cells at a rate that balances energetic costs and adaptability to varying environmental conditions (Hui et al., 2014).

This study assessed the ability of zooplankton metabarcoding to provide data comparable to that produced by using morphology-based identification. We also compared the ecotoxicological effects on the zooplankton community of two different methods for oil-spill remediation, a shoreline cleaner and nutrient enhancement using shoreline mesocosms. The study was conducted in the summer of 2019 in a boreal lake and specific objectives were to: 1) compare the relative abundances or biomass of zooplankton taxa in communities as determined by the use of DNA or RNA metabarcoding and morphologically identified taxonomic (morph-taxa) techniques; 2) determine and compare ecotoxicological effects of remediation practices on zooplankton communities in mesocosms; 3) compare the performance of the three zooplankton identification methods (DNA metabarcoding, RNA metabarcoding, and morphological taxonomy) to elucidate the effects of oil-spill remediation practices.

2.3 Materials and Methods

2.3.1 Experimental Design

The experiment was conducted at the IISD Experimental Lakes Area (IISD-ELA), an area that contains 58 boreal lakes located in northwestern Ontario, Canada that have been set aside for whole-ecosystem experimentation (49°41′45.0” N, 93°46′03.4” W) (Kidd et al., 2007; Schindler et al., 1996). In June 2019, seven mesocosms (enclosures of 15 x 5 m) were established along the shoreline of Lake 260 at the IISD-ELA in a wetland habitat type. On June 21st, 2019, after enclosure construction and baseline measurement completed, six randomly selected enclosures were treated with model spills of dilbit (mean applications = 1300 g/enclosure) applied to the surface of the water approximately 50cm from the shore. One enclosure remained untreated to serve as a reference.

The oil was allowed to interact with the shoreline soil, sediment and vegetation for 4 days to conservatively simulate spill response times, after which any oil remaining on the surface of the water was removed using pre-weighed oleophilic absorbent pads. Additionally, each enclosure, including the reference, was rinsed with 1200-L of water pumped from the interior of the enclosure over the oiled sections of the confined shoreline to mimic oil spill clean-up procedures typically used following a spill. Water was pumped under low pressure and returned to the interior of each enclosure. Any additional oil dislodged by flushing was also captured using absorptive pads.

Enclosures treated with dilbit were then randomly selected to receive one of two different remediation treatments to determine their effectiveness for promoting the longer-term recovery from residual oil contamination. The first method, enhanced monitored natural recovery (eMNR; $n = 3$), included addition of nutrients designed to promote the decomposition of remaining oil products. The second method consisted of active cleaning of the shoreline by use of the oil surface cleaning agent COREXIT® EC9580A (Nalco, Co., Illinois, USA) (SCA; $n = 3$) (Fig. B.1; Appendix B). One shoreline enclosure remained untreated serving as the reference (REF; $n = 1$).

2.3.2 Collection of Zooplankton

Triplicate 20-L water samples for DNA and RNA metabarcoding were collected consecutively from each experimental enclosure three days before the simulated spill of bitumen, and then 11 and 38 days after the spill (Fig. B.1). Pre-installed tubing, with a funnel on the end inundated within the enclosure, was used to collect representative zooplankton samples without disturbing the water surface. Zooplankton were enriched by two-step filtering by use of a pump with an in-line 53 µm mesh filter and washed off with NanopureTM water (Thermo Fisher Scientific, USA) for final enrichment with a 5 µm Durapore® PVDF membrane filter (Millipore, Germany). Samples were preserved in LifeGuard Solution (Qiagen, Germany) and stored at -80 °C before extraction of nucleic acid. To avoid cross-contamination, use of filter pumps specified for each treatment, single-use

filter-units, changing of gloves at each enclosure, and strict protocols were enforced. Equipment was also decontaminated between each replicate using 15% bleach and 70% ethanol, while field blanks were collected frequently during sampling. Field blanks consisted of an opened, decontaminated 500 mL NalgeneTM bottle (Thermo Fisher Scientific, USA) containing NanopureTM water during the period of sampling, for each treatment.

Samples of zooplankton for morphological identification were collected simultaneously with one 60-liter water sample being collected using the same protocol for metabarcoding collection, minus the final enrichment step. Taxonomic identification was conducted following procedures detailed previously (Paterson et al., 2010). Briefly, identification of zooplankton was completed using the taxonomic key of Balcer et al. 1984, as well as several other guides to North America's freshwater zooplankton (Balcer et al., 1984; Brandlova et al., 1972; Smith and Fernando, 1978; Witty, 2004). Biomass was determined using length-weight regression based on historic zooplankton weights (Schindler and Novén, 1971) and regression equations (Lawrence et al., 1987; Malley et al., 1989) obtained from IISD-ELA lakes.

2.3.3 Co-isolation of DNA and RNA, PCR amplification, and Next-Generation Sequencing (NGS)

Zooplankton were thawed on ice and pelleted by centrifugation (8000 x g for 5 min.). LifeGuard Solution was removed with a sterile pipette. DNA and RNA were co-isolated by use of AllPrep DNA/RNA Mini Kit (Qiagen, Germany) following the manual. DNA contamination of extracted RNA were digested with RNase-Free DNase (Qiagen, Germany). The extracted DNA and RNA were measured and checked for quality using Qubit 4 Fluorometer (Thermo Fisher Scientific, USA) and purity by use of NanoDrop Spectrophotometers, respectively (Thermo Fisher Scientific, USA). One extraction blank was conducted at each batch for quality control (QC). Concentration of DNA and RNA from extraction blanks were less than the limit of detection. Complementary DNA (cDNA) was synthesized using SuperScript IV Reverse Transcriptase (Invitrogen, CA, USA) along with ezDNase to remove residual DNA.

PCR amplification was performed on normalized cDNA and DNA samples (10 ng/µL) using unique dual tagged primers targeting a 313 bp region of the cytochrome oxidase subunit region 1 (COI) using the primers mICOIintF (GGWACWGGWTGAACWGTWTAYCCYCC) and jgHCO2198R (TAAACTTCAGGGTGACCAAAAAATCA) with a "touchdown" cycle program (Leray et al., 2013; Yang et al., 2017a). To minimize potential bias during amplification, PCR was performed in triplicate using Platinum Taq Hot Start II High-Fidelity DNA Polymerase (Invitrogen, USA), with plate set-up containing multiple PCR blanks for QC. PCR products were checked with agarose gel electrophoresis and purified using the QIAquick PCR Purification kit (Qiagen, Germany). No bands of blanks for extraction and PCR were observed visually. Construction of the sequencing library and next-generation sequencing by use of Illumina chemistry were performed as described previously (DeBofsky et al., 2020). Sequencing data can be accessed at https://doi.org/10.20383/101.0313.

2.3.4 Bioinformatics

Raw reads were demultiplexed based on dual tags of both forward and reverse primers for each sample, with sequences of the forward and reverse primers being removed thereafter. Paired-end sequences were merged using VSEARCH (version 2.14.2), after filtering out lesser quality (ee > 1.0), chimeras, and shorter length (< 300 bp) sequences (Rognes et al., 2016). Zero-radius operational taxonomic units (ZOTUs) were generated using Unoise3, with a minimum frequency of 5 (Edgar, 2016) and their open reading frames (ORF) were searched via NCBI ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/). Pseudogenes and short open reading frames (< 300 bp) were discarded, with features occurring in only one sample subsequently removed.

To gain confidence in identifying species and genera referred to jointly as taxa, features were classified using several steps. BOLD was used to assign features using a percent similarity of greater than or equal to 98%, 95%, and 90% for species, genera, and family-level annotation, respectively (Ratnasingham and Hebert, 2007). Independently, taxonomical

annotations were by use of an in-house curated database for zooplankton plus six barcoded taxa (e.g., *Diaphanosoma birgei, Epischura lacustris, Daphnia mendotae, Leptodiaptomus minutus, Holopedium glacialis*, and *Diacyclops thomasi*; Xie and Giesy, Unpublished) with VSEARCH (percent identity = 0.98; query coverage = 0.8) being used to taxonomically assign ZOTUs (Bolyen et al., 2019). Taxonomic identification output from BOLD and VSEARCH were combined according to consensus, with lowest-level identification superseding. Unidentified sequences or annotations at a higher-level than family, underwent megablast searching, by use of the NCBI Nucleotide Blast Tool using the standard nucleotide database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), returning up to 100 hits per query sequence (e-value = 1e-20, percent identity = 99%, and word size = 24). Taxonomy was assigned to the best attainable level by use of the lowest common ancestor (LCA) implemented using MEGANv6 (Default settings except for min score = 150, top percent = 2), with the highest assignable level allowed being genera.

ZOTUs that remained unassigned or that were nontarget taxa, with target taxa being Phylum Rotifera or select orders in Subphylum Crustacea (Orders Calanoida, Cyclopoida, and Cladocera), were removed (See Table A.1 for sequence read counts). Replicate samples for each enclosure taken for metabarcoding at each time point were merged before downstream analyses. Singleton taxa and taxa found to occur in only one sample were subsequently removed. After collapsing features to the taxa-level for data analyses, unassigned sequences were removed and samples were rarefied to 9985 sequences per sample to avoid bias introduced by uneven sequencing depth (Weiss et al., 2017). Rarefied read count data and raw morphological abundance and biomass data can be found in the supporting information for family, genera, and species-level (Table F.1-F.3). Further details on MiSeq sequencing output can be found in appendix. Bioinformatics was conducted under QIIME2 (version 2020.2) and R environment (version 4.0.0) (Team, 2013).

2.3.5 Statistics

All statistics and graphics were performed in the R environment (version 4.0.0) by use of the Vegan package (version 2.5.6) (Oksanen et al., 2007) unless otherwise stated. Venn diagrams were applied to present the agreement and difference among identification methods. Spearman rank correlation was used to determine relationships between loge-transformed biomass and loge-transformed relative abundance for shared genera between morphological identification and DNA/RNA metabarcoding. Relative abundance refers to rarefied metabarcoding count data. Differences in genera richness between treatments at each time point were estimated by use of ANOVA, as sample size was not sufficient for interpretation, with Welch's t-test used to test between treatments SCA and eMNR. A random intercept model using packages lme4 and lmer Test was used to discern differences in richness between treatment groups while controlling for the effects of time (e.g., -3, 11 and 38 days), with differences for least squares means of respective treatments used for post-hoc testing (Bates et al., 2014; Kuznetsova et al., 2017). Differences in genera richness over time for each remediation practice (e.g., eMNR and SCA) was tested using ANOVA. Principal Coordinates Analysis (PCoA) was performed on genera-level count data to visualize β-diversities of zooplankton communities, with function envfit used to project genera with high correlation with sample ordination as vectors ($p < 0.01$; 9999 permutations). Treatment group differences of β-diversities within each time point were tested using adonis2 (PERMANOVA; 9999 permutations), with a pairwise test being conducted on complete distance matrix, including all samples for each identification method, testing differences between treatments while controlling for the effects of time (Martinez Arbizu, 2017).

2.4 Results

2.4.1 Validation of zooplankton metabarcoding with morphologically identified taxonomy (morph-taxa)

Metabarcoding inferred zooplankton taxonomy was consistent with morphological identification at the family and genera-level. The agreement between mb-taxa (taxa identified

by both DNA and RNA metabarcoding) with morph-taxa decreased from 89.5% at family level (Fig. 2.1A), to 77.4% at genus level (Fig. 2.1B), to 30.0% at species level (Fig. 2.1C). Portion of shared taxa between mb-taxa with morph-taxa decreased from almost 100% at family level (Fig. 2.1D), to 98.9% at genus level (Fig. 2.1E), to 38.0% at species level (Fig. 2.1F). Within classified species, relative abundances (average ± standard deviation (SD)) of *Keratella cochlearis* and *Mesocyclops edax* determined by metabarcoding (*K. cochlearis*: 18.6 ± 22.0 %, *M. edax*: 0.0323 ± 0.0720 %) were much different from those identified by morphology (*K. cochlearis*: 0.512 ± 0.820, M. edax: 25.0 ± 18.3 %). Rare zooplankton genera inferred from morphological identification, for instance, *Lepadella* (Lepadellidae), *Macrocyclops*, *Microcyclops*, *Monostyla*, *Diacyclops*, and *Gastropus* were mis-detected by metabarcoding. Fourteen species were detected by morphological identification but not metabarcoding, specifically, *Bosmina longirostris, Chydorus sphaericus, Diacyclops thomasi, Diaphanosoma birgei, Keratella crassa, Keratella serrulata, Keratella taurocephala, Kellicottia longispina, Macrocyclops albidus, Microcyclops rubellus, Polyarthra vulgaris, Trichocerca cylindrica, Tropocyclops extensus*, and *Trichotria tetractis*. Within those, eight species, *Diacyclops thomasi, Kellicottia longispina, Keratella crassa, Keratella taurocephala, Microcyclops rubellus, Tropocyclops extensus, Trichocera cylindrica*, and *Keratella serrulate* were not found to be represented in the GenBank database (searched 2020-07-21). DNA and RNA metabarcoding revealed similar profiles of zooplankton communities (Fig. 2.1 and C.1). Mismatched taxa between DNA and RNA metabarcoding were rare (relative abundances < 0.01%).

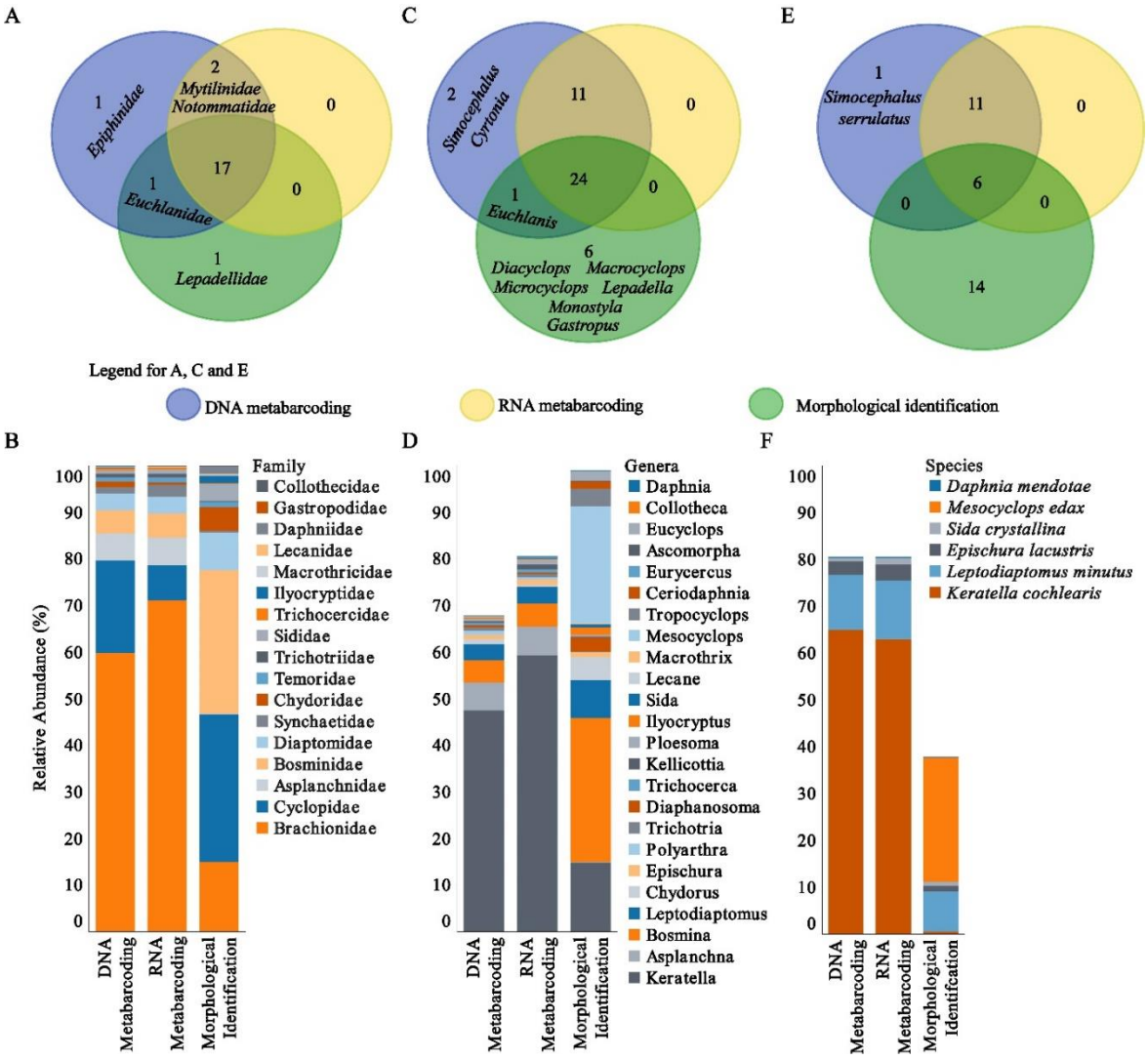

Fig. 2.1 Comparison of zooplankton metabarcoding with morph-taxa. (A) Shared families among identification methods; (B) Relative abundance of shared families; (C) Shared genera among identification methods; (D) Relative abundance of shared genera; (E) Shared species among identification methods; (F) Relative abundances of shared species. Unclassified species were filtered out, with relative abundance being adjusted accordingly.

Metabarcoding based relative abundance of shared genera of Rotifera and Arthropoda phyla revealed the distribution of morphology-based densities. Relative abundances ($\log_e$-transformed) of shared genera for both phyla's Rotifera and Arthropoda had similar trends with that of morphology-based densities ($\log_e$-transformed). For Arthropoda genera, both DNA and RNA metabarcoding based relative abundances ($\log_e$-transformed) of zooplankton were significantly correlated with loge-transformed biomass (Spearman rank correlation,

Fig.2.2B, r = 0.66, p = 0.013; Fig. 2.2C, r = 0.60, p = 0.028). For Rotifera, both DNA (Fig. 2.2B, r = 0.52, p = 0.11) and RNA (Fig. 2.2C, r = 0.52, p = 0.13) metabarcoding based relative abundances were moderately correlated with Rotifera biomass, although the level of significance was marginal. Significant correlations between DNA and RNA metabarcoding of shared Arthropoda and Rotifera genera showed that the two methods gave similar estimates for relative abundances of target taxa (Fig. 2.2A, $r \geqslant 0.93$, $p \leqslant$ 3.1e-06).

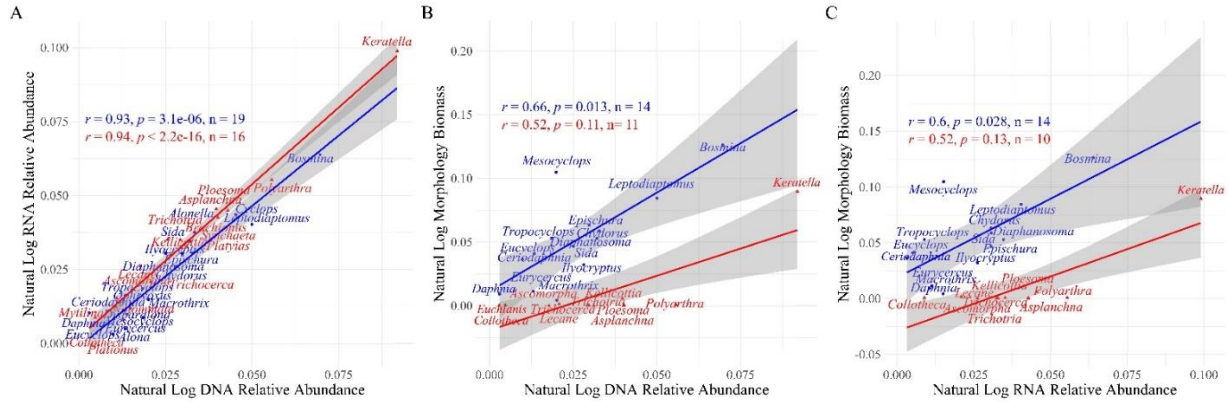

Fig. 2.2 Correlations of shared genera of the $\log_e$-transformed metabarcoding relative abundance data and $\log_e$-transformed morphology biomass using Spearman rank correlation. Blue text indicates phylum Arthropoda and red text indicates phylum Rotifera.

2.4.2 Zooplankton metabarcoding revealed effects of remediation practices on communities of zooplankton

SCA and eMNR caused two different outcomes on zooplankton genera richness in the enclosures over time. No significant differences were observed among enclosures prior to the application of oil spill and cleaning practices (Fig. 2.3, T-test, $p \geqslant 0.425$) and day 11 post-spill (Fig. 2.3, T-test, $p \geqslant 0.570$). At day 38 post-spill, DNA metabarcoding showed an increase in zooplankton genera richness for eMNR relative to SCA (Fig. 2.3A, T-test, p = 0.0357) while for RNA metabarcoding, observed genera richness in SCA declined significantly relative to eMNR (Fig. 2.3B, T-test, p = 0.0478). For remediation practices, for instance, eMNR and SCA, genera richness for eMNR increased over time for DNA metabarcoding (Fig. 2.3A, ANOVA, p = 0.026), while genera richness of SCA practice decreased for RNA metabarcoding over time (Fig. 2.3B, ANOVA, p = 0.00418). Shannon

diversity of zooplankton genera had similar trends as genera richness for DNA and RNA metabarcoding (Fig. D.1). The random intercept model and corresponding computed least square means determined differences for REF vs SCA (Table G.1, p = 0.00968) and eMNR vs SCA (Table G.1, p = 0.0129) for DNA metabarcoding, while difference was determined for REF vs. SCA (Table G.1, p = 0.00203) and REF vs eMNR (Table G.1, p = 0.0473) for RNA metabarcoding.

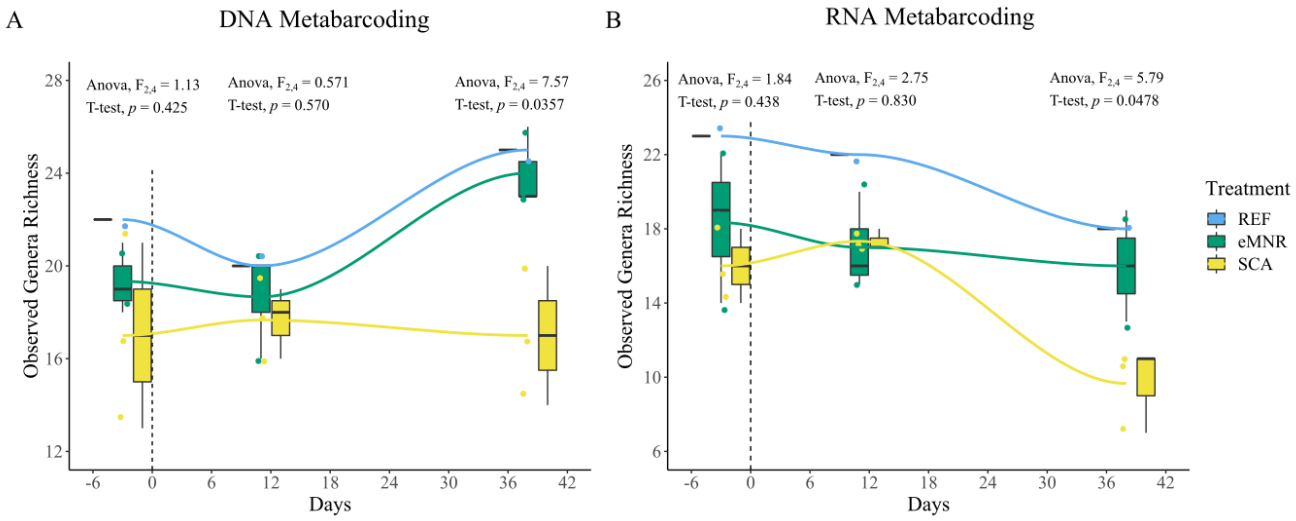

Fig. 2.3 Observed zooplankton genera richness over time for (A) DNA and (B) RNA metabarcoding. Treatment groups consisted of enhanced monitored natural recovery (eMNR; n = 3), shoreline cleaner application (SCA; n = 3), and reference (REF; n = 1). ANOVA was computed between remediation practices at each time point of interest with Welch's t-test used to test observed genera richness between treatments eMNR and SCA. Dashed line represents beginning of simulated dilbit spill and treatment trend is represented by local polynomial regression.

Differences in structures of zooplankton communities between SCA and eMNR at 38 days post-spill was greater than that of day 11 post-spill. Results for PERMANOVA based on DNA and RNA metabarcoding showed that treatments did not differ significantly at 11 days post-spill (Fig. 2.4B, C, $p \geqslant 0.171$). Reference was more closely related to SCA relative to eMNR for both DNA and RNA metabarcoding according to PCoA plots at day 11 post-spill, with *Keratella* being significantly correlated with eMNR sample ordination (Fig. 2.4A, B). At

38 days post-spill, PERMANOVA for DNA and RNA metabarcoding were significant (Fig. 2.4C, $p \leqslant 0.0429$). PCoA plot for DNA and RNA metabarcoding indicated strong clustering for SCA whereas eMNR was more variable in its community composition (Fig. 2.4C, D). From sample position on the PCoA plots at 38 days post-spil1, reference seemed more closely related to eMNR relative to SCA, with *Kertella* being significantly correlated with SCA sample location (Fig. 2.4C, D). Pairwise comparison between all samples, blocking the effect of time, determined differences for REF vs eMNR (Table G.2, $p = 0.173$) and REF vs SCA (Table G.2, $p = 0.0174$) for DNA metabarcoding, while difference was determined for treatment combination REF vs SCA (Table G.2, $p = 0.0142$) for RNA metabarcoding.

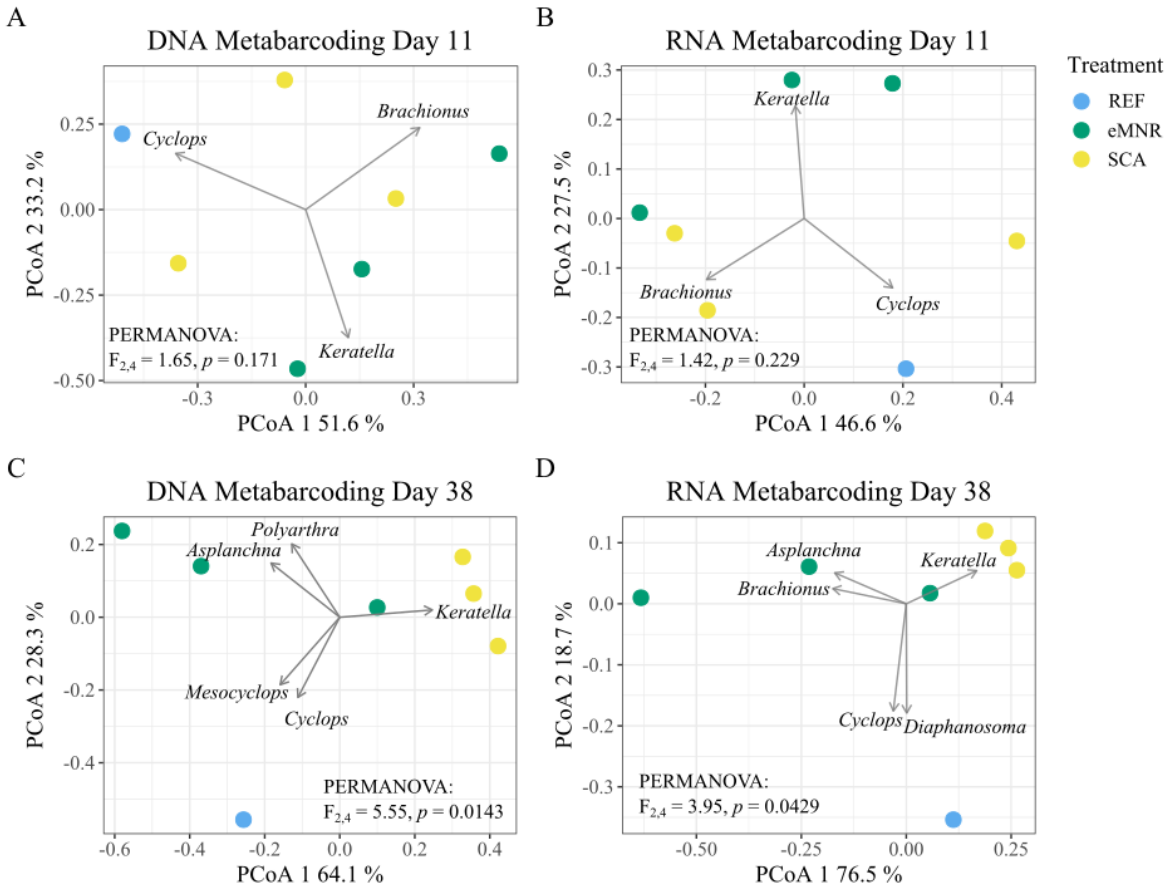

Fig. 2.4 PCoA plots between treatment groups for genera-based matrix. (A) DNA metabarcoding at 11-days post-exposure; (B) RNA metabarcoding at 11-days post-exposure; (C) DNA metabarcoding at 38-day post-exposure; (D) RNA metabarcoding at 38-days post-exposure. Treatment groups consisted of enhanced monitored natural recovery (eMNR; n = 3), shoreline cleaner application (SCA; n = 3), and reference (REF; n =1). Associated

PERMANOVA statistic is shown on the respective plots. Genera plotted inferred to be highly correlated with sample ordination ($p < 0.01$).

2.4.3 Performance to distinguish ecological effects of remediation practices

Morphological identification differed from metabarcoding in the ability to determine ecotoxicological effects of remediation practices. No statistical differences were observed among remediation practices for genera richness based on morphology. A trend towards lesser richness was observed for SCA on day 11 relative to eMNR (Fig 2.5A, T-test, $p = 0.183$). Morphological identification indicated that zooplankton richness increased for SCA on day 38 (Fig 2.5A, T-test, $p = 0.519$). The random intercept model and corresponding computed least squares means determined no differences between treatment groups (Table G.1, $p \geqslant 0.114$). Based on morphometry, treatment groups differed at 11-days post-exposure for community composition (Fig. 2.5B, PERMANOVA, $p = 0.0143$). Treatment groups did not differ in centroid position at day 38 (Fig. 2.5C, PERMANOVA, $p = 0.486$), with eMNR being closer in distance to reference at relative to SCA, indicating a stronger relationship between the two communities at day 38 (Fig. 2.5C). Pairwise comparison of the distance matrix based on all samples, blocking the effect of time, determined differences for REF vs eMNR (Table G.2, $p = 0.047$).

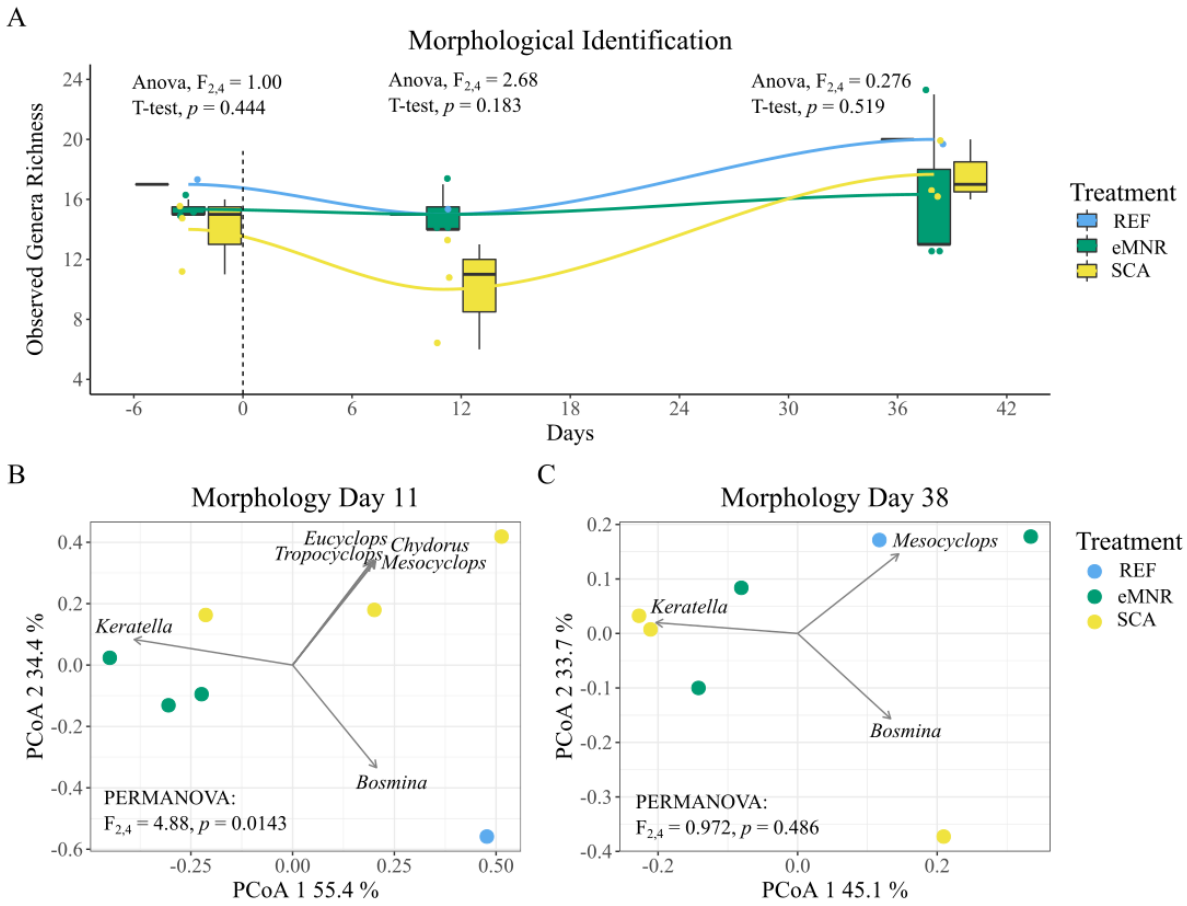

Fig. 2.5 Observed zooplankton genera richness for treatments over time and β-diversity analyses of selected time points, (B) 11-days post-exposure and (C) 38-days post-exposure, for morphological identification. Treatments consisted of enhanced monitored natural recovery (eMNR; $n = 3$), shoreline cleaner application (SCA; $n = 3$), and reference (REF; $n = 1$). ANOVA was computed between treatment groups, with Welch's t-test used to test observed genera richness between treatments eMNR and SCA. Dashed line represents beginning of simulated dilbit spill and treatment trend is represented by local polynomial regression. Associated PERMANOVA statistics are shown on the respective plots PCoA plots. Genera plotted inferred to be highly correlated with sample ordination ($p < 0.01$).

2.5 Discussion

2.5.1 Overall agreement between metabarcoding and morphological identification

Zooplankton taxonomy as determined by metabarcoding was analogous to morph-taxa at the family- and genera-levels. Overall, similar trends in the responses of zooplankton

communities to differing remediation practices were observed, even with differences existing between the profiles of relative abundances of zooplankton among methods of identification. Metabarcoding of zooplankton has previously been shown to effectively capture spatial and temporal trends determined by morphological identification, even when differences existed in community profiles of zooplankton between the two methods (Abad et al., 2016). Furthermore, monitoring of macroinvertebrates in boreal stream ecosystems by use of DNA metabarcoding was consistent with results based on morphological metrics at family and genera level (Emilson et al., 2017). Abundant zooplankton genera, *Keratella*, *Bosmina*, *Leptodiaptomus*, *Mesocyclops*, and *Polyarthra*, detected with metabarcoding were consistent with results of previous papers examining other local boreal lakes as well as the lake used in this study (Drouin et al., 2009; Kidd et al., 2014). Abundant zooplankton genera, *Keratella*, *Bosmina*, *Polyarthra*, *Asplanchna*, and *Diaphanosoma* additionally had global comparability with a boreal lake in Finland (Arvola et al., 1996). Metabarcoding provides a high throughput method for analyzing various ecological communities but has yet to be optimized for zooplankton communities in boreal ecosystems.

Use of DNA and RNA metabarcoding, or present and active taxa, respectively, resulted in similarly measured zooplankton communities; however, they differed from morph-taxa in species composition, with only six shared species. Different taxonomic levels can be used to assess the status of aquatic ecosystems and classifying individuals to the level of family, with 17 shared families, or genera level, with 24 shared genera, was shown to be somewhat sufficient for comparing the two identification methods: metabarcoding and morphological identification. Relative abundances as determined by use of metabarcoding could be an acceptable proxy for biomass of zooplankton inferred from morphological identification at genera level. Results of previous studies have shown that eDNA/DNA copies can be correlated with organism biomass (Elbrecht and Leese, 2015; Takahara et al., 2012; Yang et al., 2017c). Taxa richness is a useful metric to measure temporal dynamics of zooplankton communities. Even with the relatively great discrepancy between taxonomic compositions at the species level between zooplankton identification methods, comparatively

similar temporal changes in genera richness were detected, although differences did exist (Fig. E.1). Mitochondrial COI has been shown previously to be a valuable metabarcoding marker for zooplankton biodiversity assessment (Clarke et al., 2017).

2.5.2 Potential reasons for discrepancy between zooplankton identification methods

Several underlying factors could have affected results inferred from metabarcoding when comparing to morphological identification. Some boreal freshwater zooplankton species are not yet barcoded and represented in public databases, explaining the number of species not detected by metabarcoding. To better determine taxonomic composition through next-generation sequencing, more representative taxa of typical watersheds should be barcoded (Yang et al., 2017d). Some of the species missed by metabarcoding were included in the in-house curated database (e.g., *Diacyclops thomasi*). This seems to indicate that even in local watersheds, zooplankton can have sequence divergence and adequate number of individuals need to be barcoded in order to accurately detect at the species-level. Naming conventions of zooplankton, which can be highly variable, can also lead to differences in the species detected due to database limitations of the corresponding metadata (Visco et al., 2015), including *Keratella cochlearis.* a species complex that morphological taxonomy has not been fully worked out. More examples of variable naming conventions or validation of morphological identification consensus include *Trichotria tetractis* (e.g. *Dinocharis tetractis*), *Chydorus sphaericus*, *Diacyclops thomasi* (e.g. *Diacyclops/Cyclops bicuspidatus thomasi*), *Polyarthra vulgaris* (e.g. *Polyarthra trigla*), *Kellicottia longispina* (e.g. *Anuraea longispina* or *Notholca longispina*), *Bosmina longirostris, Trichocerca* (e.g. *Acanthodactylus, Coelopus, Diurella, Mastigocerca, Monocerca, Rattulus, or Vaginaria*), *Tropocyclops extensus* (e.g. *Tropocyclops prasinus mexicanus*), *Keratella crassa* (e.g. *Keratella cochlearis*), *Microcyclops rubellus* (e.g. *Microcyclops varicans rubellus*), and Diaphanosoma birgei (e.g. *Diaphanosoma leuchtenbergianum* or *D. brachyurum*). Metabarcoding can potentially detect taxa not from target habitat (e.g. pelagic) due to residual DNA, and possibly RNA, adhered to the zooplankton tissue and within the gut or adhered to algae likely collected by the 53 µm mesh

filter used, leading to differences in species composition relative to morphological identification (Barnes et al.; Siegenthaler et al., 2019). This could explain the greater detection of zooplankton not primarily planktonic but associated with surfaces or sediments via metabarcoding methods (e.g., *Eurycercus*, *Macrothrix*, *Eucyclops*, *Ilyocryptus*, and *Chydorus*).

Relative abundance of detected taxa also varied between identification methods. One reason for the discrepancy of differential portions between methods is zooplankton species could have shed DNA, in the form of exoskeleton or sloughed tissue, at variable rates into the water column or differ in target gene copies within individuals, resulting in potential biases of relative abundance (Harvey et al., 2017; Sassoubre et al., 2016). Total DNA amounts of zooplankton could have also influenced the inferred relative abundance. Taxa within zooplankton communities can have various life-histories, with rotifers and cladocerans being opportunistic and other plankters, such as copepods, exhibiting longer life cycles and fewer generations (Allan, 1976). This variability could have direct impacts on the inferred activity of select taxa (Blazewicz et al., 2013), while differences in zooplankton habitat preference could impact the suggested presence upon collection (Leduc et al., 2019). RNA "production" could also vary according to the life-history and biology of the target zooplankton genera, influencing the relative detection and abundance. Zooplankton DNA and RNA could also have been extracted with varying levels of recovery for different taxa, potentially affecting relative abundances inferred (Liu et al., 2019). Biases in PCR and body-size have been shown to impact inference of species presence and relative abundances in target ecosystems, affecting total species detected and their relative portions in zooplankton communities (Elbrecht and Leese, 2015; Gibson et al., 2014; Harvey et al., 2017; Polz and Cavanaugh, 1998).

Comparing to biomasses of genera determined by morph-taxa, results of metabarcoding indicated that genera in the phylum Rotifera had greater relative abundances compared to genera in the phylum Arthropoda. Traditional morphological estimates for biomasses of genera within the phylum Rotifera may not be representative due to too large of

filter mesh utilized when collecting in the field (Chick et al., 2010), or due to small sizes. Differences in life history of the two phyla could also have impacts on inferred biomass. However, additional reasons could be due to sampling, extraction, and PCR steps within the metabarcoding pipeline.

2.5.3 DNA and RNA metabarcoding: advantages and disadvantages for ecotoxicological assessment

Metabarcoding-based genera richness can be a sensitive method to measure the ecotoxicological response of communities to environmental disturbances. SCA had the greatest negative impact on richness of the zooplankton community based on both DNA and RNA metabarcoding. COREXIT® EC9580A has been shown to be acutely toxic to pelagic copepod species *Acartia tonsa*, and a mysid, *Americamysis bahia*, with a 48-h LC50 of 50.4 ± 4.47 mg/L and 32 mg/L, respectively (Bi et al., 2020; Fingas, 2013; Hansen et al., 2014). Nutrient enrichment has been found to increase richness of zooplankton, due primarily to increases in rotifer species (Azevêdo et al., 2015), which can be seen with increases in total dissolved phosphorus at 38 days post-spill for eMNR (Table B.2). Too large of an increase in primary productivity, however, can lead to an overall decrease in zooplankton species richness (Dodson et al., 2000). Since RNA metabarcoding can measure response of communities at the time of sampling, without the common issue of persistence of DNA in the environment (Cristescu, 2019) or identification of nonviable zooplankton (Zetsche and Meysman, 2012), it could be advantageous for measuring changes in community structure due to exposures to stressors. Results of previous studies have shown that RNA can decipher more significant changes in taxa richness due to treatment relative to DNA metabarcoding (Laroche et al., 2017). RNA metabarcoding could be influenced by the variability in production due to life-history traits of different zooplankton genera. The variability of the enclosures at day -3 could have also impacted inferred differences between treatments for RNA metabarcoding.

Monitoring changes in community composition is a powerful method to measure effects of environmental stressors. Over time, SCA seemed to have an overall negative effect on the composition of the zooplankton community at the genera level as measured with metabarcoding techniques (Parsons et al., 1984), which could explain the greater distance between SCA and REF at day 38, relative to eMNR, and the closer clustering of the SCA samples for RNA metabarcoding. Due to the variable tolerance of zooplankton taxa to nutrient enrichment, eMNR could have had contrasting magnitudes of effects on zooplankton communities over time (Yang et al., 2017b). With variable zooplankton communities in enclosures at day -3, greater dispersion between eMNR treated enclosures over time could occur (Strecker and Arnott, 2005). Overall, DNA metabarcoding, relative to RNA metabarcoding, may be more reliable for assessing treatment effects on community composition (Laroche et al., 2017), which was observed in the current study with a larger magnitude of differences in zooplankton community composition between remediation practices for DNA metabarcoding.

DNA metabarcoding can commonly be the result of legacy contamination in the ecosystem, as DNA is typically more stable and persistent than RNA in the environment (Cristescu, 2019). RNA metabarcoding can act as an effective method to depict responses of active communities (Baldrian et al., 2012), however, variation in activities of organisms and life history can generate taxonomic biases as well as PCR artifacts formed from cDNA synthesis (Blazewicz et al., 2013; Brandt et al., 2020; Houseley and Tollervey, 2010). It has been suggested to use both DNA and RNA metabarcoding when assessing ecosystems for these reasons, among others (Laroche et al., 2017; Pochon et al., 2017). Coupled DNA and RNA metabarcoding could serve as a stand-alone assessment of ecosystem status or can be used as a complementary method to morphology-based monitoring (Laroche et al., 2018).

2.5.4 Comparison of metabarcoding and morphological identification for ecotoxicological assessment of remediation practices of oil spills

Metabarcoding methods overall were more sensitive relative to morphology in measuring changes in genera richness caused by various remediation practices. There are however limitations in the statistical power of statistical tests conducted, due to small sample sizes. Alpha diversity as determined by metabarcoding has been shown to be consistent with that calculated from taxa defined by morphology, although typically more sensitive to spatial or environmental differences (Frontalini et al., 2020; Mauffrey et al., 2020). Regardless of identification method, SCA seemed to have the largest negative impact on richness of the zooplankton community over time. Morph-genera community composition shifted significantly on day 11, however, on day 38 no difference was seen between treatment groups, which disagreed with metabarcoding methods. A previous study determined that zooplankton metabarcoding can be a more sensitive method for analyzing community composition differences relative to morphology, which was seen at 38 days post-spill (Yang et al., 2017c).

2.6 Conclusions

This study revealed that identification of zooplankton based on ZOTUs from metabarcoding and morphological identification were relatively consistent in their ability to identify the presence of each zooplankter to the genera-level and detect changes in zooplankton communities over time due to remediation practices. Metabarcoding could be more sensitive relative to morphological identification for detecting changes in zooplankton genera richness over time due to remediation practices. There were limitations in inferences of results when comparing between methods due to small sample sizes, including having only one reference enclosure and variability of enclosures at day -3. For the metabarcoding methods, DNA metabarcoding was the most sensitive in detecting changes in zooplankton community composition due to remediation practices. For all identification methods, SCA had the greatest impact on zooplankton genera richness relative to eMNR and REF. β-

diversity analyses showed that both shoreline cleaner application and nutrient enrichment can cause changes in zooplankton community composition. Overall, as shown by both α- and β-diversity analyses, while surfactants can release stranded petroleum constituents of dilbit from shoreline substrates to be mechanically removed, shoreline cleaner application has the greatest, acute effect on the zooplankton community.

CHAPTER 3

GENERAL DISCUSSION

3.1 Project Focus and Objectives

Current rates of ecosystem change and the rise of anthropogenic impacts on aquatic ecosystems warrants next-generation methods to assess these effects and the resulting ecological health more quickly and efficiently. A recently developed method, metabarcoding, has been developed along with the overall increase in capabilities for next generation sequencing to assess community composition of organisms from nucleic acids collected from an ecosystem. This toolset is launching many new possibilities for adding in metabarcoding methods to current biomonitoring programs and using these methods for assessing the ecotoxicological impacts from chemical stressors. Oil spills have been a recurring problem worldwide, with the use of petroleum products only expected to increase. Furthermore, the impacts of the remediation practices utilized to help recover an impacted freshwater ecosystem are not well understood *in-situ*. Unique to Canada, diluted bitumen (dilbit) is a complex petroleum mixture with potential to impact low-energy freshwater ecosystems, with the resulting outcomes not well understood, including the use of remediation practices in these settings after a spill would occur.

The focus of this project was to utilize DNA and RNA metabarcoding to assess the response of the zooplankton community to two currently used remediation practices, shoreline cleaner application (SCA), and enhanced monitored natural recovery (eMNR), of a simulated dilbit spill in a boreal lake. Metabarcoding methods were also compared to traditional morphological identification to assess the ability to detect zooplankton taxa and measure the response of ecotoxicological impact on the zooplankton community. The first objective was to compare the detection and the relative abundances of zooplankton taxa in communities as determined using DNA and RNA metabarcoding or using morphologically identified taxonomic techniques. The second objective was to determine and compare the ecotoxicological effects of remediation practices on zooplankton communities in shoreline enclosures. The final objective was to compare the performance of the three zooplankton identification methods (DNA metabarcoding, RNA metabarcoding, and morphological taxonomy) to elucidate the effects of oil-spill remediation practices. This research aimed to

benchmark the use of zooplankton metabarcoding in a freshwater boreal ecosystem to assess the response of the zooplankton community to ecotoxicological impact.

3.2 Summary of Findings

This study used DNA and RNA metabarcoding and morphological identification of zooplankton to determine the response of the community to different oil-spill remediation practices. This study also compared the different identification methods in their ability to detect zooplankton taxa and measure the response of the community to ecotoxicological effects. Overall, SCA coupled with dilbit exposure had the greatest impact on the zooplankton community relative to nutrient enrichment. DNA and RNA metabarcoding was able to detect zooplankton taxa up to the genus and family-level when compared to morphological identification, however several genera were missed by metabarcoding methods and variability in relative abundance was inferred. Metabarcoding methods could be more sensitive overall compared to morphological identification for measuring the zooplankton community's acute response to the ecotoxicological effects due to the coupled oil-spill and remediation practices.

3.2.1 Acute toxicity of shoreline cleaner application (SCA) and enhanced monitored natural recovery (eMNR)

Changes in zooplankton community composition were observed during both remediation practices, eMNR and SCA. However, SCA had the greatest effects on the zooplankton community, which can be observed for both alpha and beta diversity index analysis with statistical tests utilized. It has been previously shown that SCA and the corresponding SWA used in this study (e.g., COREXIT® 9580A) can be acutely toxic to pelagic invertebrates (Bi et al., 2020; Fingas, 2013; Hansen et al., 2014). Ecosystem-level experimentation was required to gain a greater understanding of the potential effects posed by SWA at the community level *in-situ*. A recent study conducted in outdoors mesocosms determined 100% immobility of water striders (*Metrobates sp.*) to COREXIT® 9580A application within freshwater microcosms (Black et al., 2020).

The use of chemicals to treat petroleum spills can be argued to have a greater negative impact relative to the benefits gained due to the inherent toxicity of these anthropogenic compounds (e.g., COREXIT® 9580). Development of green chemicals or solvents is of priority to reduce the use of hazardous products while still attaining the same performance (Capello et al., 2007). These green chemicals or solvents can serve as a replacement to the conventional toxic solvents used in shoreline washing agent products historically (Chen et al., 2019). These emerging green solvents are commonly derived from plants, animals, and microorganisms. An example of a green solvent includes CytoSol®, a solvent composed of vegetable oil methyl esters and bioremediation agents (von Wedel, 2000). Several different plant oils have been tested for use as an SWA, including vegetable oil, peanut oil, and sunflower oil (Chen et al., 2019). The use of green solvents can assist in the reduction of energy consumption while also accelerating the biodegradation of contaminants due to natural biodegradability (Chen et al., 2019).

Nutrient enrichment is used to stimulate hydrocarbon-degrading microorganisms and can be an effective approach in remediating oil-contaminated shorelines and aquatic ecosystems, which was the purpose of implementing enhanced monitored natural recovery as a remediation practice (Atlas and Hazen, 2011; Bragg et al., 1994; Prince, 1993). Excess nutrients in aquatic ecosystems, however, can lead to eutrophication, with harmful algae bloom formation and expanding water hypoxia arising (Pretty et al., 2003; Watson et al., 2016). Long-term nutrient enrichment in a boreal lake of low-energy has been shown to negatively impact the biomass of planktonic zooplankton (Malley et al., 1988; Paterson et al., 2011). Nutrient enrichment can lead to a rise in the richness of rotifer species, sometimes leading to an overall increase in zooplankton richness (Azevêdo et al., 2015). Too large of an increase in primary productivity can, however, lead to decreases in zooplankton species richness (Dodson et al., 2000).

3.2.2 Zooplankton community measurement via DNA, RNA metabarcoding and morphological identification

DNA and RNA metabarcoding were able to detect zooplankton up to the family and genus-level. Family and genus-level identification can be employed for the biomonitoring of macroinvertebrates and zooplankton (Emilson et al., 2017; Machado et al., 2015). At the species-level, many taxa were misidentified by both methods, which can be quite common if databases have not been curated for the local region of the taxonomic group of study (Schenk et al., 2020; Yang et al., 2017d). Two different barcode regions could be used, such as coupled 18S rRNA and COI metabarcoding, to help increase identification of zooplankton species (Zhang et al., 2018a). For this experiment, we wanted to compare metabarcoding to morphological identification using a single primer to save money and time, as using a single primer can be a common practice in DNA metabarcoding biomonitoring studies. Adding in an additional primer can lead to greater difficulties with interpreting metabarcoding data output and lead to complications when inferring relative abundance and presence of target taxa. Naming conventions of zooplankton can also lead to differences in the shared species detected due to database limitations of the corresponding metadata, as naming conventions of taxa can be variable (Visco et al., 2015). Metabarcoding can also detect non-target taxa from residual DNA, and possibly RNA, adhered to organisms or on non-target collected algae, leading to differences in species composition (Barnes et al.; Siegenthaler et al., 2019). PCR bias has been shown to influence detection of species and the inferred relative abundance, affecting total shared zooplankton species between methods and the relative portions (Elbrecht and Leese, 2015; Gibson et al., 2014; Harvey et al., 2017; Polz and Cavanaugh, 1998).

Relative abundances of zooplankton determined by metabarcoding was an acceptable proxy for biomass inferred from morphological identification at the genera-level. Previous studies have shown that eDNA/DNA copies can be correlated with organism biomass (Elbrecht and Leese, 2015; Takahara et al., 2012; Yang et al., 2017c). Relative abundance of detected species, however, varied between identification methods. Zooplankton species could

have shed DNA at variable rates into the water column or differ in target gene copies within individuals, resulting in biases of relative abundance (Harvey et al., 2017; Sassoubre et al., 2016). Life-history variability of zooplankton could impact the inferred activity of select taxa or the relative abundance inferred from RNA metabarcoding (Blazewicz et al., 2013), while differences in zooplankton habitat preference could influence presence upon collection (Leduc et al., 2019). Zooplankton DNA and RNA may have been additionally extracted with varying levels of recovery for different taxa, leading to biases in inferred relative abundance (Liu et al., 2019).

Comparing biomasses of genera determined by morph-taxa with metabarcoding relative abundance, phylum Rotifera had greater relative abundances compared to genera in the phylum Arthropoda relative to what was inferred from biomasses. Traditional estimates for the biomass of genera within phylum Rotifera may not be representative due to too large a size of filter mesh utilized during collection (Chick et al., 2010) or due to small sizes. Differences in the life history of the two phyla, Rotifera and Arthropoda, could have impacted inferred biomass, with additional reasons due to sampling, extraction, and PCR within the metabarcoding pipeline. Finally, it should be noted that the zooplankton communities had high genetic variability for each assigned specific taxon using the COI gene. This was observed when looking at the number of ZOTUs assigned to each respective species. The concept of species has been evolving, especially in the context of amplicon sequencing, with intragenomic heterogeneity causing issues with inferences of diversity (Janda and Abbott, 2007; Sun et al., 2013). This can lead to inherent differences between comparisons made between morphological and genetic assessments, as genomics continues to resolve how we classify taxonomy and help assist in deciphering species complexes. Bioinformatics methods and databases will need to be continuously updated to consider new information gained from this area of research.

3.3.3 Comparison of zooplankton identification methods to measure ecotoxicological effects

Different zooplankton identification methods were used to determine the ecotoxicological effects of the two different remediation practices. RNA metabarcoding has the potential to measure the response of the active zooplankton community at the time of sampling, without the issue of DNA persistence in the environment (Cristescu, 2019) or identification of nonviable zooplankton (Zetsche and Meysman, 2012). RNA metabarcoding could be advantageous for measuring changes in community structure due to exposures to stressors, especially in the case of changes in taxa richness (Laroche et al., 2017). DNA metabarcoding may be more reliable for assessing treatment effects on community composition relative to RNA metabarcoding (Laroche et al., 2017), which was observed in the current study. One of the reasons for this outcome is DNA metabarcoding can commonly be the result of legacy nucleic acid contamination in the ecosystem due to the greater stability of DNA in the environment (Cristescu, 2019), leading to more taxa detected driving differences in communities measured in different habitats or settings.

Even though RNA metabarcoding can be effective at depicting responses of the active community (Baldrian et al., 2012), variation in organism activity and life history can generate taxonomic biases, with cDNA synthesis adding to the biases due to artifacts generated during the procedure (Blazewicz et al., 2013; Brandt et al., 2020; Houseley and Tollervey, 2010). cDNA synthesis using reverse transcriptase, unlike PCR with DNA polymerase, does not undergo proofread during the procedure. DNA and RNA metabarcoding have been suggested to be both performed when assessing ecosystems (Laroche et al., 2017; Pochon et al., 2017). Previous studies have indicated that metabarcoding could serve as a stand-alone biomonitoring tool or as a complementary method to morphology-based monitoring (Laroche et al., 2018); however from this study we determined that differences can exist between the identification approaches and the monitoring conclusions made.

Metabarcoding methods, overall, could be more sensitive relative to morphology in measuring changes in genera richness caused by various remediation practices. Alpha diversity, as determined by metabarcoding, is consistent with morphology-based alpha

diversity but can be more sensitive to spatial or environmental differences (Frontalini et al., 2020; Mauffrey et al., 2020). SCA for all methods had the largest negative impact, seen as declines, on richness of the zooplankton community. Morph-taxa community composition shifted significantly on day 11, but no difference was determined on day 38, which disagreed with metabarcoding methods. Zooplankton metabarcoding can be more sensitive for analyzing community composition differences relative to morphology-based identification, which was determined at 38 days post-spill (Yang et al., 2017c).

3.3 Recommendations for Future Work

Metabarcoding of ecological communities will serve as a valuable tool for assessing at-risk ecosystems and for development of timely and efficient methods for determination of potential ecotoxicological effects. There are many areas for improvement with this newly developed method. First, more research on the best sampling and collection methods should take place. This will allow the use of standardized protocols when collecting in the field. In the case of tissue metabarcoding, the same sampling effort can result in greater detection of non-indigenous species for metabarcoding approaches relative conventional sampling methods (Zaiko et al., 2015). Environmental DNA metabarcoding sampling effort can be more tricky, as the ecology of the eDNA will need to be accounted for (Deiner et al., 2016); however, in the case of zooplankton, tissue metabarcoding could be the best method as more quality DNA is obtained and better inference of the community dynamics could be detected.

Second, research in the use of coupled RNA and DNA metabarcoding should continue to occur, as the relationship between the nucleic acids needs to be explored in a metabarcoding context. It has been shown that eRNA can have a similar decay rate as eDNA, with greater stability in the environment than was expected (Wood et al., 2020); however, in most lines of thought eRNA degrades more rapidly than eDNA in the environment. Using tissue DNA and RNA could avoid the issue of the stability of the nucleic acids in the environment, but more information on the fluxes of these nucleic acids in response to life-history changes and stressors needs to be accounted for. Overall, RNA could serve as a useful and valuable

method for aquatic biomonitoring and biodiversity assessment (Laroche et al., 2017), with limitations due to the natural fluxes of RNA within organisms. Third, the development of PCR-free methods, better primers, or improved correction methods can help deal with the issues of PCR biases (Leese et al., 2021; Liu et al., 2016; McLaren et al., 2019).

Fourth, the ecology of eDNA and eRNA will have to be continued to be researched. Many ecologists are currently working in this area, as this information is crucial to the benchmarking of eDNA/eRNA and tissue DNA/RNA metabarcoding within government monitoring programs. The similarities and differences in what tissue DNA and RNA metabarcoding measure will also need to be researched further, as eRNA and RNA metabarcoding are a relatively new idea in terms of biomonitoring and ecological response to stressors. Finally, databases of target communities need to be continuously improved and updated with more curated species to allow the accurate identification of molecular sequences (Ruppert et al., 2019). Large limitations still exist in enabling accurate identification of metabarcoding data to the species level, as indicated by this study. For taxonomic groups not well annotated, such as freshwater nematode communities, the disconnect can be just as great (Schenk et al., 2020). More effort will need to be put into taxonomically classifying taxa via traditional methods and building comprehensive databases using the corresponding genomic data.

Oil spills will continue to have impacts on aquatic ecosystems as petroleum is continued to be extracted, transported, and consumed. Ecosystem studies help provide necessary information on the potential impacts of these anthropogenic stressors on aquatic ecosystems in a relevant context. More research needs to be done to better understand the underlying drivers and the long-term impacts of oil spills on freshwater ecosystems (Black et al., 2020; Cederwall et al., 2020), as this study determined the relatively acute response of the zooplankton community to remediation practices coupled with a simulated dilbit spill. Additional research on the most effective and least disruptive remediation practices of oil-spills needs to be conducted. The impacts of these remediation practices on oil-spills have not been previously examined in boreal lakes of low-energy. In the case of SWAs, more research

needs to be conducted in order to fully assess these compounds prior to use in freshwater ecosystems (Black et al., 2020; Stroski et al., 2019). Alternative methods for remediating oil-contaminated freshwater ecosystems needs to be closely examined to provide best methods to regulators and spill-responders. Science and technology are continuously evolving and more efficient and less invasive methods for remediation oil-spills are likely to arise. Research in this area will allow the selection of the best remediation practice and approach for the problem at hand.

3.4 Conclusion

This thesis research was conducted to examine the potential value of zooplankton metabarcoding to assess the impacts of different remediation efforts of a simulated oil spill. The results demonstrated that metabarcoding could detect zooplankton up to the genera-level and can serve as an acceptable proxy for the biomass of zooplankton at the genus-level. Zooplankton metabarcoding could also be the more sensitive method for the detection of the impacts of the different remediation practices of oil-spills relative to morphological identification. This thesis also shows the differences between using DNA and RNA metabarcoding for the assessment of ecological communities' response to stressors. While metabarcoding can be a powerful approach, it still requires substantial effort to benchmark the method, including building a comprehensive taxonomic database. SCA coupled with the simulated dilbit spill was shown to have the greatest overall impact on the zooplankton community. This suggests that the use of the SWA COREXIT® EC9580A, while effective, can have acute negative effects on the zooplankton community. Overall, this research highlights the ability of zooplankton metabarcoding to assess the impacts of acute environmental stressors and the short-term impacts of different remediation practices of simulated oil-spills.

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APPENDICES

Appendix A: MiSeq sequencing output

Metabarcoding consisted of a total of 1,637,206 sequences after demultiplexing from the two MiSeq runs, with run 1 having 698,592 and run 2 having 938,614 sequences. Technical replicates had sequence counts of 8798 ± 6914 (mean ± standard deviation (SD)), while blanks had sequence counts of 322.4 ± 455 (mean ± SD) prior to merging of the two libraries, with some samples being re-sequenced on run 2. After denoising and merging technical replicates, a total of 1,301,361 sequences were assigned to target metazoan (Phylum Rotifer and Orders Calanoida, Cyclopoida, and Cladocera) to at least the family level, with merged technical replicates having sequence counts of 30984.8 ± 18821.9 (mean ± SD) (Table S1). After collapsing features to the taxa-level and removing unassigned taxa or taxa occurring in only one sample, samples were rarefied to equal read depths of 9985 (Figure S2).

Appendix A: MiSeq sequencing output

Table A.1: Resulting annotated sequence read output post-denoising and ORF correction. Non-target indicates non-metazoan sequence reads, metazoan indicates all sequence reads assigned to metazoan, and target indicates zooplankton annotated sequence reads.

Total Reads	Non-target Reads	Metazoan Reads	Target Reads
1,552,241	57458	1494783	1301361

Appendix A: MiSeq sequencing output

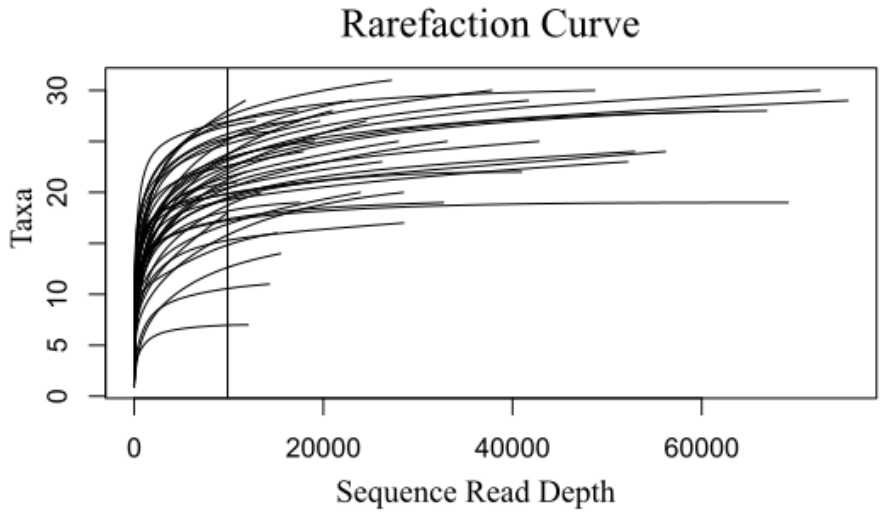

Figure A.1: Rarefaction curve of number of detected taxa for each sample. The vertical line indicates the rarefied read depth of 9885.

Appendix B: Experimental shoreline enclosure descriptions

Beginning in April 2019, when water temperatures had warmed enough to allow work to begin (>8°C), enclosures (15 X 5m) (Curry Industries, Winnipeg) were deployed in Lake 260 shorelines of organic wetland type sediment. The enclosures' consisted of a polystyrene foam floatation collar encased in a polyvinyl shell. The floatation collar suspended an impermeable polypropylene curtain that extended to the bottom of the lake, where it was sealed to the aquatic and terrestrial sediment/soil using a double row of sandbags. A total of six enclosures were treated with oil in shoreline areas of organic/wetland sediments. One enclosure, not treated with oil, was included to serve as a reference (a total of seven enclosures). Water depth measurements were obtained at 1m intervals from the shoreline to determine slope of the lake bottom and estimate enclosure volumes (28,500 ± 1650L). Enclosures were assigned to a given treatment, or to reference designations, randomly. Table S1 indicates the specific locations for each enclosure.

Appendix B: Experimental shoreline enclosure descriptions

Figure B.1: Aerial photo of experimental design used in comparing metabarcoding and traditional morphological identification of zooplankton in assessing the ecotoxicological effects of two remediation practices – enhanced monitored natural recovery (eMNR) using the addition of nitrogen and phosphorous, and a shoreline cleaner, COREXIT EC9580A (SCA) – relative to a reference enclosure (REF). Diluted bitumen was applied to enclosures on June 21st, with the selected remediation practices being applied on June 25th. Unlabeled enclosures are not part of this select experiment.

Appendix B: Experimental shoreline enclosure descriptions

Enclosure	Treatment	Latitude	GPS Latitude	Longitude	GPS Longitude
wR1	REF	N	49.69983	W	93.76760
wEMNR1	eMNR	N	49.69994	W	93.76750
wSC1	SCA	N	49.69997	W	93.76740
wSC2	SCA	N	49.70000	W	93.76730
wEMNR2	eMNR	N	49.70000	W	93.76721
wEMNR3	eMNR	N	49.70041	W	93.76715
wSC3	SCA	N	49.70048	W	93.76711

Table B.1: Experimental shoreline enclosure locations within Lake 260.

Appendix B: Experimental shoreline enclosure descriptions

Date	Total Dissolved Phosphorus			Chlorophyll *a*		
	REF	**eMNR (avg. ± SD)**	**SCA (avg. ± SD)**	**REF**	**eMNR (avg. ± SD)**	**SCA (avg. ± SD)**
6/18/2019 (Day -6)	6.00	6.03 ± 1.10	5.80 ± 0.889	4.10	3.83 ± 0.953	4.05 ± 1.27
7/02/2019 (Day 11)	6.80	6.87 ± 1.66	6.60 ± 0.458	2.60	3.60 ± 1.16	4.57 ± 1.38
7/29/2019 (Day 38)	4.90	6.27 ± 1.10	4.60 ± 0.794	1.43	1.92 ± 0.0874	3.68 ± 1.65

Table B.2: Total dissolved phosphorus and chlorophyll *a* measurement for treatments (average ± SD) over time points sampled. Day -6 was used as pre-spill measurement. Treatment groups consisted of enhanced monitored natural recovery (eMNR), shoreline cleaner application (SCA), and reference (REF).

Appendix C: Shared taxa relative abundance between DNA and RNA metabarcoding.

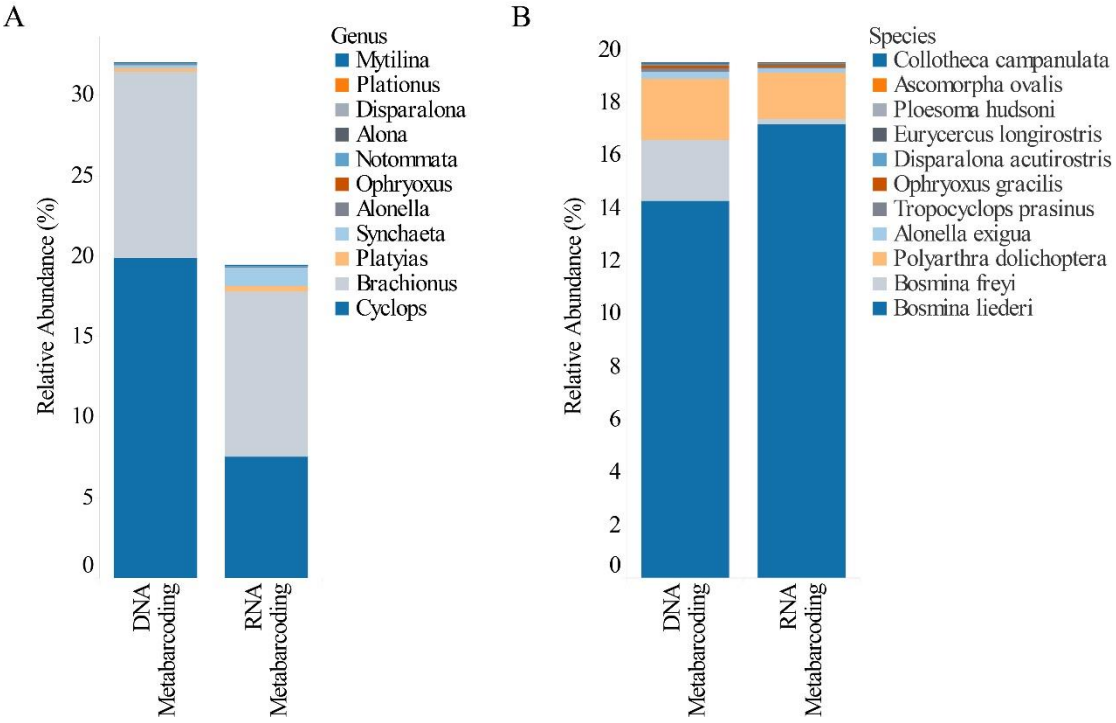

Figure C.1: Shared taxa between DNA metabarcoding and RNA metabarcoding at (A) genus level and (B) species level. Unclassified species were filtered out.

Appendix D: Shannon diversity for identification methods between remediation practices

ANOVA was used to estimate differences between Shannon diversity of treatments at each time point, as sample size was not sufficient for interpretation, with a student's t-test used to test for differences between eMNR and SCA. No differences in Shannon diversity were seen between treatment groups, eMNR and SCA, at day -3 (Fig. S4, T-test $p \geq 0.598$). Welch's t-test was significant at day 11 for morphological identification (Fig S4, T-test, $p = 0.0356$). SCA and eMNR differed significantly for DNA and RNA metabarcoding at day 38 (Fig. S4, T-test, $p \leq 0.0457$).

Appendix D: Shannon diversity for identification methods between remediation practices

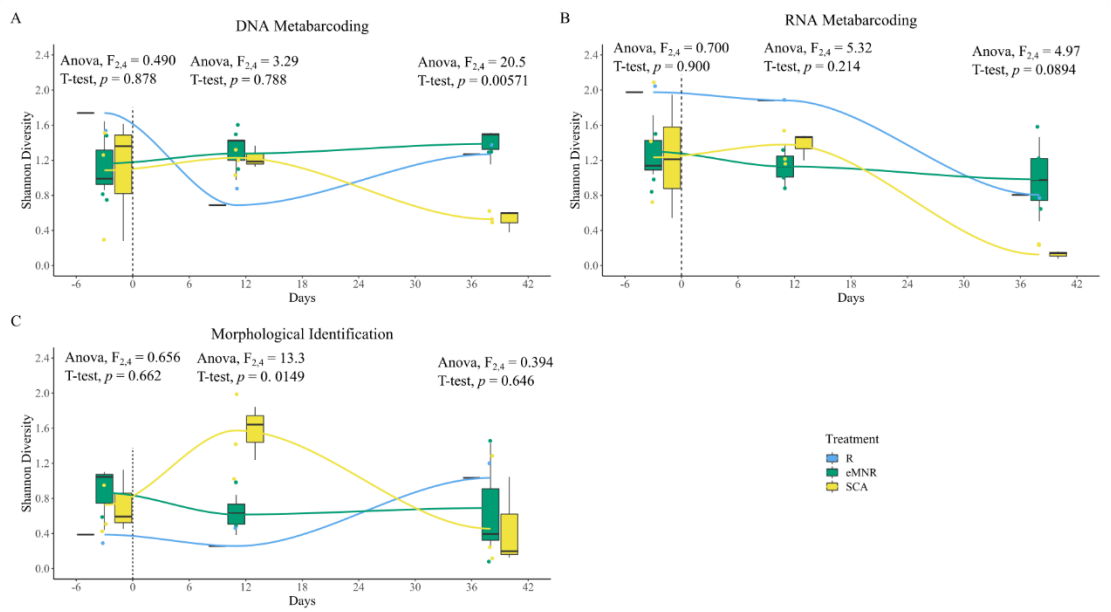

Figure D.1: Zooplankton Shannon diversity for treatments over time for each zooplankton identification methods applied. Treatment groups consisted of enhanced monitored natural recovery (eMNR; *n* =3), shoreline cleaner application (SCA; *n* = 3), and reference (REF; *n* =1). ANOVA was used to estimate differences between treatment diversity at each time point, with a Welch's t-test being used to test between eMNR and SCA. Dashed line represents beginning of simulated dilbit spill and treatment trend is represented by local polynomial regression.

Appendix E: Temporal zooplankton taxa richness between identification methods

Differences in taxa richness between identification methods at each time point were tested by use of Kruskal-Wallis (KW) with Dunn's Kruskal-Wallis multiple comparison with p-values adjusted with the Holm method for post-hoc testing. No difference was observed between identification methods at days -3 (Fig. S5, Kruskal-Wallis, $p = 0.0740$), however, significant differences were observed at day 11 and 38 post-spill (Fig. S5, Kruskal-Wallis, $p \leq 0.0191$). At day 11 post-spill, DNA and RNA metabarcoding were found to have a greater richness relative to morphological identification (Fig. S5, Dunn's test, $p \leq 0.0185$), while at day 38 post-spill, DNA metabarcoding was found to have a greater zooplankton richness than RNA metabarcoding (Fig. S5, Dunn's test, $p = 0.0147$).

Appendix E: Temporal zooplankton taxa richness between identification methods

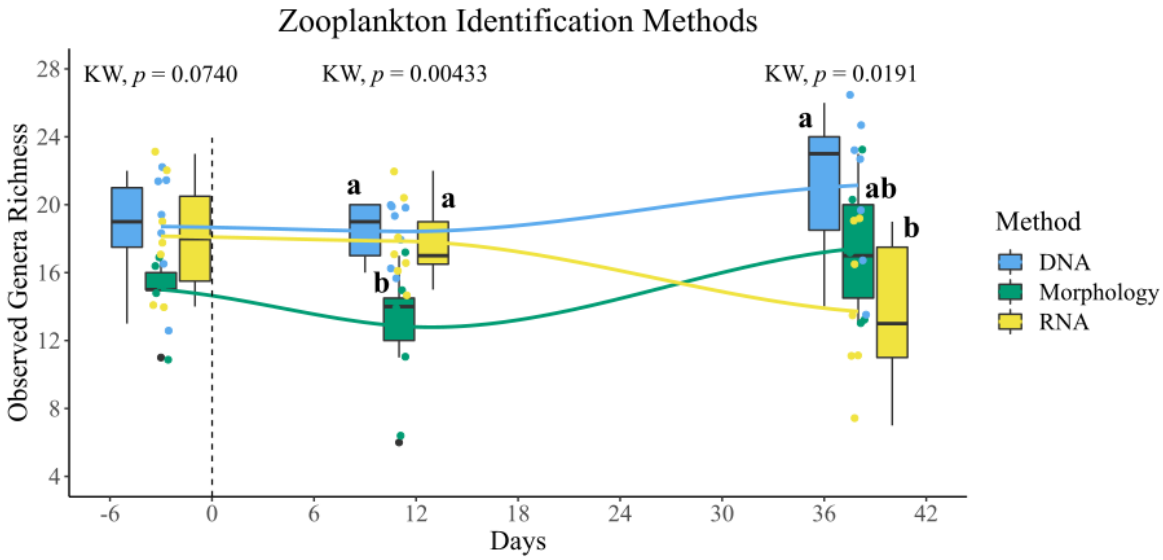

Figure E.1: Overall genera richness over time for zooplankton identification methods. Kruskal-Wallis was computed between identification methods at each sampling time point. Identification methods consisted of morphological identification (Morphology), DNA metabarcoding (DNA), and RNA metabarcoding (RNA) with sample size $n = 7$ for each method. Lowercase letters indicated significance level of $\alpha < 0.05$ inferred from Dunn's Kruskal-Wallis multiple comparison with p-values adjusted with the Holm method. Dashed line represents beginning of simulated dilbit spill and treatment trend is represented by local polynomial regression.

Appendix F: Resulting count data from metabarcoding and morphological identification.

Table F.1: Family-level rarefied (9885) metabarcoding count data and raw morphological abundance and biomass data summed for each identification method.

Family	DNA Metabarcoding	RNA Metabarcoding	Morphological Abundance	Morphological Biomass
Asplanchnidae	12274	12578	1.944	0.02527
Bosminidae	10165	10408	1597.7	264.8
Brachionidae	123956	147648	3772.8	51.89
Chydoridae	2416	1044	20.77	12.46
Collothecidae	3	7	0.5556	0.03056
Cyclopidae	41467	15635	125.89	121.91
Cyprididae	2	0	0	0
Daphniidae	34	13	5.277	5.635
Diaptomidae	7248	7500	30.51	41.014
Euchlanidae	2	0	0.3162	0.009486
Eurycercidae	21	12	0.3299	0.6597
Gastropodidae	20	53	1.994	0.03408
Lecanidae	131	102	4.541	0.14212
Lepadellidae	0	0	0.1325	0.0006630
Macrothricidae	917	413	3.475	3.377
Notommatidae	34	20	0	0
Sididae	1429	1708	14.44	22.33
Synchaetidae	3079	5595	8.497	0.3292
Temoridae	1850	2098	4.335	9.367
Trichocercidae	1051	1298	0.2792	0.01954
Trichotriidae	1486	1450	0.2406	0.007219

Appendix F: Resulting sequence reads and count data from metabarcoding and morphological identification.

Table F.2: Genera-level rarefied (9885) metabarcoding count data and raw morphological abundance and biomass data summed for each identification method.

Genus	DNA Metabarcoding	RNA Metabarcoding	Morphological Abundance	Morphological Biomass
Alona	32	14	0	0
Alonella	182	152	0	0
Ascomorpha	20	53	0.4529	0.006341
Asplanchna	12274	12578	1.944	0.02527
Bosmina	10165	10408	1597.7	264.8
Brachionus	23934	21300	0	0
Ceriodaphnia	23	3	5.127	5.127
Chydorus	2176	869	20.77	12.46
Collotheca	3	7	0.5556	0.03056
Cyclops	41274	15598	0	0
Cyrtonia	2	0	0	0
Daphnia	2	10	0.1500	0.5087
Diacyclops	0	0	7.062	4.772
Diaphanosoma	1075	852	13.98	15.41
Disparalona	26	9	0	0
Epischura	1850	2098	4.335	9.367
Euchlanis	2	0	0.3162	0.009486
Eucyclops	10	2	3.064	3.999
Eurycercus	21	12	0.3299	0.6597
Gastropus	0	0	1.541	0.02774
Ilyocryptus	732	324	1.582	3.1630
Kellicottia	956	2362	2.096	0.03144
Keratella	98530	123168	3770.7	51.86
Lecane	131	102	1.961	0.06472
Lepadella	0	0	0.1325	0.0006630
Leptodiaptomus	7248	7500	30.51	41.014
Macrocyclops	0	0	0.2480	1.171

Macrochaetus	0	6	0	0
Macrothrix	111	27	1.894	0.2143
Mesocyclops	106	28	83.79	102.1
Microcyclops	0	0	0.2817	0.3934
Monostyla	0	0	2.580	0.07739
Mytilina	13	30	0	0
Notommata	34	20	0	0
Ophryoxus	74	62	0	0
Plationus	20	6	0	0
Platyias	503	782	0	0
Ploesoma	928	1961	7.307	0.2923
Polyarthra	1742	1230	1.191	0.03691
Sida	354	856	0.4615	6.923
Simocephalus	9	0	0	0
Synchaeta	409	2404	0	0
Trichocerca	1051	1298	0.2792	0.01954
Trichotria	1486	1444	0.2406	0.007219
Tropocyclops	77	7	31.45	9.421

Appendix F: Resulting sequence reads and count data from metabarcoding and morphological identification.

Table F.3: Species-level rarefied metabarcoding count data and raw morphological abundance and biomass data summed for each identification method. Note, due to taxa not assigned to species-level being filtered out, the read counts do not equal to the rarefied depth of 9885.

Species	**DNA Metabarcoding**	**RNA Metabarcoding**	**Morphological Abundance**	**Morphological Biomass**
Alonella exigua	167	117	0	0
Ascomorpha ovalis	4	7	0	0
Bosmina freyi	1429	134	0	0
Bosmina liederi	8736	10274	0	0
Bosmina longirostris	0	0	1597.7	264.8
Chydorus sphaericus	0	0	20.77	12.46
Collotheca campanulata	3	7	0	0
Daphnia mendotae	2	10	0.1500	0.5087
Diacyclops thomasi	0	0	7.0618	4.772
Diaphanosoma birgei	0	0	13.98	15.41
Disparalona acutirostris	26	9	0	0
Epischura lacustris	1850	2098	4.335	9.367
Eurycercus longirostris	21	12	0	0
Kellicottia longispina	0	0	2.0962	0.0314

Keratella cochlearis	39751	37656	201.06	2.011
Keratella crassa	0	0	28.920	0.2892
Keratella serrulata	0	0	1.5176	0.01518
Keratella taurocephala	0	0	3539.16	49.55
Leptodiaptomus minutus	7231	7500	30.508	41.01
Macrocyclops albidus	0	0	0.2480	1.171
Mesocyclops edax	106	28	83.79	102.1
Microcyclops rubellus	0	0	0.2651	0.3686
Ophryoxus gracilis	74	62	0	0
Ploesoma hudsoni	12	12	0	0
Polyarthra dolichoptera	1407	1026	0	0
Polyarthra vulgaris	0	0	1.1905	0.03691
Sida crystallina	354	856	0.4615	6.923
Simocephalus serrulatus	9	0	0	0
Trichocerca cylindrica	0	0	0.2792	0.01954
Trichotria tetractis	0	0	0.2406	0.007219
Tropocyclops extensus	0	0	31.449	9.4214
Tropocyclops prasinus	77	7	0	0

Appendix G: Resulting statistical output from pairwise comparisons.

Table G.1: Resulting t-test statistics from pairwise comparison of computed least square means for treatments based on random intercept model. Treatment groups consisted of enhanced monitored natural recovery (eMNR), shoreline cleaner application (SCA), and reference (REF).

	Comparison	Estimate	Std. Error	df	t value	Pr(>|t|)
DNA Metabarcoding	REF - eMNR	1.67	1.74	16	0.958	0.353
	REF - SCA	5.11	1.74	16	2.94	0.00968
	eMNR - SCA	3.44	1.59	16	2.80	0.0129
RNA Metabarcoding	REF - eMNR	3.89	1.81	16	2.15	0.0473
	REF - SCA	6.67	1.81	16	3.69	0.00203
	eMNR - SCA	2.78	1.28	16	2.17	0.0613
Morphological identification	REF - eMNR	1.78	2.06	16	0.864	0.401
	REF - SCA	3.44	2.06	16	1.67	0.114
	eMNR - SCA	2.67	1.46	16	1.15	0.269

Appendix G: Resulting statistical output from pairwise comparisons.

Table G.2: Pairwise comparison between treatment groups based on total distance matrix for all samples, while blocking the effects of time. Treatment groups consisted of enhanced monitored natural recovery (eMNR), shoreline cleaner application (SCA), and reference (REF).

Comparison		DNA Metabarcoding			RNA Metabarcoding			Morphological Identification		
	DF	F-Statistic	R2	Pr(>F)	F-Statistic	R2	Pr(>F)	F-Statistic	R2	Pr(>F)
REF -eMNR	1,10	2.91	0.226	0.0173	1.58	0.136	0.0974	2.01	0.168	0.0478
REF - SCA	1,10	4.13	0.292	0.0174	2.16	0.178	0.0142	1.96	0.164	0.0796
eMNR - SCA	1,16	1.10	0.0642	0.203	0.979	0.0576	0.267	0.568	0.0343	0.498