The effects of a simulated spill of diluted bitumen on invertebrates in a boreal lake environment

by

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Abstract

To bring bitumen from Canada's Oil Sands to market requires transportation over sensitive boreal environments via rail, truck, and pipeline. With proposed expansion of pipeline infrastructure, there is a need for whole-ecosystem research evaluating fate and toxicity of oil spills specific to freshwater environments; the Boreal Lake Oil Release Experiment by Additions to Limnocorrals (BOREAL) aimed to address this. The BOREAL study was conducted in an oligotrophic lake (Lake 260) at the IISD-Experimental Lakes Area in Summer 2018. Nine 10-metre diameter, ~ 100-m^3, limnocorrals were deployed, with seven treated with different volumes of a diluted bitumen product in a regression design accompanied by two reference limnocorrals. Dilbit volumes ranged from 1.5 L to 180 L, which is representative of historical oil:water ratios for pipeline spills in North America between the 50th and 99th centile (2008-2018). Zooplankton, emerging insects, and benthic invertebrates were monitored pre- and post-spill for abundance and community composition. By 13 days post-spill, zooplankton abundance had decreased in all limnocorrals and did not recover to pre-treatment values, with rotifers becoming the dominant phylum. No discernable impact based on treatment to zooplankton community diversity was observed. No impact was observed to resident benthic invertebrate communities relative to control limnocorrals; however, a concentration-response decline was observed in total insect emergence. Emergence rate declines were confounded by benthic impacts and presence of submerged oil and will require further work to elucidate drivers of long-term impacts. The physical component of oil was observed to be the likely driver of pleuston (water striders) immobility and mortality.

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Introduction

Preface

Much of Canada's political and economic discourse is currently focused on our energy sector, with interest in expanding pipeline infrastructure to transport unrefined crude oil to refineries across the continent. Canada's landlocked oil, primarily contained within Alberta's oil sands, requires transportation via pipeline or other means. The bitumen product within the oil sands is highly viscous and must be diluted using low molecular weight petroleum products such as natural gas condensates to allow its transportation via pipeline (Paskey et al, 2013). This modification poses a challenge for spill response: the resulting product known as diluted bitumen (dilbit) characteristically changes almost immediately when exposed to the natural environment, in contrast to conventional oil. Volatile components within the diluent are lost, increasing viscosity and density of the compound. This is a direct result of weathering processes including ultraviolet (UV) radiation exposure, wind and wave action, presence of suspended solids within the water column, temperature, and numerous other factors that affect weathering and breakdown rates of the dilbit. The resulting product behaves and acts much differently than fresh dilbit. The extent of this difference and the conditions to which this occurs in freshwater environments is not yet fully understood (Lee et al, 2015).

The Royal Society of Canada released a report in 2015 entitled *The Behaviour and Environmental Impacts of Crude Oil Released into Aqueous Environments* (Lee et al, 2015). This report identified gaps in our understanding of crude oil spills and identified

the need for field-based research to understand impacts of oil spills in freshwater environments. Stemming from this work, a multi-year, interdisciplinary project was developed called BOREAL that sought to fill in these knowledge gaps and reduce uncertainty around the fate and effects of dilbit. BOREAL stands for **B**oreal lake **O**il **R**elease **E**xperiment by **A**dditions to **L**imnocorrals and served to improve our understanding of spills of diluted bitumen into freshwater environments. The four main objectives, informed by the report, included:

1. **Fate and Behaviour.** Knowledge on spills of crude oil into freshwater environments is limited as most research and investment has gone into understanding impacts on marine systems. Marine oil spill knowledge has been expanded by spills like Deepwater Horizon (Gulf of Mexico, 2011) and Exxon Valdez (Prince William Sound, Alaska, 1989). Diluted bitumen, a form of unconventional crude oil, is a complex compound and its behaviour relative to conventional crudes is not well characterized. Diluted bitumen may sink or float in freshwater environments, dependent on a multitude of factors discussed later in this introduction. Understanding how diluted bitumen behaves immediately following a spill and how it changes over time will be key in developing tools to best respond to spills of this nature, while also helping develop risk assessments around exposure (e.g. priority polycyclic aromatic hydrocarbons, metals, physical fouling) for freshwater biota.

2. **Impacts on Ecosystem Structure and Function**. Little is known about how diluted bitumen interacts with freshwater biota (e.g. algae, microbial

communities, zooplankton, benthic communities, fish). Each of these play important roles in aquatic ecosystems, and changes to them may have cascading impacts on the rest of the ecosystem. Understanding how diluted bitumen spills impact each component and how they, in turn, induce changes in other components will be vital to understanding the risk and scope of oil spill impacts, and the potential for recovery.

3. **Bioaccumulation and Uptake**. Polycyclic aromatic compounds (PACs) and other oil components may bioaccumulate and move through food webs. Understanding how these petrogenic compounds behave in biota will help develop biomarkers of toxicity and oil exposure for assessment and clean-up responses.

4. **Direct Toxicity to Select Freshwater Biota**. Diluted bitumen contains complex compounds of which many have been observed to be independently toxic to freshwater biota (e.g. fish, amphibians). Understanding the toxicity of complex mixtures of oil components that may be present in the water column and within the sediments will be important in developing our understanding of the toxicity of oil spills to individual biota. As well, toxicity tests will provide an understanding of molecular and cellular responses of freshwater biota to oil exposure for risk assessment purposes (e.g. exposure assessment).

Each of these objectives contributes to a detailed look at the impact of oil spills on freshwater environments. These were addressed using (a) a pilot-scale land-based mesocosm study in 2017 to inform (b) a large-scale lake-based limnocorral study in 2018, both at the International Institute for Sustainable Development's Experimental

Lakes Area (IISD-ELA). The IISD-ELA is a remote freshwater research laboratory located in the Kenora district of Northwestern Ontario, Canada (49°39'36.3"N, 93°43'40.0"W). The research facility encompasses 58 freshwater lakes and their watersheds set aside for whole ecosystem research. Consequently, this served as a one-of-a-kind facility to conduct the necessary whole ecosystem research needed to evaluate impacts of oil spills into sensitive freshwater environments. Lake 260, a small boreal lake (49°41'56.4"N, 93°46'01.2"W), was chosen as the site for the limnocorral study in 2018.

This thesis is one component of the larger BOREAL project and sought to understand how ecosystem structure and function changed (**Objective #2**), with a focus on the invertebrate communities, following simulated spills of diluted bitumen into the lake-based limnocorrals. The invertebrate community consisted of zooplankton (planktonic crustaceans including the copepods, cladocerans, and rotifers) and benthic macroorganisms (sediment-dwelling organisms present within the benthic zone that include aquatic insects, polychaetes, and other crustaceans). This thesis outlines how these communities changed following simulated spills of diluted bitumen. Communities were assessed for composition and abundance and were evaluated alongside other components of the food web, including fish and primary producers. Each component was also evaluated against changes in oil volumes and the presence of various oil components, as well as environmental variables (e.g. light, pH, dissolved oxygen, nutrients, etc.).

A second set of experiments, informed by the BOREAL limnocorral study, was developed to evaluate how surface oil would impact water strider (Family: Gerridae) communities. Oil spills are known to cause physical impacts, including physical smothering and inhibition of movement in freshwater biota and terrestrial organisms (e.g. birds). This work helped inform some observations made throughout the limnocorral study pertinent to water surface dwelling organisms (pleuston).

In addition to the work described above, an additional component was included to look at the use of chitobiase, an invertebrate moulting enzyme, as a tool for evaluating how arthropod (i.e. organisms with an exoskeleton) secondary production changes in stressor-impacted aquatic systems. Measures of invertebrate productivity in aquatic systems are difficult and time consuming due to the need to identify and count hundreds to thousands of organisms. The chitobiase assay relies on a direct correlation between arthropod biomass and enzyme activity in the water column. Its use, however, has not been evaluated extensively in lakes, and its usefulness as a tool for assessing arthropod secondary production as a measure of impacts was evaluated in conjunction with the BOREAL project.

The BOREAL project pilot study was conducted from the 1st to 17th of August, 2017 at the IISD-ELA. The large-scale limnocorral study was then conducted in 2018 with initial limnocorral installation from 1st May to 5th June, followed by pre-spill monitoring between 6th June and 19th June, oil addition on 20th June, and post-spill monitoring continuing until 5th September. Prior to discussing the study design and results of the BOREAL project, this introduction will discuss: (a) the physical and chemical properties of diluted

bitumen and how this will influence this study; (b) a brief assessment of the pathways by which dilbit may affect freshwater invertebrates; and (c) an overview of what we know about the chitobiase method for assessing arthropod secondary production and how this may be employed in a setting like the BOREAL project.

1.1 Diluted Bitumen

1.1.1 Overview

Many challenges surround the expansion of pipelines in Canada and North America as a whole. There are concerns around Indigenous independence and sovereignty associated with the lands through which pipelines pass. This was at the forefront in protests surrounding the Dakota Access Pipeline (DAPL) in the Summer of 2017, which continue to this day (Shoemaker, 2017). The future of energy from safe, green, and renewable sources, and as an invaluable economic driver is also a polarizing issue - partisan ideologies and proximity to pipelines have shaped public opinion (Gravelle & Lachapelle, 2015). This stems from opinions on climate change (Huber & Bowe, 2014; Shoemaker, 2017). Differences in ideology/opinion have manifested themselves under the Trudeau government as a nationwide carbon pricing debate and interprovincial conflict between provinces with energy-based economies (i.e. Alberta) and those reliant upon tourism and environmental affluence (i.e. British Columbia; MacNeil & Paterson, 2018). The public is highly invested in the topic of oil transportation in Canada given how multifaceted an issue it is and how rooted it is in Canada's economy. Although concerns around oil spills are present in all aqueous environments, there is a recognized and significant knowledge gap with respect to the effect of diluted bitumen (dilbit) on boreal lake ecosystems (Lee et al, 2015).

Diluted bitumen is an unconventional crude oil developed to facilitate pipeline transportation of bitumen from Canada's Oil Sands Region, Alberta (Paskey et al, 2013). Bitumen cannot be transported in its raw, highly viscous state and thus relies on

the addition of diluents, such as naphtha (a natural gas condensate) or other low molecular weight (LMW) light petroleum products (Alsaadi et al, 2018). These are added in varying concentrations dependent upon provincial and federal regulations and seasonal changes (Lee et al, 2015). Bitumen's viscosity is temperature dependent – this means that diluent composition must vary with season or geographic location to meet standards for pipeline transport (Lee et al, 2015). Petroleum producers have developed proprietary dilbit blends that vary based on the type and amount of diluent added. This limits access to dilbit composition information, making spill planning and response more difficult than conventional crudes (Madison et al, 2015). As diluent proportions change, so too will the viscosity, density, and surface tension – these properties dictate the fate and behaviour of spilled dilbit subject to weathering processes. In turn, this influences bioavailability and toxicity to organisms in the event of an accidental release.

Crude oil is composed generally of four main components: saturates, aromatics, resins, and asphaltenes (SARA). Saturates and aromatics are the greatest concern for aquatic toxicity given their relatively small size and high bioavailability - these include the BTEX (benzene, toluene, ethylbenzene, xylenes) compounds and polycyclic aromatic compounds (PACs; Madison et al, 2015). The LMW components are highly volatile relative to high molecular weight (HMW) compounds (asphaltenes/resins/alkylated-PACs) and are lost quickly upon release to the environment because of weathering processes (ultraviolet (UV) radiation, wind action inducing mixing of the water column, oil emulsification, microbial breakdown, etc.). As LMW components are lost, this increases the density of the residual oil, whereby it may exceed that of water (~1.00 g/cm3) and sink to the sediments (Lee et al, 2015). When in the sediments, further

chronic toxicity is of concern given the presence of larval life stages, and the potential for exposure long after the initial clean-up of the oil spill associated with alkylated PACs (Lee et al, 2015).

1.1.2 Physical and Chemical Properties

Dilbit's physical and chemical properties are dictated by the composition of diluent relative to bitumen, and the diluent used. Density is key to its sinking behaviour – a density below 1.00 g/mL (commonly measured as API gravity; American Petroleum Institute) or an API below 10.0 results in sinking of oil in water at 15.5°C (Demirbas et al, 2015). Viscosity describes its ability to spread in water – viscosity is greater when bitumen (rich in asphaltenes and dense/heavy hydrocarbons) content is greater (Lee et al, 2015; Environment Canada, 2013). The two blends most commonly transported in Canada (Cold Lake Blend, CLB; Access Western Blend, AWB) show varying degrees of density as a result of chemical composition (i.e. amount of diluent added that makes up the majority of light hydrocarbons and the BTEX components) (**Table 1.1**). These physical properties are also affected by evaporation and weathering processes in the event of a spill.

Table 1.2 outlines the change in physical properties associated with CLB as a result of evaporative loss – a clear increase in density and viscosity is noted. In a pilot study to the BOREAL project, Stoyanovich et al (2019a) evaluated the fate and behaviour of CLB-W using two volumes of dilbit – they observed that dilbit can sink after eight days of weathering under the conditions they reported.

These physical properties are a result of the chemical changes associated with the diluent – bitumen mixture. Bitumen is rich in high molecular weight (HMW) compounds such as asphaltenes and resins (Lee et al, 2015; Dew et al, 2015). Conversely, diluents comprise low molecular weight (LMW) aromatics like BTEX and the PACs – these

compounds are of most concern given their high acute toxicity to aquatic organisms, as discussed below (Dew et al, 2015). BTEX and LMW PACs are highly soluble and will volatilize quickly. With this loss (

Table 1.2) density and viscosity will increase as HMW components remain (Lee et al,

2015).

Table 1.1: Physical properties of Cold Lake Blend and Access Western Blend diluted bitumen (+/- SD). Adapted from Environment Canada (2013).

Product	Density (g/mL)	Light ends (vol%) (C-2 to C-10 alkanes)	BTEX (vol%)
Cold Lake (CLB)	0.9277 +/- 0.005	20.4 +/- 1.5	1.06 +/- 0.17
Access Western (AWB)	0.9229 +/- 0.0046	24.1 +/- 1.7	1.20 +/- 0.15

Table 1.2: Physical properties of Cold Lake Blend (CLB) dilbit at 15.5°C at various stages of evaporative loss (+/- SD). Shading denotes point at which CLB will tend to sink at 15.5°C in freshwater. Adapted from Environment Canada (2015).

Evaporative Loss	0.0%	8.50%	16.90%	25.30%	26.50%
Density (g/mL)	0.9249	0.9537	0.9816	1.0034	1.0085
API gravity	21.0	16.5	12.5	9.5	8.8
Dynamic viscosity, 15°C (mPas)	29	1.3×10^3	1.8×10^4	3.9×10^5	3.2×10^5
Light Ends (vol%) (C-2 to C-10 alkanes)	20.4 +/- 1.5				
BTEX (vol%)	1.06 +/- 0.17				

1.1.3 Effects of Weathering on Oil

Weathering refers to the physical and chemical changes to dilbit in an aquatic environment because of physical, chemical, and biological actions. Density and viscosity of oil play a role in the properties of oil in water, affected by the relative composition of diluent to bitumen. However, when oil is spilled into freshwater, a number of other parameters must be considered including the effect of temperature and other water quality parameters, presence of organic matter, including algae and microbes, wind action, UV radiation, etc. – all of which can affect the rate of deposition of oil to the sediments and the volatilization rate of the LMW components of oil (Lee et al, 2015).

The formation of oil-particle aggregates (OPAs) is one driver in dictating the fate and behaviour of spilled oil; the National Academies of Science noted that temperature and salinity can impact OPA formation – these factors change given season, water turbidity, and a variety of other factors specific to any given water body (NASEM, 2016; Gustitus and Clement, 2017). An OPA forms when oil droplets interact with particles in the water, such as soil, sediments that have been disrupted, and algae. Oil droplets form because of weathering processes – UV radiation can induce photodegradation and increase the volatilization rate of LMW components of oil (Dew et al, 2015). This was evident following a study that looked at oil droplet formation under direct UV versus no UV light – the density of CLB increased from 0.945 mg/L to 0.998 mg/L following UV radiation exposure (Ross Environmental Research Ltd., 2012). If oil droplets are present, aggregation of oil droplets with suspended solids may create a mass great enough to

sink to the sediments. Given its dependence on water quality, conditions at the time of a spill, and amount of particulate matter in the water column, this makes it difficult to predict what will happen in any specific body of water on any given day (Lee et al, 2015; Environment Canada, 2013).

1.1.4 Transportation of Diluted Bitumen

Alberta's Oil Sands contain approximately one third of the world's reserves of bitumen, acting as a powerhouse for Alberta's economy (Dew et al, 2015). In 2011, daily extraction of bitumen in Canada was estimated at 1.7 million barrels; this number is expected to reach 4.25 million barrels per day by 2035 (NRC, 2017; CAPP, 2019). Following recovery of bitumen from the Oil Sands, transportation to refineries across the continent is supported via pipeline, truck, and rail. Transportation via pipeline accounts for the largest means of transportation of oil in the country, with hopes of expansion of Canada's pipeline infrastructure soon (Lee et al, 2015; NEB, 2016). Current pipelines traverse major tributaries and sensitive freshwater habitats across Canada –**Figure 1.1** demonstrates the extent of the present pipeline network and details future expansion efforts.

With expansion of oil transportation comes increased risk of spills, such as the Kalamazoo River diluted bitumen spill in Michigan in 2010. The Kalamazoo River spill saw a release of 3,320 m^3 of diluted bitumen (dilbit) over a 17-hour period, presenting several concerns on the fate and behaviour of dilbit in freshwater, while also calling into question preparedness for dealing with spills of dilbit in freshwater. Similarly, route approval of TransCanada's Keystone XL pipeline in the Midwest United States was

complicated by a spill in South Dakota, USA by its parent company (Keystone). The Keystone XL expansion would see an additional 830,000 thousand barrels of oil transported from Alberta to Nebraska, per day, pending approval (NEB, 2016; Lee et al, 2015).

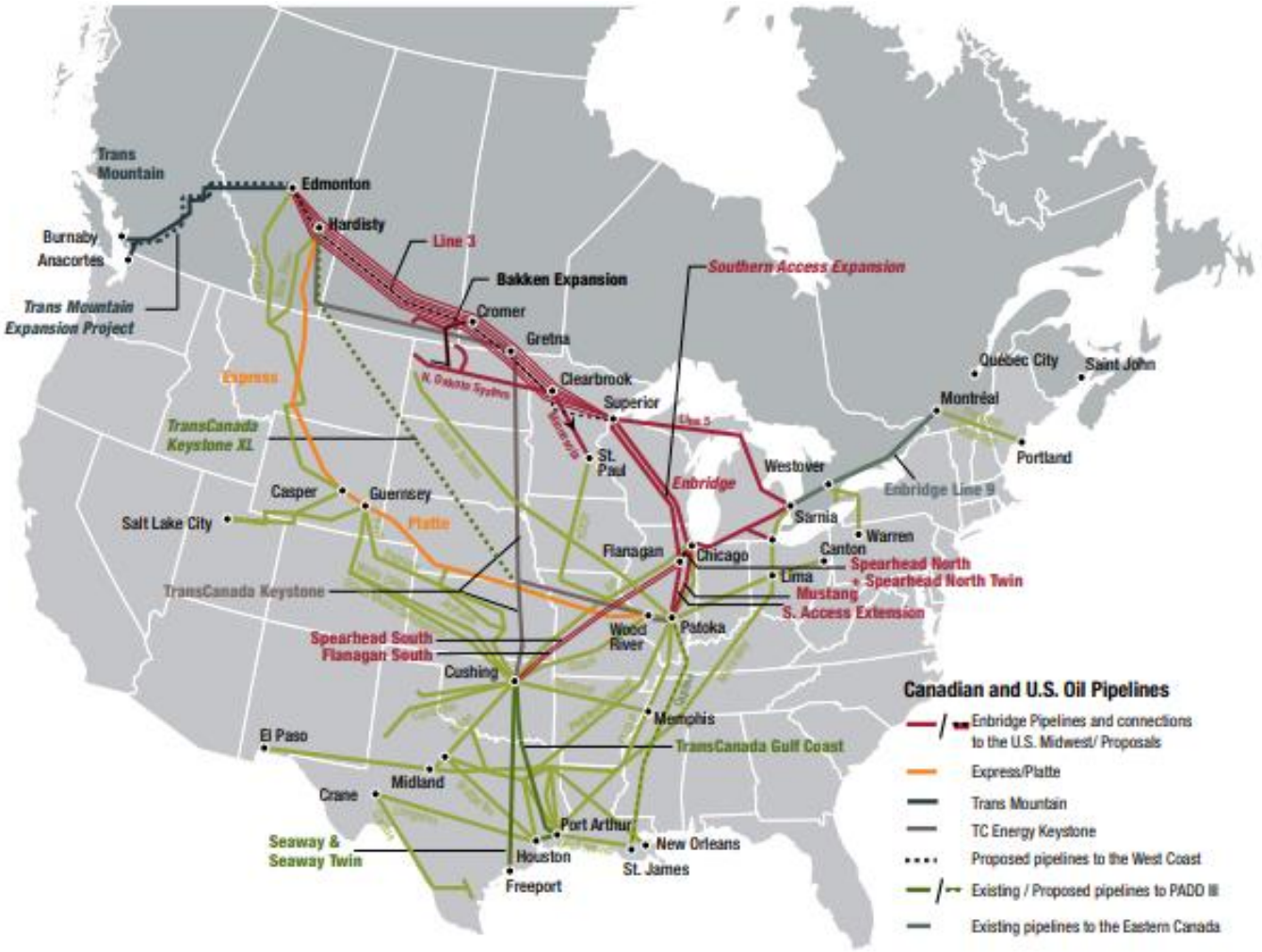

Figure 1.1: Current and proposed pipeline infrastructure in Canada and the United States (Adapted from CAPP, 2019).

1.1.5 Freshwater Pipeline Spills in North America

In North America, accidental spills of diluted bitumen and other crude oil products are anticipated, especially considering the expansive pipeline infrastructure needed to bring these products to market. The average spill size in North America between 2008 and 2017 was 8.98 m^2, calculated as the 50th centile corresponding to a cumulative frequency distribution of 149 crude oil spills in Canada and the United States between 2008 and 2017 (**Figure 1.2**). An understanding of the impact of even relatively small spills into freshwater environments are not well known; however, studies following spills like the Kalamazoo River spill are adding to our knowledge.

Spills may become more frequent as pipeline infrastructure expands, and although the average spill size is small and will likely have impacts contained within a small area, implications may vary dependent on the environment and conditions. Spills may occur on land or surface water, where pipelines cross, wetlands, streams, and rivers. The area where a spill occurs will have implications for the environmental fate and impact of the spill – spills on land may be more contained and have fewer impacts on wildlife because they are more easily contained, whereas spills into aquatic environments may impact areas downstream and are typically more complex in response (Lee et al, 2015).

The Kalamazoo River spill in 2010 was the largest in-land spill of diluted bitumen recorded. It was the result of a 2-metre fracture of Enbridge's Line 6B, causing the release of 3,320 m^3 of a dilbit mixture consisting of Cold Lake (CLB) and Western Canadian Select (WCS) blends (Michigan Department of Community Health, 2014). The

latter was a diluted synthetic bitumen (dilsynbit; a blend of bitumen, synthetic petroleum products, and condensate; Lee et al, 2015), with CLB being the most commonly exported dilbit in Canada (Crude Quality Inc., 2017; Madison et al, 2017). The source of the spill was not identified and shut off until 17 hours after it began. High precipitation resulted in the flow of oil into a surrounding wetland, running off into Talmadge Creek and eventually reaching downstream Kalamazoo River where it impacted over 60 km of shoreline (Lee et al, 2015).

The Kalamazoo spill posed an interesting question – how much do we know about the behaviour of weathered dilbit? Of note were the effects of particulates in the water on the fate of the oil; that is, the formation of OPAs brought about by the association of oil with particulate matter (such as organic matter or soil) in the water column (Fitzpatrick et al, 2015). Intense rain conditions resulted in high turbulence that increased levels of particulate matter suspended in the water column (Lee et al, 2015; Environment Canada, 2013). This led to high levels of OPA formation and deposition of weathered dilbit to the sediments, posing risk to benthic organisms. The conditions at Kalamazoo may represent a unique scenario, confounding our ability to understand the behaviour of weathered dilbit in other situations (Fitzpatrick et al, 2015).

The spill also highlighted issues surrounding responses to inland, freshwater oil spills, and how we monitor changes to biological communities, post-spill. Much of the damage to Talmadge Creek and the Kalamazoo River watershed was a result of clean-up efforts including dredging, which severely disrupted benthic habitats, and inhibited the ability to define actual risk of submerged oil to benthic habitats (Fitzpatrick et al, 2015; Lee et al,

2015). Consequently, little information exists surrounding the potential for weathered dilbit to affect benthic communities. Spills of opportunity, such as the Kalamazoo spill, have not appropriately captured the risk to benthic communities as a result of the effects of the ensuing response and cleanup methods – of benefit would be the study of the effects of weathered dilbit (i.e. dilbit following OPA formation with respect to benthic communities) on benthic invertebrates in a controlled, field-based setting.

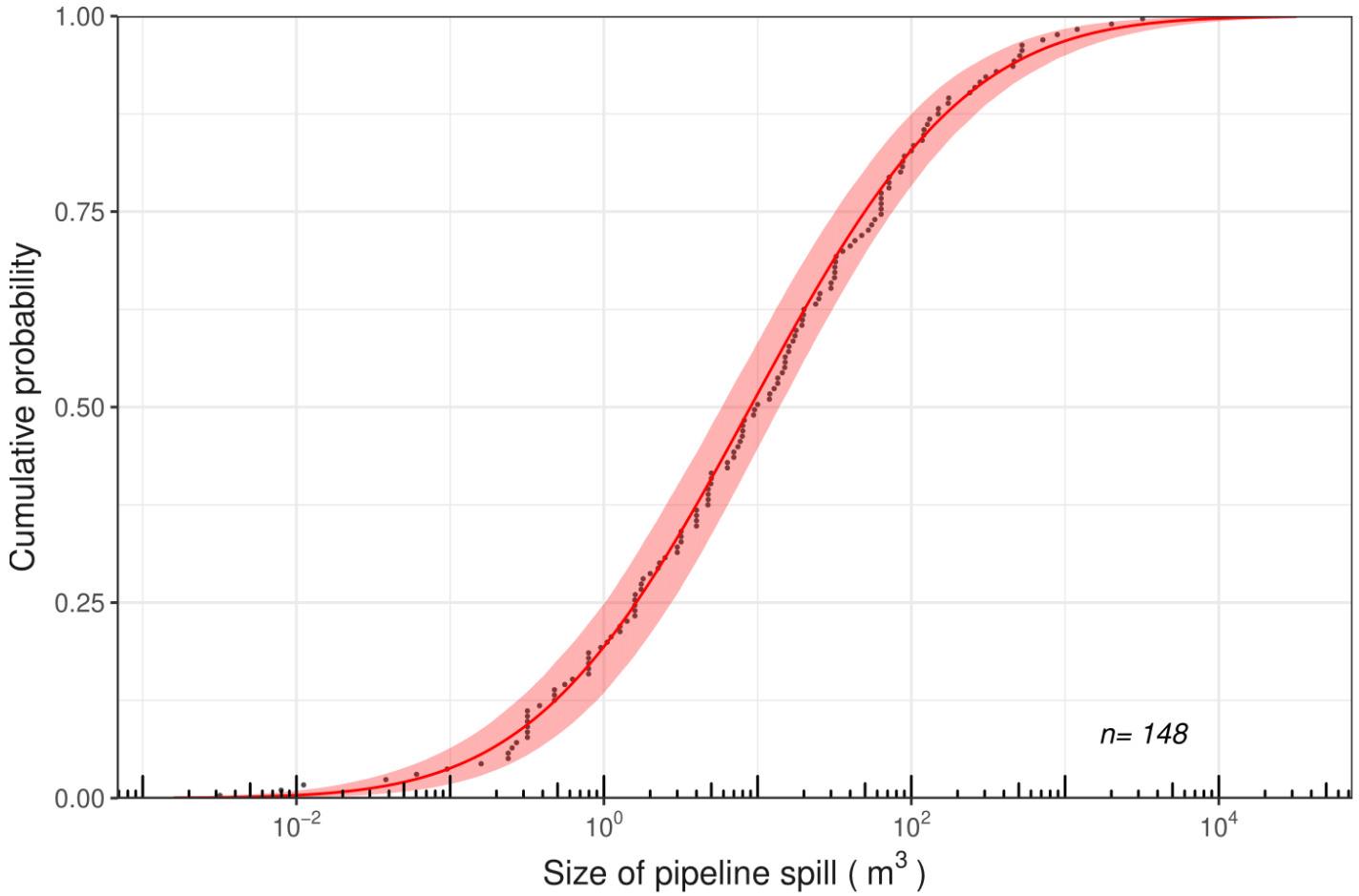

Figure 1.2: Cumulative probability distribution for sizes of in-land pipeline crude oil spills affecting freshwater (n=148) in the United States and Canada between 2008 and 2017. (Adapted from the BOREAL project's application to IISD-Experimental Lakes Area's Research Advisory Board; created by Jose Luis Rodriguez-Gil, 2017).

1.1.6 Toxicology of Diluted Bitumen

Dilbit's main constituents include PACs and BTEX – these are known to be carcinogenic, inducing narcosis and mutations in aquatic organisms in low amounts (Lee et al, 2015; Madison et al, 2015; Madison et al, 2017). The PACs offer a challenge for assessing dilbit toxicity – there exists thousands of PAC congeners, all with varying solubilities, bioavailability, volatilization rates, and subsequently, risk for inducing toxic effects. As such, our knowledge of the toxicity of dilbit to freshwater invertebrates is limited. Gerner et al (2017) set out to develop a species sensitivity ranking for freshwater invertebrates exposed to petroleum hydrocarbons but could not include PACs in the assessment given little work done on acute and chronic lab-based testing of invertebrates exposed to various PAC mixtures.

Laboratory-based toxicity testing of crude oils has relied on using water-accommodated fractions (WAFs) and chemically-enhanced water-accommodated fractions of oil (CEWAFs) that do not capture the complexities associated with dilbit's weathering or properly assess the toxicological risk associated with dilbit exposure (Lee et al, 2015; Adams et al, 2017). Water-accommodated fractioning involves the mechanical mixing of an oil layer with water such that hydrocarbons go into solution (Adams et al, 2017). When spilled into freshwater, exposure of organisms to dilbit may come from a variety of sources, including surface slicks (oil present on the water's surface directly following a spill – typically rich in LMW, acutely toxic substances), from oil droplets suspended in the water column, and from weathered oil that is submerged and coats the sediments (typically high in HMW components, like alkylated-PACs, asphaltenes, and resins). As

dilbit can be weathered and its chemical composition drastically changed within hours of a spill, this brings into question the realism of laboratory-based studies using WAFs and CEWAFs. As such, WAF-based lab-studies are used mainly in providing direction in evaluating effects associated with PAC exposure under natural conditions.

LMW saturates and aromatics are of greatest concern for toxicity to aquatic biota (Lee et al, 2015). Among these, polycyclic aromatic hydrocarbons (PACs) are known to have carcinogenic and reproductive impacts (Abbriano et al, 2011; Almeda et al, 2013; Madison et al, 2017. PACs consist of a variety of aromatic structures with 100s of isomers, creating a diverse mixture of compounds, each of varying degrees of bioavailability and toxicity to aquatic biota. Given the loss of LMW compounds during weathering processes (e.g. naphthalene, anthracene), 3- to 5-ringed alkylated PACs are of most concern for toxicity in field environments (Bellas & Thor, 2007). Alkyl-PACs (i.e. PACs with alkyl side-chains) are more persistent than their non-alkylated counterparts, and therefore may be present in the sediments following sinking of the oil as a result of their greater molecular weight (Lee et al, 2015). Hodson et al (2007) and Adams et al (2014) identified alkyl-PACs as the primary driver of chronic toxicity in juvenile rainbow trout exposed to a CEWAF of a heavy fuel oil. However, alkyl-PACs are rarely assessed given their complexity and difficulties associated with analytical detection (Lee et al, 2015). This was observed in the Kalamazoo River spill where only the 16 priority PACs as determined by the U.S. EPA were analyzed at the spill site (Andersson and Achten, 2015); yet, alkyl-PACs constitute the greatest proportion of PACs in Cold Lake Winter Blend (CLB-W; a blend of dilbit most commonly transported in Canada) and are present

in far greater proportions in dilbit relative to conventional crude oils (Environment Canada, 2013).

PAC-induced toxicity is a result of interaction between several pathways, including induction of the aryl hydrocarbon receptor (AhR), and cytochrome P450 enzyme induction (Adams et al, 2017). This can have reproductive, developmental, and necrotic effects on fish, plankton, and benthic organisms. Toxicological data on dilbit exposure to aquatic biota is inconsistent and limited; efforts have been made to streamline the study of toxicity of dilbit to aquatic biota (Adams et al, 2017), but whole ecosystem characterisation is lacking.

1.1.6.1 Zooplankton

Given how little research has been conducted pertaining to oil spills in freshwater environments (e.g. Kalamazoo River spill), understanding of the effects of oil on zooplankton communities is informed primarily by research on marine species and marine spills (Exxon Valdez, Deepwater Horizon) and laboratory-based studies, often with more conventional crude oils. However, Barron et al (2018) demonstrated that the acute and chronic toxicities associated with exposure to two dilbit blends, Cold Lake and Western Canadian Select, had similar toxicity profiles to conventional crude oils. Therefore, marine organisms exposed to PACs via the water-accommodated fraction or through direct crude oil exposure (not specific to dilbit) have been used as a surrogate in elucidating impact to freshwater zooplankton. In marine oil spills, the zooplankton response has been observed to be transient – in Deepwater Horizon, rapid recovery of the zooplankton community was observed as a result of high fecundity and rapid

reproductive rates (Abbriano et al, 2010), as has been reported in other marine spills (Johansson et al, 1980; Dean et al, 1996). Avoidance of oiled patches has also been observed in *Temora longicornis* and *Eurytemora affinis*, two species of calanoid copepods (the dominant copepod taxa in Lake 260; Kidd et al, 2014) in a lab-based environment (Seuront 2010). Community response by zooplankton seems also dependent on species size, duration of exposure to oiled environments, and the chemical composition of the spilled oil (Abbriano et al, 2011). LMW PAHs can be acutely toxic to zooplankton (Calfee et al, 1999; Holst & Giesy, 1989); however, LMW components are quickly lost due to volatilization and weathering within hours of a spill occurring (Lee et al, 2015). As such, an oil spill may result in the short-term loss of portions of the zooplankton community initially, but long-term effects may be limited due to increases in opportunistic zooplankton species and a recovery of species diversity (Linden et al, 1987; Suchanek, 1993; Abbriano et al, 2011).

Exposure to PACs by zooplankton can occur via two main routes: particle-associated PACs and oil contained within the water column (water-accommodated, oil droplets, etc.). Sedimentation of oil is an important pathway of exposure for zooplankton (Johansson et al, 1980; Verrhiest et al, 2001). Sedimentation of 3- to 5-ringed PACs is to be expected following an oil spill, and sediment-bound PACs thus must be evaluated in considering acute and long-term risk to zooplankton following an oil spill (King et al, 2017; Lee et al, 2015). Most studies have focused on the water-accommodated fraction (WAFs), dispersed oils, or oil droplets and do not capture an important exposure pathway (Almeda et al, 2016; Barron et al, 2018; Berrojalbiz et al, 2009; Dupuis & Ucan-Marin, 2015).

Figure 1.3 outlines a proposed adverse outcome pathway (Ankley et al, 2010) for zooplankton following a freshwater oil spill. Moulting is an important process in arthropod development. This process results in the shedding of the exoskeleton and generation of a new one. As moulting processes are vital to arthropod development, disruption of these pathways may be attributed to changes in community composition observed following oil spills (Abbriano et al, 2010). Aryl hydrocarbon receptor (AhR) agonism and formation of DNA adducts have been attributed to some of the moulting impacts observed in arthropods (Oberdorster et al, 1999; Nebert & Karp, 2008; Dupuis & Ucan-Marin, 2015). Polycyclic aromatic compounds – particularly benzo[a]pyrene, benzo[b]fluoranthene, pyrene, and chrysene – have been observed to encourage ecdysone receptor (EcR) activation, inducing changes in ecdysis in arthropods (Oberdorster et al, 1999). Following modification of EcR and AhR-mediated pathways, changing in ecdysis success, heart failure, and other embryonic impacts are possible (**Figure 1.3**). Molecular and cellular based changes can cascade to the population level, affecting overall community structure (i.e. certain species may be selectively susceptible to PACs more than others), recruitment and fecundity (associated with changes in reproductive capacity and fitness) (Lee et al, 2015; Dupuis & Ucan-Marin, 2015).

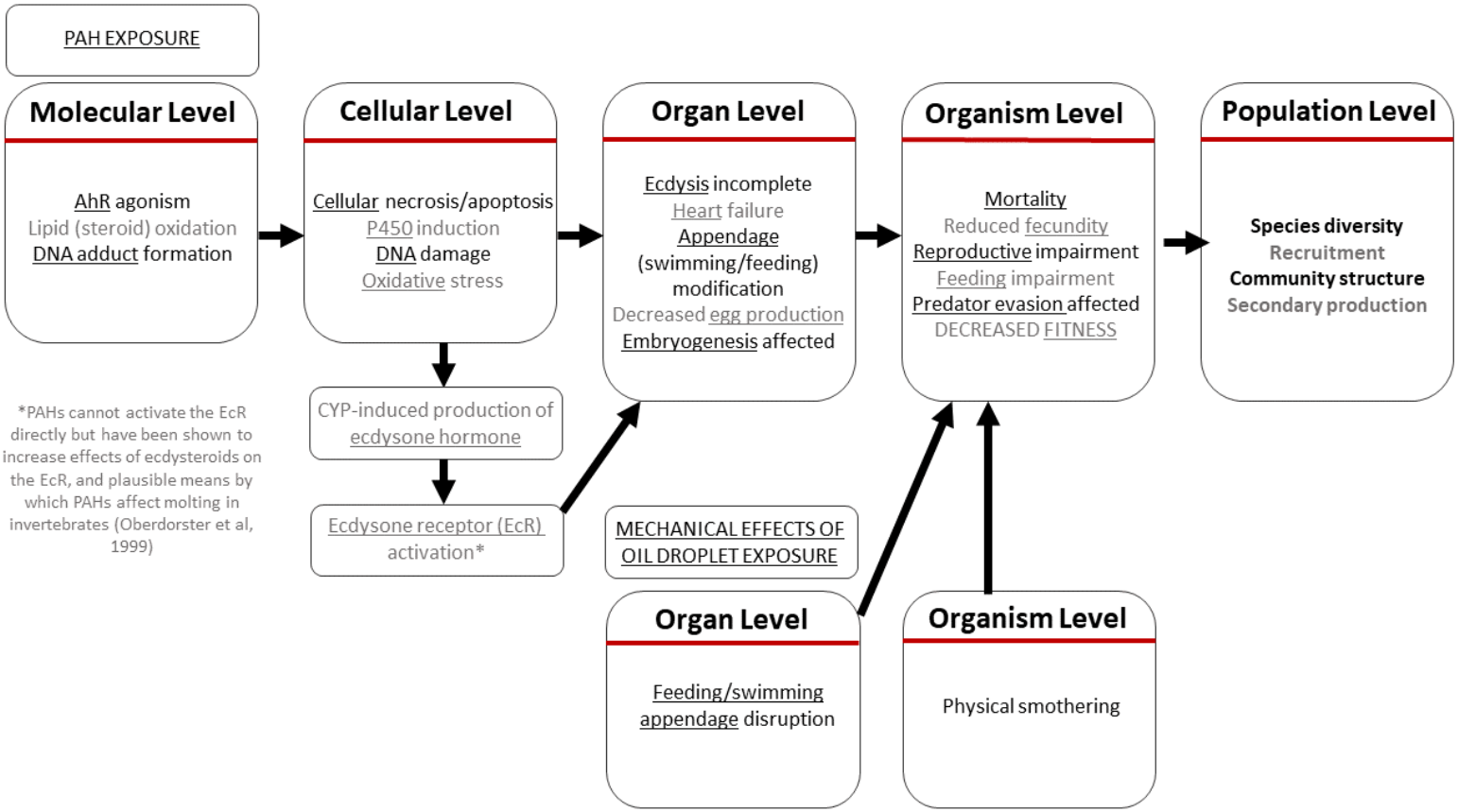

Figure 1.3: Proposed adverse outcome pathway for zooplankton exposed to oil droplets and associated acute polycyclic aromatic compound toxicity following an oil spill (Abbriano et al, 2010; Dupuis & Ucan-Marin, 2015; Lee et al, 2014; Lee et al, 2015; Nebert & Karp, 2008; Oberdorster et al, 1999; Song et al, 2017).

1.1.6.2 Benthic Invertebrates

The benthic community is an important aspect of the aquatic ecosystem, providing a key link between higher trophic levels (fish) and bacterial and algal communities. The benthic zone is also important for ecological function in aquatic systems, helping to regulate nutrients and energy flow. Unfortunately, our understanding of the impacts on the benthic community following an oil spill are limited. The Kalamazoo River spill offered an opportunity to evaluate the impact of a dilbit spill on this community. However, much of the damage brought about to Talmadge Creek and the Kalamazoo River watershed following the spill was a result of clean-up efforts including dredging, which severely disrupted benthic habitats, and inhibited the ability to define actual risk of submerged oil to benthic habitats (Fitzpatrick et al, 2015; Lee et al, 2015). Consequently, little information exists surrounding the potential for weathered dilbit to affect benthic communities and given the ability for dilbit to sink following weathering, chronic effects following a spill are of most concern to benthic habitats and organisms that rely on sediments for larval development (Lee et al, 2015). Sediment-bound hydrocarbons tend to be rich in higher molecular weight PACs (3 to 5-ringed PACs) and are the main source of exposure to HMW PACs in aquatic environments (Dupuis & Ucan-Marin, 2015; Lee et al, 2015). Benthic organisms are less mobile than planktonic organisms and fish and may be unable to avoid oiled patches of sediment, especially in larval life stages (Dupuis & Ucan-Marin, 2015; Abbriano et al, 2010).

Vicentini et al (2017) assessed the toxicity of benzo(a)pyrene (BaP; 5-ringed PAC) to *Chironomus sancticaroli* larvae and found genotoxic and neurotoxic effects as a result of oxidative stress, DNA damage, and acetylcholinesterase inhibition following a 72-hr exposure at a concentration of 4.73 µg/L. Although BaP will likely be present in minute amounts in the sediments, this presents a possible pathway of toxicity for benthic invertebrates exposed to sediment-bound PAHs (Vicentini et al, 2017; Crude Quality Inc, 2017). Moulting of invertebrates during larval development may also be affected following exposure to sediment-bound PAHs (Oberdorster et al, 1999; Song et al, 2017). Ecdysteroid hormones regulate molting behaviour in invertebrates, as previously discussed with zooplankton. As such, PACs may be able to increase the effect of ecdysteroids (Oberdorster et al. 1999) (**Figure 1.3**; **Figure 1.4**). Changes in moulting capacity and other organism-level impacts are outlined in the adverse outcome pathway in **Figure 1.4**. As with zooplankton, PAHs can induce changes in AhR and EcR activity and may affect survivorship, reproductive output, feeding ability, and overall fitness.

As oil is highly viscous and may stick to a multitude of surfaces, the physical threat posed by oil needs to be considered and is outlined in **Figure 1.4**. A key component of the life cycle of many aquatic insects is emergence – larval stages may live much of their life in sediments, but reproduction occurs following emergence and oviposition of egg mases to the water's surface. No studies have evaluated the impacts of an oil slick on insect emergence or on oviposition but the potential impacts on invertebrate community composition are multiple. If organisms cannot emerge through the water column and complete their final moult into sexually mature adults, recruitment may fall. This will also occur if the presence of surface oil affects the viability of eggs or inhibits

oviposition of viable eggs. If certain species are more susceptible to the presence of oil, there may be selective changes in community composition, altering community dynamics, which will have a cascading impact on the aquatic food web.

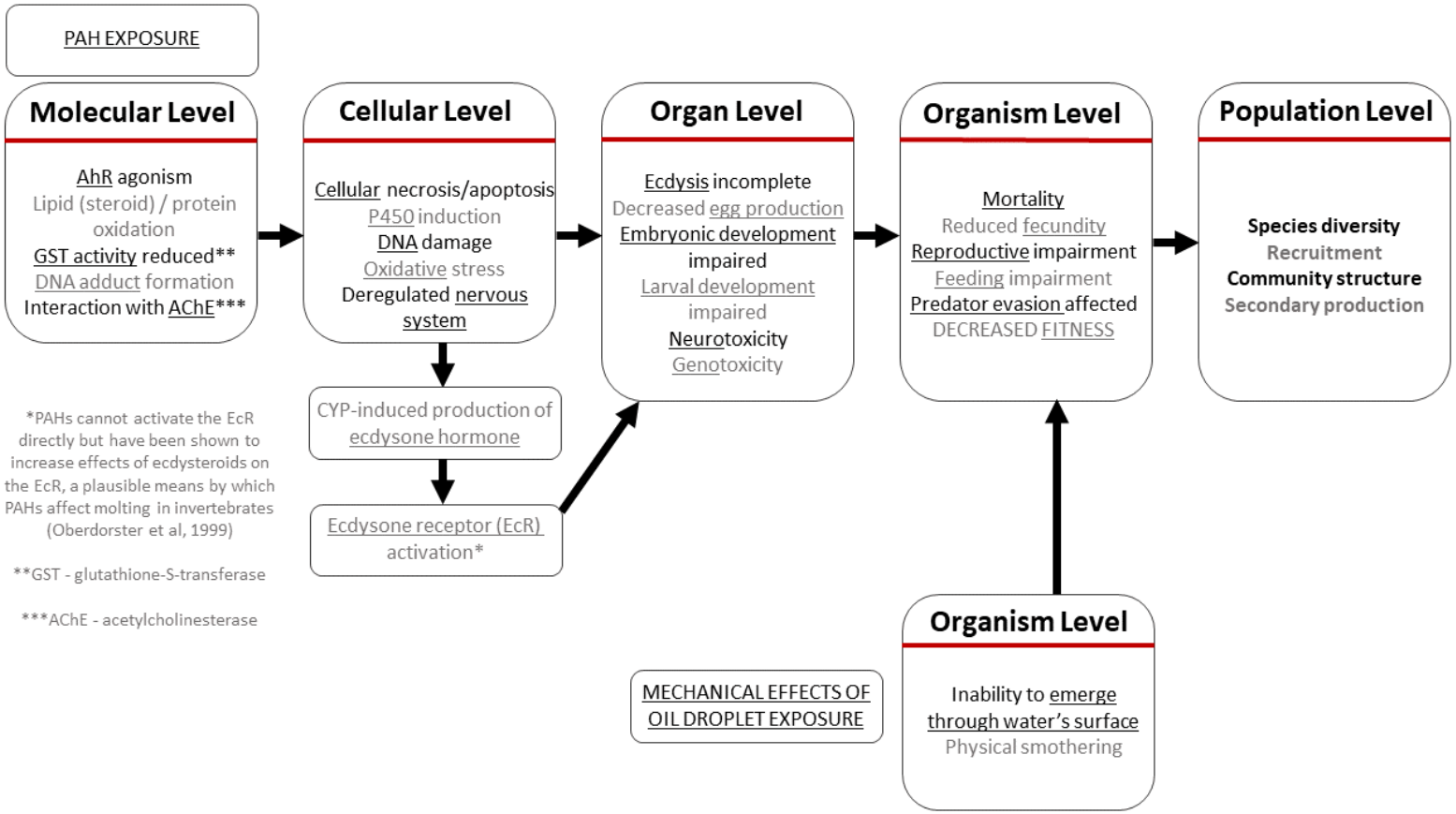

Figure 1.4: Proposed adverse outcome pathway for benthic invertebrates exposed sediment-bound polycyclic aromatic hydrocarbons (PAHs) and the presence of an oil slick on the water's surface following an oil spill (Abbriano et al, 2010; Dupuis & Ucan-Marin, 2015; Lee et al, 2015; Oberdorster et al, 1999; Song et al, 2017; Vicentini et al, 2017).

1.2 Measures of Arthropod Secondary Production in Lakes

1.2.1 Current Methods

Our understanding of energy flow in biological systems has developed from trophic level classification of organisms; primary producers derive energy from the sun that supports primary consumers and other predators up the food chain. Arthropod secondary production is the assessment of the net rate of biomass production (i.e. consumption and assimilation of energy) in primary arthropod consumers, including benthic macroinvertebrates and zooplankton (Benke, 2010; Benke & Huryn, 2010). For the purposes of this thesis, it will be referred to simply as secondary production from this point forward. Secondary production measures offer predictors of ecosystem health at the population and community level based on abundance, biomass, and parameters key to reproductive success and survivorship when evaluated in contrast with pre-impacted secondary production estimates (Valentine–Rose et al, 2011). Fish species are reliant on an abundant and diverse food source supplied by these organisms and if they were to fail, so too would the fish population. The reliance of most all other parameters of ecological importance in freshwater on secondary production is key to its use in assessing impacts from anthropogenic sources, such as diluted bitumen.

As a direct result of nutrient enrichment of a body of water, Cross et al (2006) concluded that secondary production increased by 226% ($p<0.001$). Valentine-Rose et al (2011) indicated that calculation of invertebrate secondary production using traditional methods (i.e. arthropod collection, identification, and biomass determination) offered the most

sensitive metric of ecosystem function in evaluating fragmentation effects (i.e. presence of dams or diversions in a lotic system), as compared with measures of species abundance and distribution. However, they noted that comparisons between secondary production measures cannot be made across systems – a result of variability in ecosystem structure under different environmental conditions (i.e. ecosystem size and dynamics, community structure). For an effects indicator to be useful, it must be able to predict changes at the community and ecosystem levels in impacted sites relative to reference sites while also providing detailed information that can allow identification of the stressor.

Measures of secondary production via traditional means are often difficult and labour-intensive; biomass estimates, and species identification require hours of intensive work both in field and lab only to merely offer estimates of secondary production (Conley et al, 2009). It is also difficult to extrapolate this information to whole community effects due to the variability (Sastri et al, 2013). Net selectivity and species identification may introduce biases into biomass determination, further complicating results (Suchy et al, 2016a). As such, secondary production is often disregarded from analyses and studies altogether. There are, however, emerging tools that may provide reliable, field-based applications of secondary production estimates.

1.2.2 Chitobiase as a Proxy for Secondary Production

Chitobiase (β-N-acetylglucosaminidase) is an invertebrate molting enzyme used to cleave chitin oligomers during molting (Oosterhuis et al, 2000). It is present in organisms that have a chitinous exoskeleton and include, in the context of a boreal lake,

zooplankton and benthic invertebrates. Chitobiase is released into the surrounding waters during ecdysis (shedding of the exoskeleton) as opposed to being reabsorbed by the organism (Oosterhuis et al, 2000). Vrba & Machacek (1994) found that chitobiase can be detected in levels 40 to 100 times greater post moult in *Daphnia pulicaria,* corresponding to body size and therefore may correlate with molting activity. Building off this observation, the inverse of chitobiase degradation rate was used as proxy for biomass production rates in lab studies using *Temora longicornis*. As chitobiase production could not be calculated directly due to microbial degradation of the enzyme *in situ*, it was purported that the rate of production of chitobiase, under steady state, would equal the enzyme's degradation rate (Oosterhuis et al, 2000; Sastri et al, 2013). From this work, a simple and cost-efficient fluorescence assay was developed (Sastri & Roff, 2000; Hanson & Lagadic, 2005).

Secondary production estimates for zooplankton communities have been conducted in marine and estuarine environments using the chitobiase fluorescence assay and contrasted with other methods of estimating secondary production, such as with mathematical models or direct biomass estimates (Avila et al, 2012; Sastri & Dower, 2006; Sastri & Dower, 2009). Freshwater research has been less extensive; lotic systems have been studied using chitobiase biomass estimates in determining impact from natural and anthropogenic stressors (Conley et al, 2009; Hanson & Lagadic, 2005) and in lab-based studies of *Daphnia magna* (Richards et al, 2008). The method has been studied even less in lentic systems (Sastri et al, 2013). Zou & Fingerman (1999a; 1999b) first assessed the effect of contaminants including diethyl phthalate, 4–(tert)–octophenol, 2,4,5–trichlorobiphenyl, and four estrogenic compounds, on chitobiase

activity in the fiddler crab (*Ucapugilator*). Zou & Fingerman (1999a) noted a decrease in molting rate corresponding to chitobiase inhibition, with estrogenic interference elucidated to be the cause.

Hanson & Lagadic (2005) examined chitobiase measures in freshwater lotic systems to use in an environmental monitoring context. Downstream of a sewage treatment plant in the Oir River, France, chitobiase levels corresponded to changes in water quality parameters ($p<0.0001$ $r^2=0.77$). They also noted low variability in chitobiase activity at the same sampling site and across sites of similar hydrological structure. Further evidence for the inclusion of chitobiase assessments in risk assessment was presented by Richards et al (2008) and Conley et al (2009). Pharmaceutical contamination was correlated with a decrease in standing chitobiase activity in lab, a valuable metric considering the rise of CECs in freshwater environments downstream of wastewater treatment plants (Richards et al, 2008). Under field conditions, however, negative correlation was only reported for summer months.

1.2.3 Gaps in Methodology for Chitobiase Production Estimates

Although chitobiase methods for estimating secondary production offer promising means of assessing changes to ecological function in impacted waters over time, they are limited in their ability to identify stressors and characterise their risk. To offer any valuable estimate of impact, chitobiase methods will benefit from context provided by abundance data across seasons in interpretation and characterisation of ecological changes proposed by the assay (Dolbeth et al, 2012). Few studies have considered geographical and seasonal variability. Work has been done in this regard (Suchy et al,

2016a; 2016b) but little from a contaminant response scenario and in freshwaters. Furthermore, only one study (Sastri et al, 2013) has compared secondary production estimates obtained using traditional biomass methods and the chitobiase method in a lake. Lentic and lotic systems are inherently different; flow rate, stratification, seasonal temperature changes, and organism abundance are drastically different between these systems and as such, research must be conducted independently at these sites (Dolbeth et al, 2012). Behaviour of contaminants in these systems will also vary; dilution, flow rate, and temperature must be considered. Further research on the seasonal and spatial relationship of arthropod communities to chitobiase activity and natural variability in chitobiase activity over time must be conducted before its use in toxicological studies.

Another issue surrounding the use of chitobiase analyses in a field-setting is based on the viability of water samples following collection. Often, field-work occurs in remote areas (such as IISD-ELA) where samples cannot be immediately processed. As such, storage of these samples in coolers and, eventually, a -20°C freezer, is often the best that can be done under resource constraints. Various parameters have been proposed below in the study of long-term sample storage for chitobiase analysis based on Chróst & Valimirov's (1991) study of enzyme kinetics following long-term storage at below freezing temperatures. This study showed that following substrate hydrolysis and incubation, sample fluorescence was retained with no significant loss following 10 days of storage at -20°C. This contrasts with storage of unaltered samples, in which enzyme activity decreased unpredictably. They noted that freezing and thawing can have consequences on enzyme affinity and activity. As such, it may be of relevance to

compare chitobiase activity over various storage periods and storage conditions, with water samples either subject to immediate storage or storage following substrate addition and incubation.

Chapter 2
Research Objectives and Hypotheses

This thesis has several objectives and hypotheses. These are provided below.

Objective 1: Characterize the response of invertebrate communities (arthropods) to experimental dilbit spills under field conditions at the IISD-Experimental Lakes Area through analysis of productivity, community diversity, biomass, and function by abiotic and biotic measures. Additionally, a broader lens will be applied to this research by contrasting effects on invertebrates to changes in fish populations and phytoplankton, corresponding with changes in the fate and post-spill concentration of dilbit throughout the water column.

> **H1-A:** Community diversity of invertebrates will decrease with increasing dilbit concentrations or volumes, corresponding to the dominance of the community by opportunistic species, or species that more easily adapt to the presence of dilbit in the system. Recovery of the community will occur several weeks following dilbit addition because of weathering processes, causing a decrease in acute toxicity risk. Long-term effects may be present depending on the amount of residual oil present at the surface of the sediments. Surface sheen presence will have a clear impact on emerging insect communities. Impacts to insect emergence may affect benthic reproduction as a result of impacts on fecundity.

H1-B: Arthropod secondary production will decrease with increasing dilbit concentrations, corresponding to a loss of primary production directly following dilbit additions. As dilbit sinks, and light penetration increases with the loss of surface oil, secondary production will recover as a result of increased primary productivity. Decreases in secondary productivity (assessed via benthic invertebrate and zooplankton biomass) will corresponded with a decrease in phytoplankton biomass as a result of anthropogenic stressors attributed to PAC exposure and reduced light penetration through the water's surface with increasing dilbit concentration.

H1-C: Fish presence will influence invertebrate abundance – changes in fish community health will have a top-down trophic impact on benthic and pelagic invertebrates and will follow a concentration-response change; increased PAC presence will directly impact fish health and grazing pressure. Additionally, the loss of zooplankton with increasing PACs will have concurrent bottom-up trophic impacts, reducing food sources for planktivorous fish.

Objective 2: Characterize the impacts of surface oil on water strider (Family: Gerridae) movement and survivorship using small and large tanks.

H2: The presence of a surface sheen, even in small amounts, will affect water strider mobility and will lead to immobility and death associated with surface tension reduction and physical smothering by oil.

Objective 3: Assess a new method of quantifying secondary productivity in a lake environment under field conditions at the IISD-Experimental Lakes Area.

H3: Rate of chitobiase production is positively correlated with total zooplankton and benthic invertebrate biomass in a lentic system impacted with diluted bitumen, enabling its use in assessing changes in secondary production from anthropogenic stressors.

Chapter 3
Methods and Materials

This thesis consists of three main components: evaluating the impacts of simulated oil spills on (a) zooplankton communities and (b) benthic invertebrate communities, and (c) an assessment of the impacts of simulated oil spills on organisms that live on (pleustonic) or pass through (emerging insects) the air-water interface. Concurrent with the BOREAL study, chitobiase analysis was conducted to evaluate its use as a tool for assessing arthropod secondary production in stressor-impacted environments.

These systems were evaluated via (1) a pilot-scale study conducted in August 2017 in preparation for (2) a full-scale in-lake limnocorral study the following year. Part 1 was conducted at the International Institute for Sustainable Development - Experimental Lakes Area (IISD-ELA), located near Kenora, Ontario using surface waters and sediments collected from Lake 240, an oligotrophic reference lake surrounded by Boreal forest. Water was transported into three land-based microcosms and dosed with 0 L, 0.1659 L (low), and 1.6350 L (high), applications of dilbit. The purpose was to test methods for sampling enclosures treated with dilbit and to evaluate the fate and behaviour of spilled diluted bitumen exposed to the elements. This was conducted to ensure that all methodology was sound and to troubleshoot any problems in study design prior to the larger study. Furthermore, it was useful to have an idea of what to expect in terms of dilbit weathering and behaviour to prepare for changes in the compound that would occur in the limnocorral study. The pilot study was scaled up to

nine 10-m diameter, 2-m deep *in situ* limnocorrals at IISD-ELA's Lake 260 during the open-water season in 2018, dosed with dilbit in a regression design with seven treatments ranging from 1.5 L to 180 L of dilbit and 2 controls.

The IISD-ELA is home to 58 freshwater lakes and their watersheds and serves as an important location for conducting whole ecosystem research. It is located in Kenora District, Northwestern Ontario, Canada at 49°39'36.3"N, 93°43'40.0"W. Lake 240 (49°39'24.3" N, 93°43'48.1" W) was the source lake for water and sediment used in the 2017 pilot-scale study. Lake 240's sediments are sandy and low in organic matter in the near shore areas where water and sediment were collected. Further physical and chemical properties of Lake 240 are outlined in Orihel et al (2006). Lake 260 (49°41'55.2" N, 93°46'04.7" W) is also a small boreal shield lake with a maximum depth of 14.4 metres, a surface area of 340,000 m^2 (Kidd et al, 2014). The site chosen for the limnocorrals was located along the north-western shore of Lake 260 surrounded by 3 small islands. Sediments were characterized as silty-sandy with small pebbles scattered throughout. Organic material on/within the sediments was minimal throughout the area. Further discussion on physical and chemical properties of Lake 260 are provided in Kidd et al (2014).

Data used in this thesis includes chlorophyll *a*, fish recapture numbers and size metrics, as well as information on fate and behaviour of diluted bitumen (including density and TPAH concentrations). These data were collected as part of the larger BOREAL project and the former two metrics were not assessed relative to oil volumes or concentrations. These data were collected by a multitude of other people. Sawyer Stoyanovich

(University of Ottawa) conducted oil chemistry and oil analysis to understand fate and behaviour. Lauren Timlick (University of Manitoba, IISD-ELA) collected and analyzed fish communities. Jeffrey Cederwall (Queen's University) conducted analysis on primary producers, including determining chlorophyll *a* concentrations. Methods for these analyses are not included here but will be included in future publications and respective theses/dissertations from these students. Interpretation of these data (including nutrient data) are limited to understanding direct impacts to invertebrate communities. Detailed analysis of these data will also be included in future publications when attempting to understand big picture impacts on the food web.

3.1 Pilot-Scale Mesocosm Study

3.1.1 Mesocosm Set-Up and Installation

The pilot-scale study was conducted between August 12th and August 27th, 2018 at the IISD-Experimental Lakes Area. Three circular, flat-bottomed, low-density polyethylene outer containment tanks (2.7 m diameter x 0.72 m height) were used to house the microcosms, and were originally purchased from ACE Rotomolds (Hospers, Iowa, USA; see Cardinal et al., 2014 for more details). Into these were placed 2-m diameter microcosms constructed from woven Novathene plastic (Curry Industries, Winnipeg, Manitoba, Canada), as used in previous microcosm studies (e.g. Orihel et al, 2016). Each was suspended from 2-m diameter hexagonal vinyl-wrapped Styrofoam expanded polystyrene) collars (Dow, Midland, Michigan, USA; **Figure 3.1**). Flexible PVC tubing was used to maintain the circular structure of the microcosms, attached via sleeves sewn into the top and bottom of the microcosm and secured to the collar. Prior to set-

up, microcosms were submerged for 24 hours in Lake 240 to allow leaching of any substances on the plastic that may be harmful to plankton, as recommended in O'Brien et al (1991) and Pilati and Wurtsbaugh (2003).

Water and sediment were collected from Lake 240, a reference lake at IISD-ELA. Sediment was added first to the microcosms on August 9th, followed by water on August 11th. Sediment was collected via shovel to a depth of approximately 10 cm from Lake 240, mixed in a large tank, split into three aliquots, and randomly added to each microcosm to a target height of 5 cm, amounting to approximately 169 L of sediment per tank. The sediment was covered with two layers of polyethylene plastic (vapour barrier) to limit disturbance and suspension of sediment during the water addition. Water was pumped from the end of Lake 240's dock, extending 15 m from shore. Water was collected via centrifugal water pump and suction hose into a graduated 350 U.S. gallon tank and transported to the microcosm site via truck. Water was added via gravity feed to the containment tank and the microcosm tank in 380 L intervals to limit strain on the microcosm walls. A total of 1364 L was added to the microcosms and 818 L added to the outer containment tank (**Figure 3.1**). The plastic covering was carefully removed, and tanks could settle prior to treatment on August 13th.

A secondary containment berm was constructed to contain the low and high dose tanks in the event of a breach. It consisted of a polyethylene plastic layer covered by gravel, with a sandbag berm surrounding the perimeter (three sandbags high with average height of 13 cm/sandbag;

Figure 3.2). The berm surrounded an area 7 m wide by 5 m deep. A fence 8’ (2.44 m) in height was used to contain the entire microcosm site (7 m by 14.3 m), including the control tank. To prevent birds from interacting with the water, a bird mesh covered the tanks when sampling was not occurring.

Efforts were made to limit disruption by sampling of any oil layer on the water surface through pre-positioned 1.5” ABS sampling ports mounted via a wooden 2x4 plank (**Figure 3.3**). Two ports were inserted: a centrally positioned port extending 40 cm below the water surface for water sampling, with a second port midway between the center and edge of the tank, extending 10 cm below the water surface for water quality measurements via a Yellow Springs International (YSI) multiparameter sampling sonde.

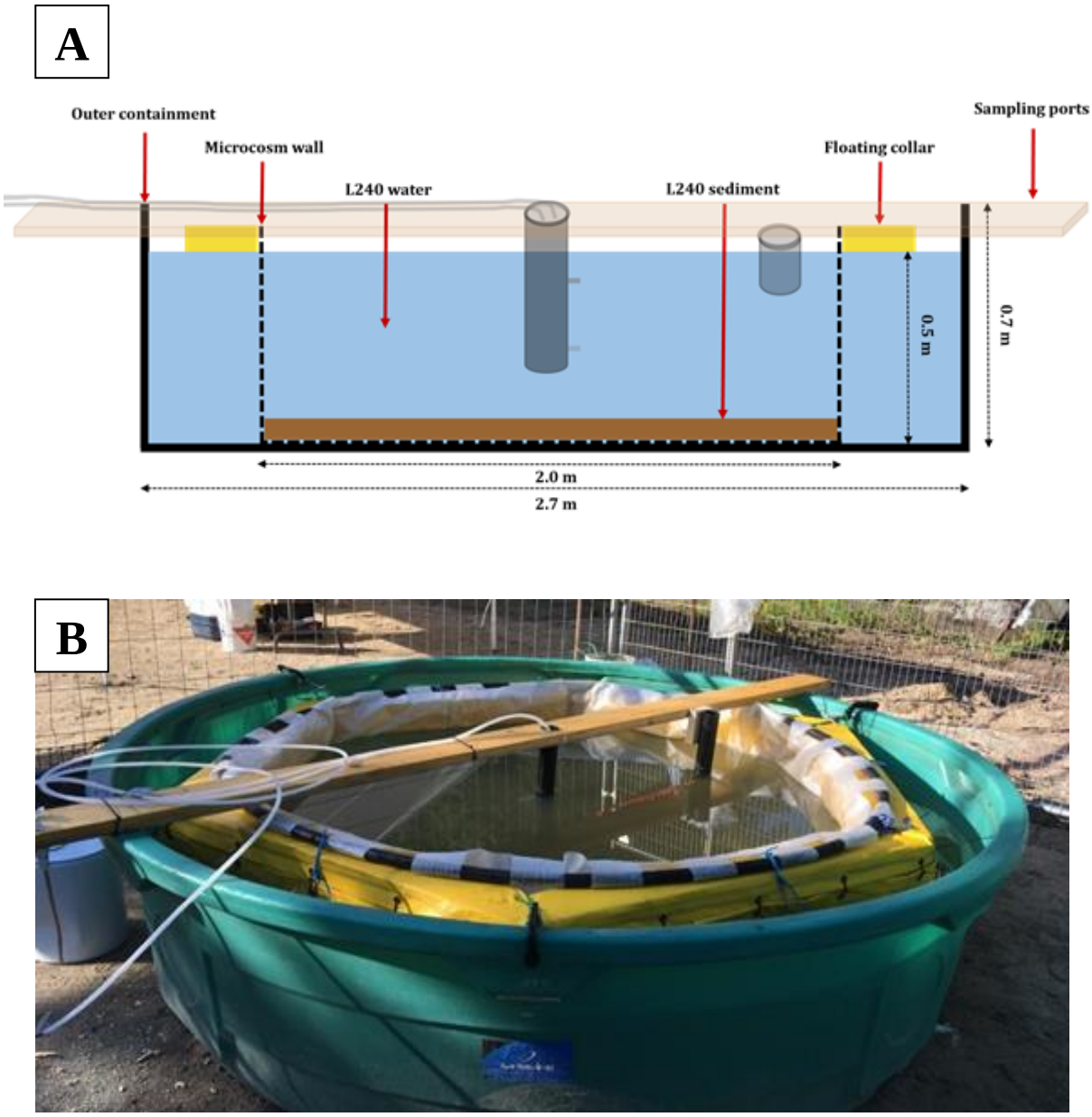

Figure 3.1: (A) Vertical profile of microcosm used in pilot-scale study in August 2017 at IISD-Experimental Lakes Area. (B) Microcosm consisting of an outer containment tank, Novathene microcosm wall secured to a yellow flotation collar via flexible tubing, with sampling ports stretching across tank.

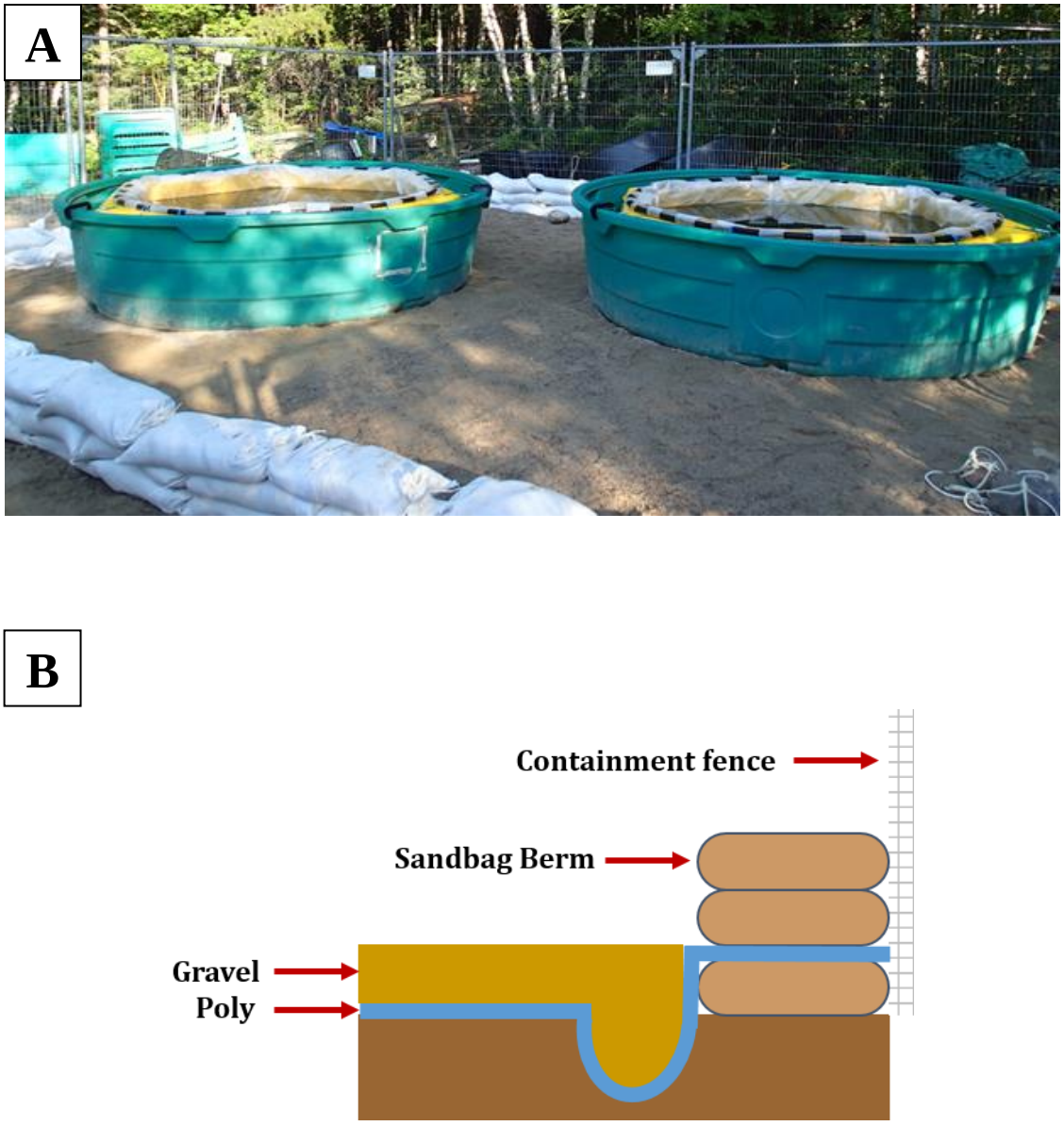

Figure 3.2: (A) Low (right) and high (left) treatment microcosms with sandbag berm and containment fence in background, and (B) sandbag berm with poly plastic layer covered with gravel.

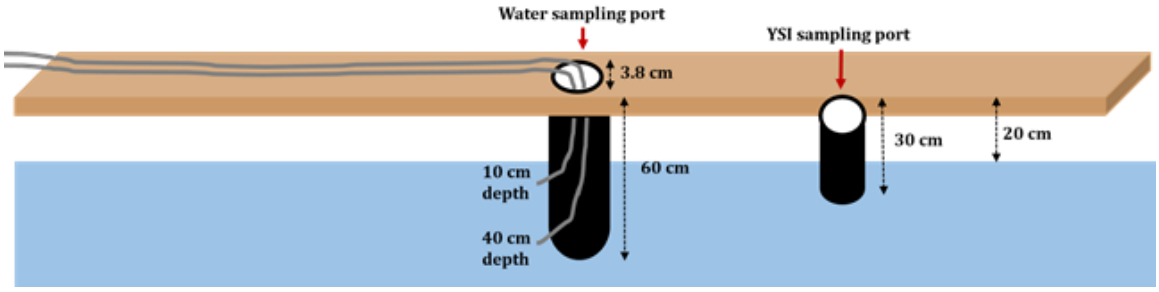

Figure 3.3: Sampling ports for water sample collection, mounted to a 2”x4” plank extending across the microcosm tank. Image not to scale.

3.1.2 Zooplankton Amendments

Amendments of zooplankton were undertaken to ensure community presence in the mesocosms. Zooplankton samples were collected via tow net at center buoy from Lake 240 one day prior to oil treatment (August 12th; Day -1). Zooplankton were collected by 15 1-m vertical hauls using a 53-µm Wisconsin plankton net with a 30-cm diameter opening (total of 1060 L of water sampled). Hauls were diluted to 15 L with Lake 240 water to match densities of Lake 240 zooplankton and the pilot mesocosms. Hauls were then mixed, split into 3 aliquots (5 L each), and randomly added to each microcosm. Baseline zooplankton Lake 240 data were collected from subsamples of each 5 L sample and analyzed for community composition and abundance. The same was done for phytoplankton by conducting 100 4-m vertical hauls using a 10-µm Wisconsin plankton net with a 12-cm diameter.

3.1.3 Oil Addition

Fresh Cold Lake Winter Blend (CLB-W) was added to the low concentration tank and high concentration tank at nominal volumes of 0.1659 L and 1.6350 L, respectively. This corresponds to a total relative concentration of 1:8,222 and 1:834.3 (v/v, dilbit:water) in the low and high tanks, respectively. Additions of dilbit and volume determinations were conducted by Environment Canada's Emergencies Science and Technology Division in Ottawa, Ontario. These concentrations were selected based on mid-range and high-range dilutions used in the full-scale study (1:10,000 and 1:1,000). Oil was added on August 13th, 2017 (Day 0).

3.1.4 Zooplankton Sampling

At 11 days post-treatment (August 24th, 2017), zooplankton were sampled using a diaphragm pump (High Flo 12V, 3.8 gpm) with in-line 53-µm Nitex mesh, and flow meter (Sotera Model 825) for accurate volume measures. 59.7, 59.4, and 59.9 L of water was filtered from the Control, Low, and High microcosms, respectively. Pump design was adapted from methods outlined in Dixon & Robertson (1986), Yocum et al (1978), and Waite and O'Grady (1979). All samples were preserved in 5% sugar-formalin solution. Counts and taxa identification were completed following procedures outlined in Paterson et al (2013). Nauplii and CI to CIII copepodites were not identified to species. Rotifers were also enumerated, but not identified. Community diversity was measured by species richness (total number of species observed), Renyi diversity (Hill number) and Shannon diversity was calculated as outlined in Preston & Rusak (2010) and Kenkel (2006).

3.1.5 Water Nutrients and Water Chemistry

Nutrients within the water column were sampled on Day -1, 2, 7, and 11, using the "top" sampling port (**Figure 3.3**). Samples were analyzed by IISD-ELA's Chemistry Laboratory following procedures outlined in Stainton et al (1997) for the following: alkalinity, anions (Cl^-, SO_4^{2-}), chlorophyll *a*, dissolved inorganic carbon (DIC), dissolved organic carbon (DOC), total dissolved nitrogen (TDN), total dissolved phosphorous (TDP), total dissolved solids (TDS), turbidity, suspended phosphorous, suspended C:N, NH_3, NO_2, NO_3^-, total suspended solids (TSS), and soluble reactive silica (SRSi). In

addition, pH, conductivity, temperature, and dissolved oxygen were measured daily using a multiparameter probe (YSI Professional Plus, Xylem, Yellow Springs, Ohio).

3.2 In-Lake Limnocorral Study

3.2.1 Limnocorral Set-Up and Installation

A total of nine limnocorrals were deployed in Lake 260 at IISD-ELA in June 2018. Limnocorrals consisted of a plastic curtain (Novathene plastic; Curry Industries, Winnipeg, Manitoba, Canada) secured and sealed to the sediments using sandbags. Each curtain was suspended from a 10-m diameter expanded polystyrene floating collar, as used in previous microcosm studies (e.g. Orihel et al, 2016). A partition was added within the collar to provide an “oil-free” area that would allow sampling equipment to enter the enclosed water column without having to pass through an oil sheen and risk contamination of equipment (**Figure 3.4**). Limnocorrals were installed along the northeast littoral zone of Lake 260 with a target limnocorral depth between 1.5 and 2 metres.

Nine limnocorrals were randomly assigned to seven dilbit treatments and two controls (near-field control (NC) and far-field control (FC)). On June 20th, 2018, Cold Lake Winter Blend (CLB-W) dilbit was added to the surface of each treatment limnocorral with volumes of dilbit added ranging from 1.5 L to 180 L (**Figure 3.5**).

Prior to oil addition, fish were removed from the limnocorrals via minnow cage traps, seine nets, and gill nets to ensure each limnocorral was as similar as possible with regards to plankton and other invertebrate grazing rates and predatory pressures. A

known number of fish were also added on 11th July (Day 21). This included seven adult female and seven adult male finescale dace (*Phoxinus neogaeus*). Subsequently, fish removals began on the 17th August (Day 58) and ended on the 31st August (Day 72) using one minnow cage trap per limnocorral. Traps were retrieved daily. Catch per unit effort (CPUE), a metric used to determine abundance of fish populations, was one minnow trap per 24-hour period in each limnocorral (i.e. one trap deployed in each limnocorral and fish collected every 24 hours).

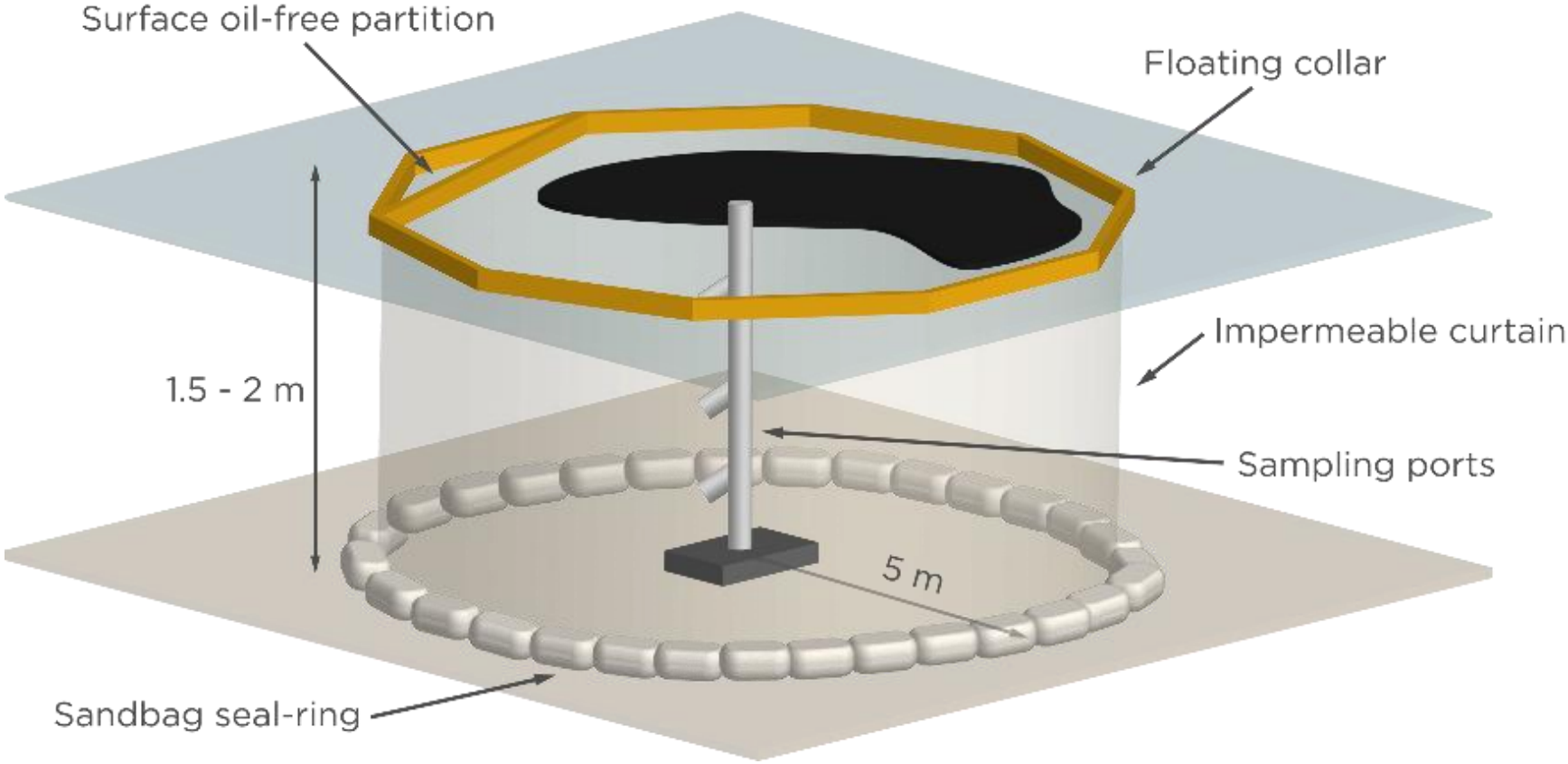

Figure 3.4: Limnocorral enclosure design as used in the in-lake limnocorral study in 2018. Graphic produced by Jose Luis Rodriguez-Gil.

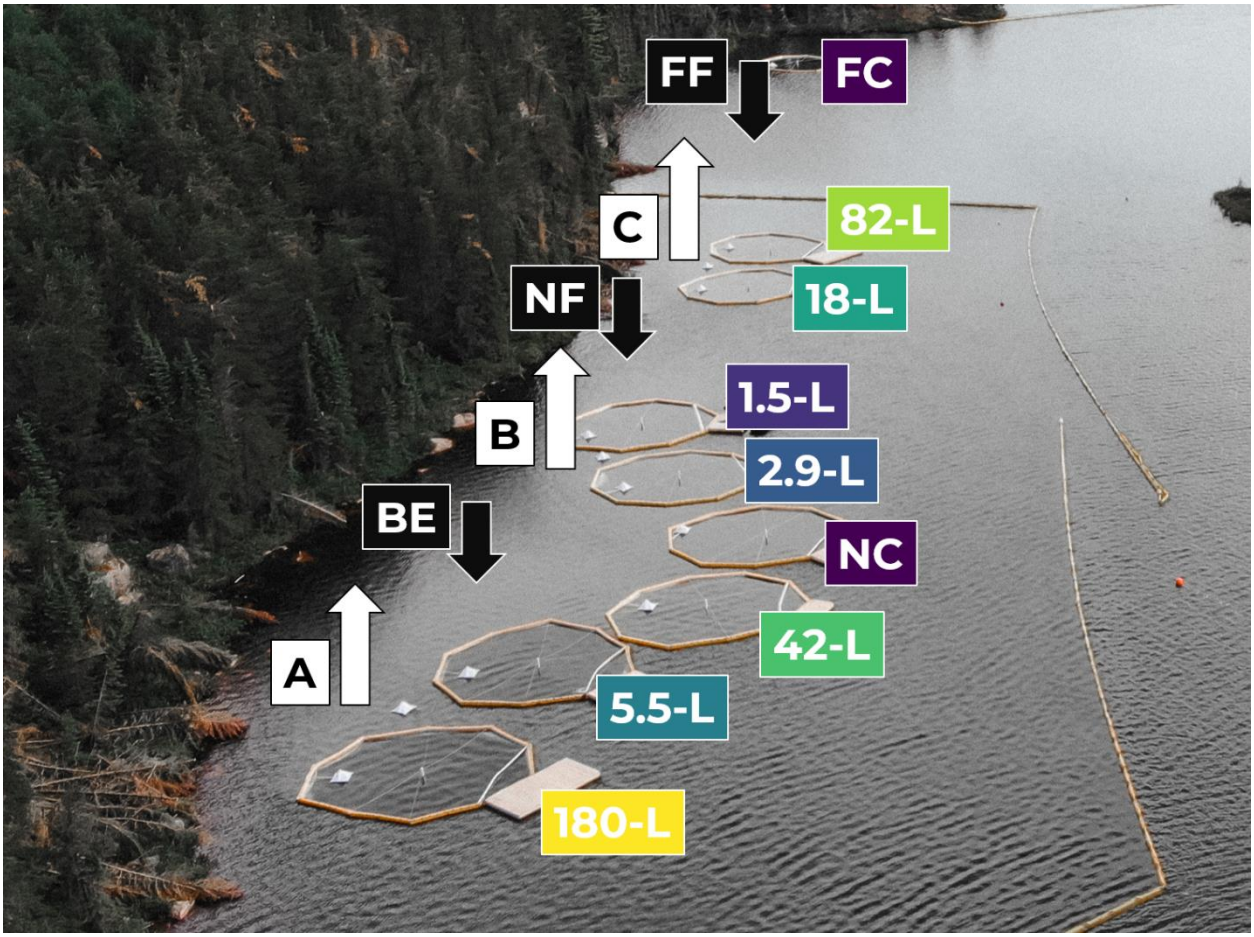

Figure 3.5: Cold Lake Winter Blend diluted bitumen was added to limnocorrals in Lake 260 at the IISD-Experimental Lakes Area. Treatments ranged from 1.5 L to 180 L of dilbit, with 2 control limnocorrals (near-control, NC; and far-control, FC). A 30-cm surface boom surrounded the limnocorrals. Downward-facing arrows indicate reference sites for benthic sampling (behind enclosures, BE; near-field, NF; and far-field, FF). Upward-facing arrows indicate emergence trap reference sites (A, B, and C).

3.2.1.1 Sampling System

Sampling was conducted from floating platforms located adjacent to each limnocorral. Our sampling system consisted of three components: (a) a vertical sampling column housing sampling ports located in the centre of the limnocorral; (b) sampling tubes extending from the sampling column to the dock; and (c) the sampling station – a plastic container housing the ends of the sampling tubes (**Figure 3.6**). The sampling column consisted of three sampling ports at depths of 30 cm below the water surface (“top”), the centre of the water column (“middle”), and 30 cm above the sediment (“bottom”) (**Figure 3.7**). Sampling ports consisted of a PVC “wye” angled 45 degrees downward to reduce the potential of falling oil contacting the sampling tubes. Oil chemistry sampling used “top” and “bottom” sampling ports, while water chemistry, zooplankton, and phytoplankton sampling used the “middle” sampling port (**Figure 3.7**).

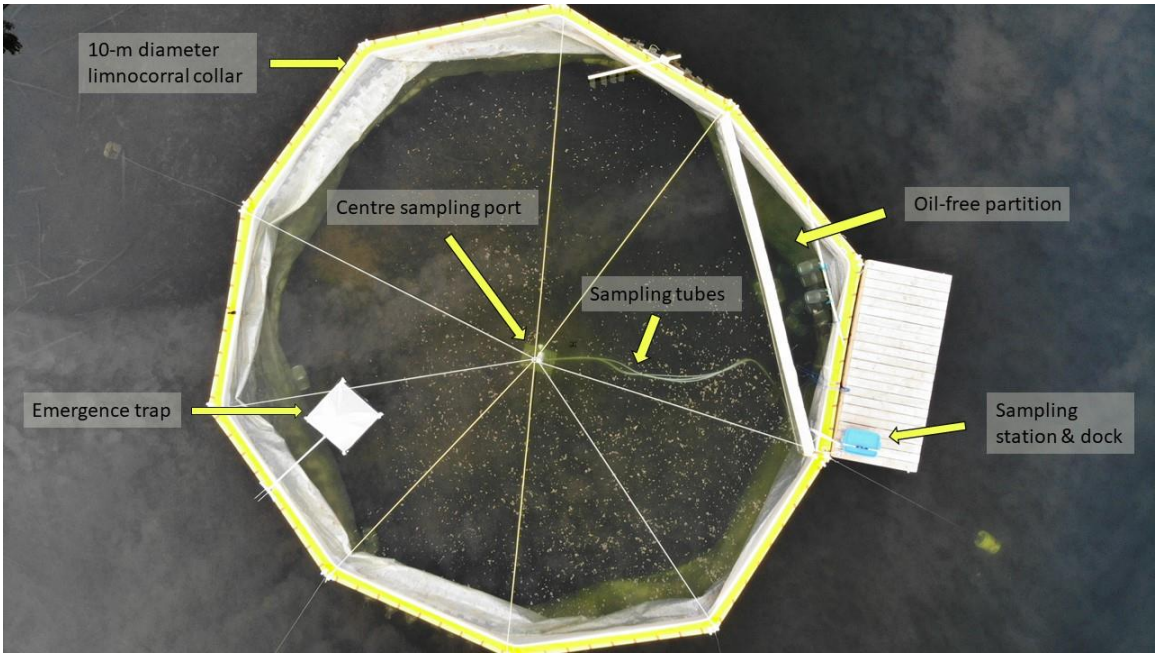

Figure 3.6: Aerial view of limnocorral indicating location of the sampling column housing the sampling ports, insect emergence trap, oil-free partition, and the sampling station used to sample zooplankton and water.

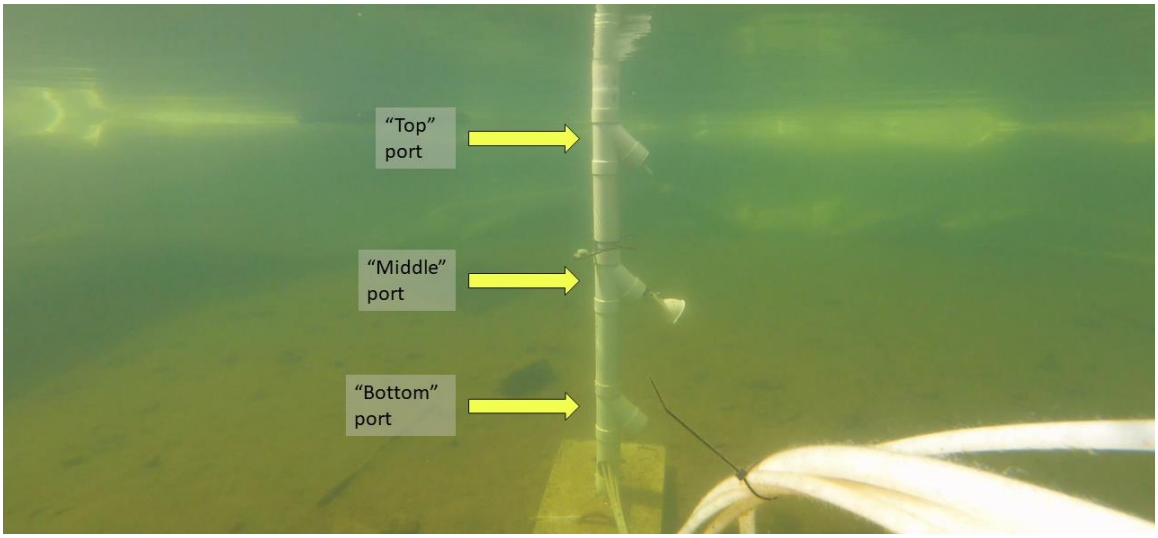

Figure 3.7: Lateral view of sampling ports and sampling column used to sample water and biota from BOREAL limnocorrals.

3.2.2 Zooplankton Sampling

Zooplankton sampling was conducted via a pump system designed specifically for use in the BOREAL limnocorrals (**Figure 3.8**). Zooplankton pump design was adapted from methods outlined in Dixon and Robertson (1986), Yocum et al (1978), and Waite and O'Grady (1979). Zooplankton located in the centre of the water column were sampled using the "middle" sampling port located at a depth between 0.6 and 1.0 m. Zooplankton were pulled through a ½" linear low-density polyethylene (LLDPE) tube via a 3.8 gallon per minute High-Flo Diaphragm pump (no impeller) powered by a 12-V deep-cycle marine battery. Water was then pushed through a 53-µm mesh housed within a removable filter container made from 1-1/2" ABS fittings. Although a 53-µm mesh does not capture all rotifers (some smaller species may pass through the mesh), it reduces the amount of phytoplankton collected and offers a balance between facilitating sample enumeration and collecting the majority of zooplankton species. From 60 to 120 L of water was filtered based on visual assessment of biomass collected on the 53-µm filter mesh; the filtered volume remained constant for all limnocorrals on each sampling day. Filter volumes were evaluated using an in-line flow meter (Sotera Fill-Rite Digital Turbine Meter), accurate to 0.01 L (+/- 1.00%). Filtrate was then returned to the limnocorrals via ½" PVC-braided vinyl tubing. After filtering the desired volume of water, the filter container housing the 53-µm mesh was removed in the field and its contents were washed into a 250-mL polyethylene bottle containing 100% methanol solution to immediately narcotize the zooplankton (Gannon et al, 1975).

All samples were then preserved in 5% sugar-formalin solution upon return to the lab. Counts and taxa identification were completed following procedures outlined in

Paterson et al (2013). Copepod nauplii and CI to CIII copepodites were not identified to species. Rotifers were enumerated and identified to species. Primarily, Balcer et al (1984) was used for zooplankton identification with the support of several other keys and guides to North America's freshwater zooplankton (Brandlova et al, 1972; Smith & Fernando, 1978; Witty, 2004).

Zooplankton biomass was also determined using length-weight regression estimates. Literature values informed zooplankton weights (Schindler & Noven, 1971) and equations used in determining biomass estimates (Lawrence et al, 1987; Malley et al, 1989). Length measurements were conducted for Lake 260 zooplankton using light microscopy and an accompanying microscope camera. Lengths were taken for n= 40 zooplankton per taxa and the mean was taken to determine average zooplankton taxa length. These lengths were then applied to the published regression equations to determine an estimate of total taxa biomass.

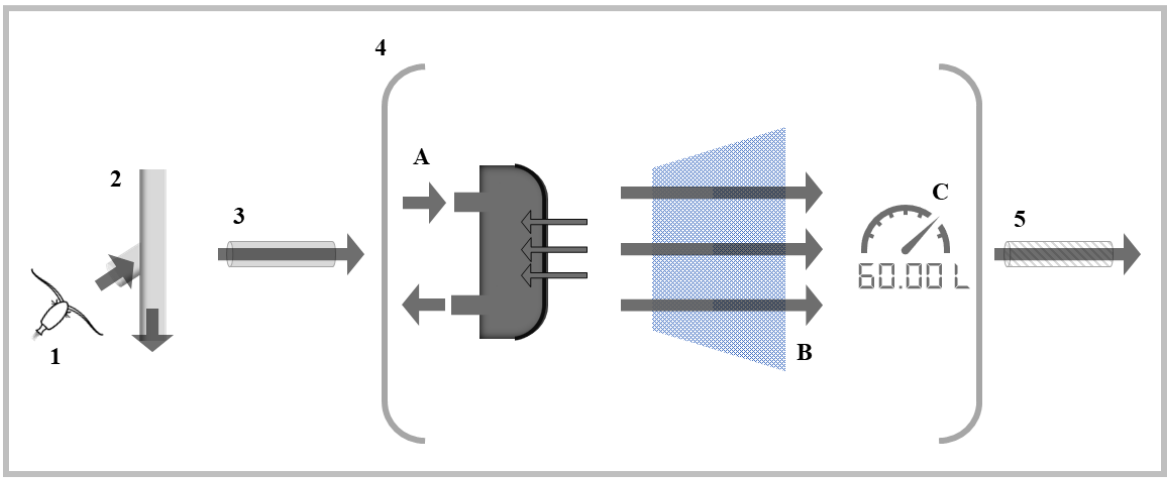

Figure 3.8: Schematic of the zooplankton pump used to quantitatively sample zooplankton assemblages within the BOREAL limnocorrals. Zooplankton located in the centre of the water column (1) were sampled using the "middle" sampling port located at a depth between 0.6 and 1.0 m (2). Zooplankton were pulled through a ½" linear low-density polyethylene tube (3) via the pump system (4). The pump system consisted of a 3.8 gallon per minute High-Flo Diaphragm pump (no impeller) powered by a 12-V deep-cycle marine battery (A), followed by the removable filter container made from 1-1/2" ABS fittings housing a 53-µm Nitex mesh to which zooplankton were captured (B), and a flow meter (Sotera Fill-Rite Digital Turbine Meter) to quantify water volume sampled (C), accurate to 0.01 L (+/- 1.00%). Filtrate was then returned to the limnocorrals via ½" PVC-braided vinyl tubing.

3.2.3 Macroinvertebrates

The macroinvertebrate community was assessed via: (a) floating emergence traps; and (b) by Ponar grab sampler at the end of the study.

3.2.3.1 Emergence Traps and Emerging Insects

One emergence trap was placed into each limnocorral (1 metre from the limnocorral wall). Emergence trap design was based on Cadmus et al (2016) and Malison et al (2010). Traps covered 75 cm^2 of the water's surface (**Figure 3.9**). A 250-mL polyethylene bottle filled with 50 mL of propylene glycol was used to preserve all trapped insects until time of sample retrieval. Traps were set directly following limnocorral installation to assess pre-exposure emergence. Before dilbit addition and for five days post-oil-addition, traps were removed as to not interfere with initial chemistry and mass-balance sampling, as well as observations of oil slick development and breakdown that occurred during the first week of the spill. Samples were retrieved, and the 250 mL bottle was swapped out with fresh preservative weekly for the duration of the study. Traps were also deployed in Lake 260 at three locations outside of the limnocorrals to assess enclosure effects; Reference-A (located behind the 180-L and 5.5-L limnocorrals), Reference-B (located behind the 2.9-L and 1.5-L limnocorrals), and Reference-C (located behind the 18-L and 82-L limnocorrals) (**Figure 3.6**). Emerging insect identification was conducted to family where possible using a key developed by Merritt et al (1978) with support from other sources (Jones et al, 2007).

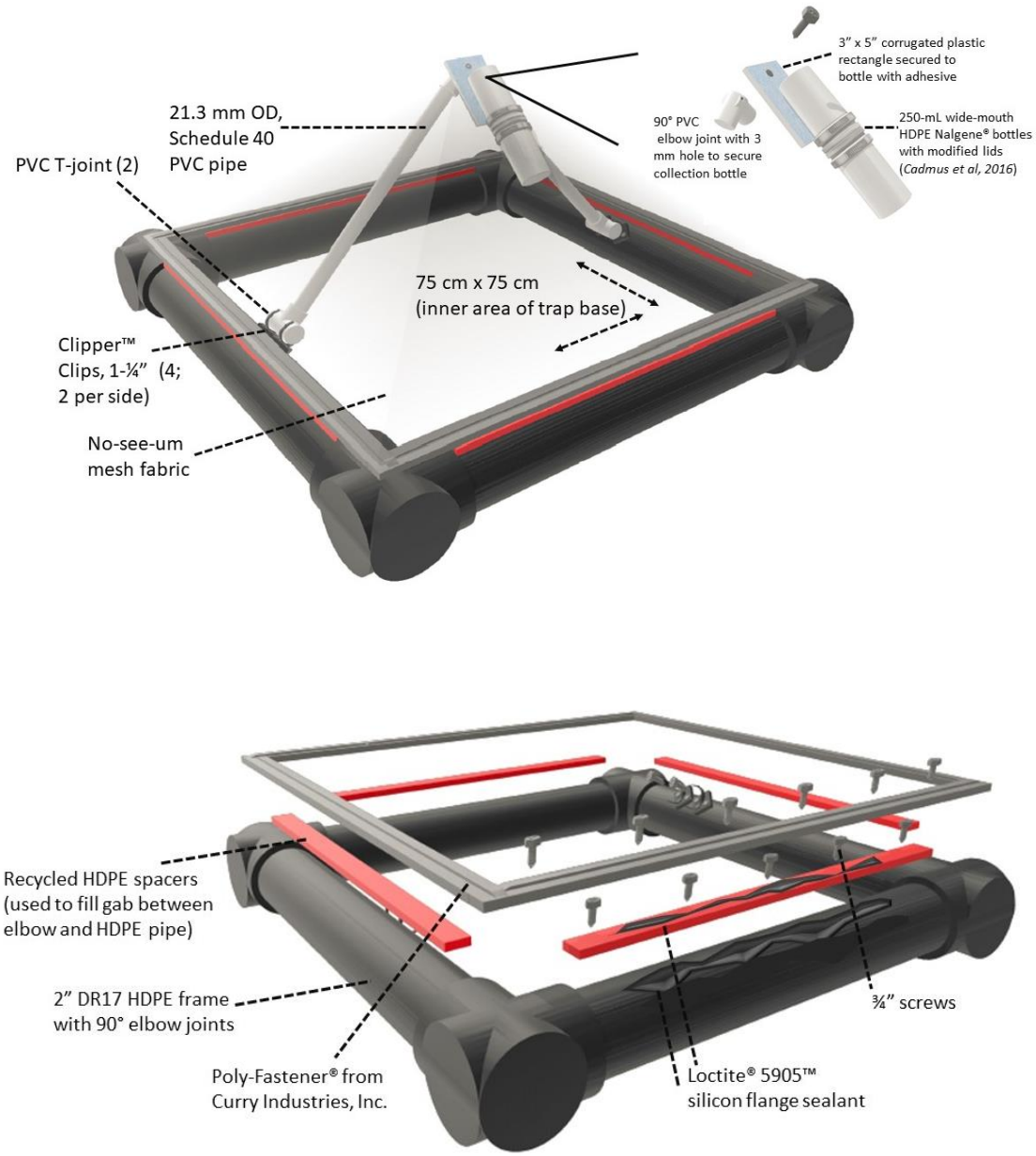

Figure 3.9: Diagrams of emergence trap used in passive sampling of the emerging insect communities within BOREAL limnocorrals.

3.2.3.2 Benthic Sampling and Benthic Macroinvertebrates

Sediment samples were taken on Day 84 via “petite” Ponar grab sampler (6” x 6”; 2.4 L). Benthic samples were taken only on the last day as a mass-balance assessing the fate and behaviour of the spilled dilbit was being completed throughout the study – sediment sampling via Ponar is highly disruptive and efforts were made to reduce disturbance of these analyses and removal of residual oil that may be present in the sediments. Three haphazardly selected sites were sub-sampled in each limnocorral and pooled to form a composite sample. Three additional samples (three composites) were taken in a randomly chosen limnocorral (1.5-L) to assess within-enclosure variability. Finally, three sites (NC – “near control”, FC – “far control”, BE – “behind enclosures”) were also sampled outside of the limnocorrals to address the impact of the enclosures themselves on the benthic communities (**Figure 3.6**). When conducting benthic sampling, caution was taken to select areas of sediment without visible tar mats or tar balls so as not to damage equipment being used and avoid sampling primarily sunken oil. This certainly introduced a degree of bias, which will be discussed later.

Ponar samples were emptied into a bucket, swirled to suspend any organic matter and to remove rocks and stones, and then sieved using a 355-µm sieve bag. The remaining sample was then washed into a sample bottle and preserved using 10% buffered formalin. Sediment samples were assessed based on the Canadian Aquatic Biomonitoring Network (CABIN) protocols for conducting aquatic biomonitoring. Samples were identified to the lowest possible taxonomic level (genus for most insects and genus or species for others).

An additional benthic survey of Lake 260 was conducted between 31st August and 7th September, 2017 by Joey Tonin (ELA staff). These data served to provide an understanding of the Lake 260 benthic community prior to limnocorral installation and the addition of diluted bitumen. As well, this provided ample time (8 months) for the benthic communities to recover from any disturbance brought about by the sampling. Ekman dredges were conducted in triplicate at three sites along the limnocorral area. The Ekman sampled an area of 231.04 cm^2 (15.2 cm x 15.2 cm) The depth ranged from 1.5 to 2 metres. Samples were sieved using a 500-µm screen and enumerated and identified to family using light microscopy.

3.2.4 Chitobiase Collection and Analysis

Chitobiase sampling was conducted at nine time points throughout the study – two pre-addition and seven post-addition – 24 to 48 hours following zooplankton sampling to compare chitobiase production rates with total zooplankton biomass. Water samples were collected from the "middle" sampling port (**Figure 3.7**) using a peristaltic pump (Waterra E-476; Hoskin Scientific Ltd.). Samples collected for the analysis of chitobiase production rate were immediately filtered into 250-mL Nalgene bottles using a 53-µm sieve to remove zooplankton and other organisms that may contribute to chitobiase production (**Figure 3.10**). Bottles were stored in a cooler in the dark at a temperature approximate to that of the lake temperature at time of sampling. At 0 hours, 1, 4, 8, and 24 hours, 20 mL subsamples were removed using a syringe and filtered into 20 mL glass scintillation vials using a 0.2 µm syringe filter to remove bacteria that may degrade the chitobiase enzyme prior to analysis. Filtered samples were then stored in a dark cooler at 4°C until transfer to a refrigerator at 4°C until analysis. A field blank was used

consisting of Milli-Q nanopure water (18MΩ-cm) in a 20-mL scintillation vial. The blank was left open for the duration of sampling.

Chitobiase analysis was conducted according to Sastri and Roff (2000), with procedures modified by MacKenzie (2011), and Randell (2015) in **Appendix C** (see also Hanson and Lagadic, 2005; Conley et al, 2009). QA/QC protocols are also outlined in **Appendix C**. Samples were analyzed using a SpectraMax M2 spectrofluorometer plate reader and degradation curves prepared using Microsoft Excel and R software. Prior to analysis, all samples were incubated with 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (MUF-NAG) substrate (>98% purity, TLC, Sigma-Aldrich Corporation) – chitobiase acts to cleave this compound, releasing the fluorescent MUF (4-methylumbelliferone) to which fluorescence is assessed via plate reader at 360 nm excitation and 450 nm emission. 100 µL samples were incubated with 100 µL of 0.3 mM MUF-NAG substrate in 0.15 M pH 5.5 citrate phosphate buffer for one hour, and the reaction stopped using 50 uL of 0.25 mM NaOH. Incubation was conducted in a 96-well black polystyrene microplate and samples were measured in quadruplicate relative to 4 blanks (MUF-NAG replaced with Milli-Q water). Results were compared to a calibration curve using 9 standards prepared from a stock MUF solution at concentrations of 0, 2, 4, 8, 16, 32, 64, 128, and 512 nM MUF. All results were expressed as nM MUF liberated per hour. Degradation curves were prepared using a log-transformed plot of time vs. MUF concentration to obtain a line of best fit. The slope of this line equals the enzyme degradation rate (which equals the production rate of chitobiase, under steady state).

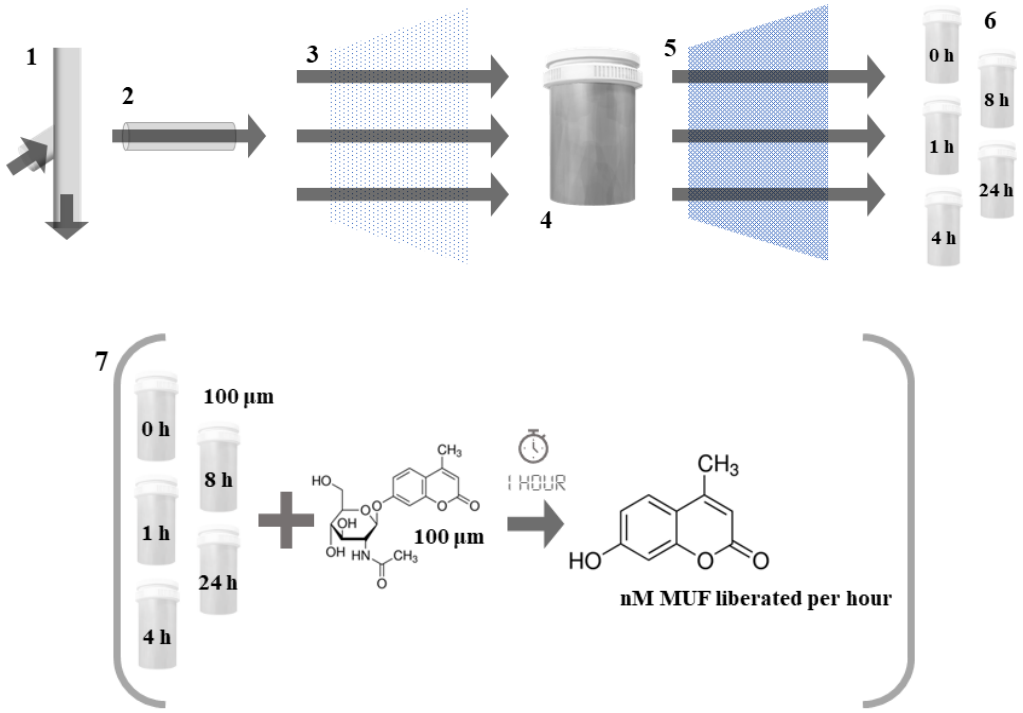

Figure 3.10: Key steps in the analysis of chitobiase in BOREAL enclosures. Water samples were collected at nine time points for chitobiase analysis to assess the ability of chitobiase production rate to predict invertebrate biomass in BOREAL limnocorrals. Water samples were collected from the "middle" sampling port (1) via peristaltic pump (2) and immediately filtered through a 53-µm mesh (3) to remove zooplankton and other organisms that may release chitobiase. The filtrate was collected in a 250-mL high density polyethylene Nalgene™ bottle (4). At times 0, 1, 4, 8, and 24 hours, a subsample was removed from the 250-mL bottle and filtered using a 20-µm syringe filter (5) into 20-mL glass scintillation vials (6). Samples were then incubated for 1 hour in MUF-NAG substrate and fluorescence was assessed using a spectrofluorometer (7).

3.2.5 Water Nutrients and Water Chemistry

Nutrients within the water column were assessed on a weekly basis (bi-weekly after July 17th, Day 27) for the same parameters assessed for the pilot-scale study. Some of these parameters will be used in assisting with the interpretation of oil impacts on the invertebrate communities and their food sources (top-down and bottom-up trophic interactions). Samples were collected using the "top" sampling port (**Figure 3.7**) and analysed by IISD-Experimental Lakes Area's Chemistry Laboratory following procedures outlined in Stainton et al (1997). In addition, pH, conductivity, temperature, and dissolved oxygen were measured weekly (Day -14 to Day 76) using a multiparameter probe (YSI Professional Plus, Xylem, Yellow Springs, Ohio). HOBO Pendant (MX2202) loggers were mounted within each limnocorral via the centre sampling port within the middle of the water column. The loggers remained for the duration of the study (Day -15 to Day 76) and recorded temperature and light intensity. An outline of all sampling events for biota and water is outlined in **Figure 3.11**.

3.2.6 Oil Additions

Cold Lake Winter Blend (CLB-W) diluted bitumen was added to each limnocorral based on nominal concentrations outlined in **Table 3.1**. Starting water volumes included in this table are based on a current tritium model and may change in future publications, thus altering dilution factors. As such, this thesis uses mainly volumes of oil and the volume:surface area ratio values in interpreting results. Dilbit was added using a diaphragm pump system and pumped onto the surface of the water within each limnocorral. Application occurred on the morning of 20th June between 08:00 and 11:00.

Prior to this, tritium (hydrogen-3), a radioactive isotope of hydrogen, was added to each limnocorral in known amounts. Subsequent modelling and estimation of tritium dilution within each limnocorral provided an estimate of limnocorral volume.

Table 3.1: Concentrations of dilbit added to BOREAL in-lake limnocorrals on June 20th 2018 and corresponding dilbit:water (v/v) dilution factors based on the approximate starting volume of water within each limnocorral estimated using tritium as a tracer to determine initial volume and subsequent leakage. Volume to Surface Area ratio indicates the amount of oil added relative to a limnocorral surface area of 78.5 m^2. The "Limnocorral" column defines the name designated for each treatment and is used when referring to each limnocorral treatment hereafter.

Limnocorral	Volume of dilbit added (L)	Starting volume of water (L)	Dilbit:water (v/v) dilution factor	Volume:surface area ratio (L/m^2)
180-L	179.78	94,100	1.91×10^{-3}	2.29
82-L	81.83	103,237	7.93×10^{-4}	1.04
42-L	42.34	107,558	3.94×10^{-4}	0.54
18-L	18.13	119,048	1.52×10^{-4}	0.23
5.5-L	5.51	95,855	5.75×10^{-5}	0.07
2.9-L	2.87	101,204	2.84×10^{-5}	0.04
1.5-L	1.45	105,714	1.38×10^{-5}	0.02
NC ("Near" Control)	0	105,714	0	0
FC ("Far" Control)	0	96,154	0	0

*Starting water volumes are based on a current tritium model and may change in future publications, thus altering dilution factors. As such, this thesis uses mainly volumes of oil and the volume:surface area ratio values in interpreting results.

3.2.7 Overview of Sampling Scheme

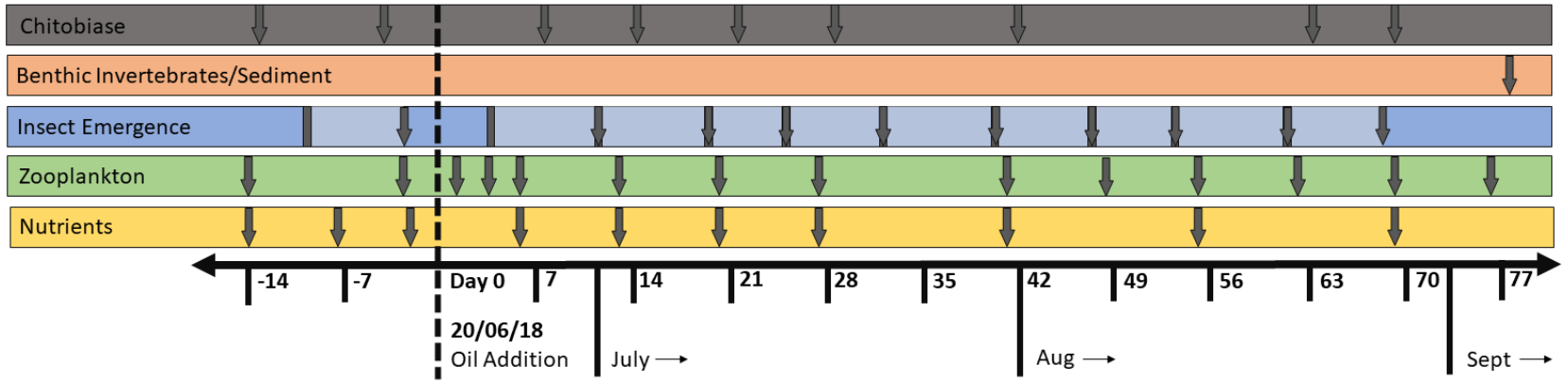

Figure 3.11: Summary of sampling events for nutrient chemistry, chitobiase, benthic invertebrate sampling of the sediments, zooplankton, and the start (solid bar) and end (arrow) of emergence sampling periods for the invertebrate component of the BOREAL 2018 study from June 6th, 2018 (Day -14) to September 4th, 2018 (Day 76).

3.3 Pleuston Bioassays

Water striders were first evaluated as part of the BOREAL project to inform the following water strider studies (Part 1 and Part 2 below). Water striders were enumerated within all limnocorrals between Day 55 (14th August) and Day 63 (22nd August). Enumeration occurred by observing limnocorrals for 5-minute periods. Counts were repeated to ensure striders were not being double counted. Upon arriving to the limnocorral, a wait of two minutes was employed as docking and exit of the boat may have disturbed the limnocorral and water surface and the 2-minute period would help ensure that any disturbance in counts was minimized. If no bugs were observed within the 5-minute window, a value of zero was assigned. Counts were conducted on the half of the limnocorral closest to the dock as observations would not have been accurate beyond this.

3.3.1 Part 1: Pilot-Scale Water Strider Semi-Field Bioassay

Concurrent with the BOREAL in-lake limnocorral study, water striders (Family: Gerridae) were exposed to fresh CLB-W dilbit in separate sets of small containers. The water striders studies were triggered based on observations made in the limnocorral study, which will be discussed later in this thesis. Test chambers consisted of 1700 mL of Lake 260 water contained within a 48.1-cm x 26.7-cm x 7.2-cm aluminum container and covered with a fine mesh to prevent escape by the bugs (**Figure 3.12**). CLB-W was added based on droplet mass. A total of 15 droplets were weighed, and the average weight was used in determining nominal volumes of oil added to each vessel

(**Appendix Table D.1**). Droplet size was calculated as 5.29 µg (+/- 0.24 µg) with a volume (based on a density of 0.9215 g/m^3 for fresh CLB-W; ECCC, 2018) of 5.74 µl. Treatments included 0, 1, 2, 4, 8, 16, and 32 drops of CLB-W (**Figure 3.13**). In comparison to the volume to surface area ratios provided in **Table 3.1** for the BOREAL study, the largest amount of oil added in the pilot-scale water strider assay was 1,600 times smaller than the amount of oil added to the 180-L limnocorral.

Water striders were collected from Lake 260 using a manta trawl (surface tow net) pulled between two boats. A total of 10 organisms were added to each test vessel prior to oil droplet addition and allowed to acclimate to the vessel for four hours. Oil was added via a small opening in the protective mesh to the centre of each vessel using a 10-mL syringe with a 25-gauge (0.5 mm x 25 mm) needle.

Striders were monitored at 0 hours, 0.25 hours, 0.50 hours, 1 hour, 2 hours, 4 hours, 18 hours, 24 hours, and every 24 hours thereafter until 96 hours (4 days). At each time point, each vessel was gently agitated for 10 seconds and activity was recorded, based on the following categories: (1) active with no visible impairment (i.e. water strider movement showed no visible difference to movement of water striders within the open lake community of Lake 260); (2) impairment (reduced movement/ability to move about the water/water surface relative to the lake community – i.e. the water striders were moving but had lagged movement or could not freely move without difficulty); (3) immobility (lack of any movement following agitation or organism has fallen to the bottom of the test vessel, indicating mortality). Mortality was not directly used as an

endpoint as it was difficult to distinguish a strider that was severely oiled, entirely limiting its ability to move, from a dead strider unless it had fallen to the bottom of test vessel.

Figure 3.12: Test vessel used in evaluating impacts of surface sheen exposure of Cold Lake Winter Blend diluted bitumen to surface-dwelling freshwater bugs (water striders; Family: Gerridae).

Control	1	2	4	8	16	32
0:0 (v/v, dilbit:water) 0 µL 0 µL/m^2	1:300,000 (v/v, dilbit:water) 5.74 µL 44 µL/m^2	1:150,000 (v/v, dilbit:water) 11.5 µL 90 µL/m^2	1:75,000 (v/v, dilbit:water) 22.9 µL 180 µL/m^2	1:37,500 (v/v, dilbit:water) 45.9 µL 360 µL/m^2	1:18,750 (v/v, dilbit:water) 91.8 µL 715 µL/m^2	1:9,375 (v/v, dilbit:water) 184 µL 1430 µL/m^2

Figure 3.13: Study design for water strider exposures to surface oil. 10 water striders (Family: Gerridae) native to Lake 260 at IISD-Experimental Lakes Area were exposed to fresh Cold Lake Winter Blend diluted bitumen ranging from 1 to 32 droplets in a semi-field study.

3.3.2 Part 2: Large-Scale Water Strider Semi-Field Bioassay

Following the 2018 pleuston study using water striders in small containers, concurrent with the BOREAL 2018 project, a scaled-up study was used to once again evaluate impacts of surface sheen on water striders. This study used the results from the first study to shape the study design and determine more appropriate treatments to understand the range of volumes at which impacts are observed. The pilot-scale volume to surface area ratios were too high and immobility was observed in among all water striders, except in the control vessel. As the oil volume could not be decreased (equipment could not produce a smaller volume of oil accurately), the next option was to increase the vessel size. As such, 1.7-m diameter tanks were chosen and the oil additions remained somewhat consistent, however decreasing the volume to surface area ratio due to increased vessel surface area. The highest treatment in the second study was between the two lowest treatments from the first study, allowing the assessment of this lower volume to surface area range.

Test chambers consisted of 226 L of Lake 240 water contained within a 1.7-m diameter tank and covered with a mesh to prevent disturbance by predators (**Figure 3.14**). CLB-W was added based on droplet mass (see above; **Appendix Table D.1**). Treatments included 0, 1, 3, 9, 27, and 81 drops of CLB-W (**Figure 3.15**)**.** In comparison to the volume to surface area ratios provided in **Table 3.1** for the BOREAL study, the largest amount of oil added in the large-scale water strider assay was 28,000 times smaller than the amount of oil added to the 180-L limnocorral.

Water striders were collected from Lake 240 using D-nets and kick nets from a dock situated on the shore of Lake 240. A total of 20 organisms were added to each test vessel prior to oil droplet addition and allowed to acclimate for four hours. Oil was added to the centre of each vessel using a 10 mL syringe with a 25-gauge (0.5 mm x 25 mm) needle. Striders were monitored at 0 hours, 0.25 hours, 0.50 hours, 1 hour, 4 hours, 8 hours, 24 hours, 32 hours, and 48 hours (study end; based on observed immobility and change between time points). At each time point, each vessel was agitated for 10 seconds by gently kicking the outside of the tanks and activity was recorded. Endpoints assessed followed the same categorization used in the pilot-scale water strider assay.

Figure 3.14: Large tanks used to conduct the large-scale exposure to fresh Cold Lake Winter Blend diluted bitumen evaluating impacts of surface sheen exposure to surface-dwelling freshwater bugs (water striders; Family: Gerridae). Tanks were 1.7 metres in diameter and contained 220 litres of water each. Oil added ranged from 0 uL to 460 uL (0 drops to 81 drops of oil).

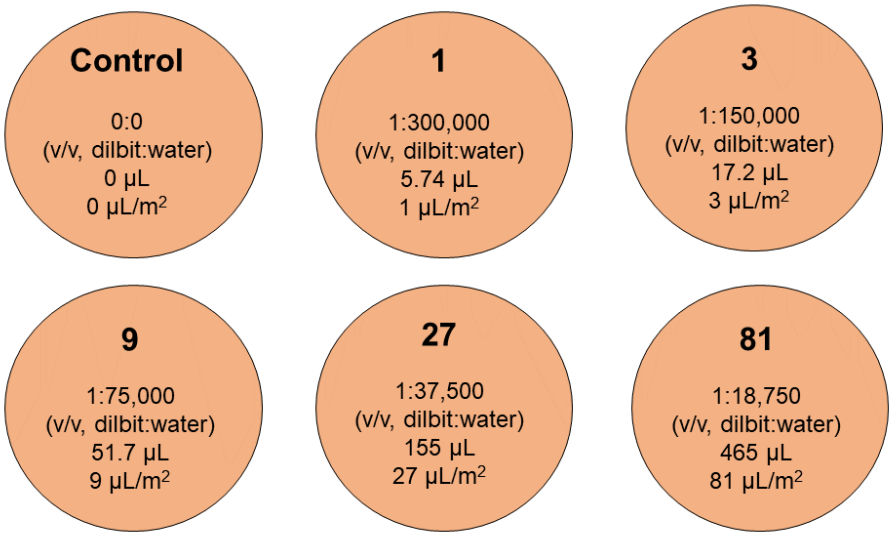

Figure 3.15: Study design for water strider exposures to surface oil. 10 water striders (Family: Gerridae) native to Lake 260 at IISD-Experimental Lakes Area were exposed to fresh Cold Lake Winter Blend diluted bitumen ranging from 1 to 81 droplets in a semi-field study.

3.4 Data Processing and Statistical Analysis

3.4.1 General

All multivariate and univariate analyses were conducted using R v. 3.5.3 and R studio v. 1.1.456 with *vegan* version 2.5-5 and *ggplot2* for data visualization. Invertebrate counts (zooplankton, emerging insects, benthic invertebrates) were converted to abundances based on the sampling method employed. Abundance data were log-transformed (natural log) prior to multivariate assessment or regression analysis, as needed. This was based on tests of normality using a Shapiro-Wilkes test on residuals of the regression output. All data was compiled using excel and processed/managed using R studio. Raw data sets are included in **Appendix A**.

All regression and statistical testing used an alpha level of 0.1 for abundance and count data and an alpha level 0f 0.05 for other data (chitobiase production rate). An alpha of 0.1 has been recommended for field-based studies previously (Hinds, 1984). Significance, although defined by these levels, was also assessed in the context of the raw data and contrasted with multivariate statistics; statistical outputs were therefore not the defining criteria of a significant response in the biological communities. As such, an alpha level of 0.1 was appropriate here given assessment of raw data and multivariate assessments alongside statistical hypothesis testing. Multiple comparisons adjustments (e.g. Bonferroni) were not conducted for these reasons, and to reduce the risk of Type II error.

3.4.2 Univariate Assessments

Univariate biodiversity metrics and community composition assessments included determination of species richness (total number of taxa observed), the Inverse Simpson Index ($1/\lambda$), Shannon's Index (H), Gini-Simpson (N_2), Species evenness (E_{21}), and overall community density (total number of organisms per unit; unit was dependent upon sampling device used). Densities and diversity metrics, individually, were linearly regressed against volume of oil added to statistically evaluate associated relationships.

In evaluating zooplankton dependence on chlorophyll *a* and abundance of phytoplankton within a size range of 1-20 µm, multiple linear regression was applied using these factors as explanatory variables. Phytoplankton within this size range were chosen as they are more edible than larger phytoplankton and accommodate the range in zooplankton food sizes (Barnett et al, 2007).

3.4.3 Non-Metric Multidimensional Scaling

Non-metric multidimensional scaling (NMDS) was used in evaluating benthic invertebrate communities on Days 76 and 77. NMDS was chosen as it can effectively represent abundance data relative to a suite of treatment (sites). NMDS reduces a complex data matrix into a 2-dimensional scale ordination of the data that is more easily interpreted. Absolute distances between objects (i.e. taxa or treatments) indicate similarities between them. NMDS functions by placing objects (i.e. taxa or treatments) on a 2-dimensional plane several times until the "stress" is reduced. Stress is a measure of how poorly objects are positioned – lower stress indicates that the ordination more accurately represents the data, with stress values below 0.1 being

optimal. To determine positioning of treatments and taxa, a data matrix was determined using the Bray Curtis dissimilarity matrix to determine dissimilarity between taxa – Bray Curtis dissimilarity is the prime choice for abundance or count data, as with the benthic data analysed here. Dissimilarity is used in developing a data matrix by which the NMDS is developed. If a given treatment has the same abundance of two species, dissimilarity is zero. If two species do not co-occur, they are given a dissimilarity of 1, and therefore may appear far apart in the ordination. The NMDS was run 100 times until stress reached a minimum associated with the final NMDS configuration.

3.4.4 Principal Response Curves

To understand changes in zooplankton and insect densities associated with time and treatment, principal response curves (PRC) were generated based on methods and suggestions outlined in Van den Brink & Ter Braak (2009) and Lawrence et al (2018). The PRC is a strong tool in evaluating impacts of a stressor on a biological community, condensing multivariate data to a simple figure with a curve developed based on treatment and species-level response, and how the community changes over time. Van den Brink et al (1999) outline this modified redundancy analysis (RDA), with Lawrence et al (2018) providing a detailed overview of how to interpret PRCs. A PRC is a representation of species abundance within a given treatment defined as the sum of abundance in the control, treatment effects related to time, and an error metric (Lawrence et al, 2018). Three values determine the PRC and include C_{dt} (coefficient of community response/effect of treatment at a given time point – i.e. the slope of the regression), b_k (species score used to interpret species-level response to a particular stressor), and t_{dtk} (the effect of the stressor/treatment at a given time point on a species)

(Van den Brink et al, 1999). All treatment-related responses were assessed relative to the control (i.e. FC and NC).

In interpreting the PRC, both the species scores (b_k) and the coefficient of community response (C_{dt}) must be considered together. The magnitude of b_k indicates the agreement between what is being observed at the community level and how the one taxon is responding relative to the entire community – taxa with a low or near-zero b_k show no or very little response to the treatment. The magnitude of C_{dt} indicates the degree of response to the treatment relative to the control – greatly negative values are indicative of a greater community response. If C_{dt} and b_k have opposite signs (i.e. positive and negative), the taxa abundance is decreasing or lower in a given treatment, relative to the control. If they have the same sign, species abundance is increasing or greater in a given treatment, relative to the control (Auber et al, 2017).

In this thesis, only the first PRC axis is reported and is evaluated based on contribution to explaining total variance of the redundancy analysis for (a) conditional variance – the variance accounted for by time, and (b) constrained variance – the variance accounted for based on the interaction of treatment with time. For this study, the PRC demonstrated changes in the seven treatments relative to the control (FC), over time and provided more resolution in understanding how dilbit directly influenced these communities. To note, the FC limnocorral did not have species-specific data generated for it on Day -3 as a result of poor sample integrity (sample was not appropriately preserved and degraded between time of collection and enumeration). As such, only one pre-spill time point (Day -14) is included in the curve.

For both zooplankton and emergence data sets, total abundance (organisms per L or organisms per m^2) was used in developing the model in R studio. As such, the curve generated is a multivariate output in terms of community response based on total abundance of each invertebrate taxon.

3.4.5 Other Data

Environmental data were evaluated using correlation matrices and a combination of principal component analysis and redundancy analysis.

Principal component analysis (PCA) was conducted at two time points (Day -3 and Day 69) to evaluate relationships between several environmental variables and the amount of oil added to each limnocorral. The two time points were chosen to understand how environmental variables changed throughout the study. A number of environmental variables showed some degree of correlation so correlation matrices were developed to assess this; if a correlation exceeded $R^2 = 0.8$, one of the two parameters was excluded from the analysis to simplify the data set and PCA (**Appendix Figure B.2**; **Appendix Figure B.3**). Correlation matrices were developed using pairwise Pearson product-moment correlation. PCA was conducted in R Studio using the prcomp() function. Variables were log-transformed prior to PCA and correlation analysis to standardize each variable.

Redundancy analysis (RDA) was performed on absolute densities (org/L) of dominant zooplankton taxa and groups relative to each limnocorral and was constrained by a subset of the environmental variables (chlorophyll *a*, pH, DOC, DO, temperature).

Variables were log-transformed prior to analysis. RDA was conducted to evaluate correlation between environmental variables and dominant zooplankton taxa at three time points of interest: Day -3, Day 13, and Day 69. The Day -3 and Day 69 time points capture the start and end of the BOREAL study, whereas the Day 13 time point was chosen as it shows a distinct change in the biological communities. RDA was performed in R Studio using the rda() function from the *vegan* package. Permutation tests were conducted on RDA outputs to assess model significance.

Chitobiase data non-detects (i.e. below method detection limits) were changed to a value of zero as negative values for chitobiase production rate are not sensical. Other chitobiase analyses are outlined in **Appendix D**.

Chapter 4
Results

The results presented herein include an in-depth review of the three main components of this thesis, including: (a) impacts on zooplankton communities, (b) impacts on benthic invertebrates, and (c) impacts on pleuston and emerging insects. Environmental parameters, including DO and chlorophyll a, were evaluated in the context of oil volume added. Additionally, they were used to explain some of the changes observed in biota (zooplankton and benthics).

Zooplankton were evaluated in both the pilot-scale mesocosm study (2017) and the large-scale limnocorral study (2018) based on community composition, abundance, and total biomass. Benthic observations include a lake survey conducted in Fall 2017 prior to the installation of the limnocorrals, as well as data collected on Day 76 and 77 of the BOREAL 2018 in-lake limnocorral study. Included is an analysis of the benthic communities outside of the limnocorrals to provide an understanding of limnocorral effects (i.e. to what degree did the limnocorrals deviate from the open lake environment?). Impacts on emergence are contrasted with the benthic data to discern if oil has an impact on specific life stages (i.e. are impacts being observed during emergence/is emergence the limiting factor in fecundity and future reproductive output or are impacts, if any, occurring during larval stages within the sediments?).

4.1 Zooplankton: Pilot-Scale Mesocosm Study

The mesocosm study evaluated the effect of added dilbit on the abundance and community composition of zooplankton at just one time point (Day 11). Other communities (periphyton, phytoplankton, microbial communities) were also evaluated in the pilot-scale mesocosm study and their response is outlined in Cederwall et al, 2019.

Following addition of the oil, spikes in PACs within the water column were observed, peaking at 1.25 ug/L and 4.59 ug/L in the low and high treatments, respectively (Stoyanovich et al, 2019a). Oil submergence occurred on Day 8, likely attributed to a rainfall event prior to this and the loss of volatile components aiding in the increase in viscosity and density (Stoyanovich et al, 2019a). Nutrients and other water chemistry parameters showed some treatment-dependent behaviour. Dissolved oxygen was markedly lower in the high treatment, ranging from 7.95 mg/L (93.8% saturation) to 7.1 mg/L (76% saturation) on Day 11. DO in the control and low treatments on Day 8 were 8.84 mg/L (93.6%) and 8.45 mg/L (91.8%), respectively. Chlorophyll *a* also dropped in the high treatment, with a 46% reduction on Day 4 (relative to the control) (Cederwall et al, 2019).

The sampled zooplankton community in Lake 240 consisted mostly of six taxa: the cladocerans *Holopedium glacialis*, *Diaphanosoma birgei*, *Bosmina* cf. *longirostris*, *Daphnia mendotae*, and calanoid copepods *Diaptomus minutus* and *Epischura lacustris* (**Figure 4.1**). Cyclopoids *Mesocyclops edax* and *Diacyclops thomasi* were not observed in the Lake 240 sample; however, they were present in the tanks on Day 11 (**Table 4.1**; richness of 6 in Lake 240 sample vs richness of 8 in the mesocosms). These species

may have either been juveniles at time of L240 sampling and therefore not identified to species (nauplii and CI to CIII) or were present in the sediment/water added to microcosms during the establishment of the experimental units. L240 composition and abundance reported here are similar to historic L240 data, as collected at centre buoy in summer; the species reported here were previously found in L240 and species dominance has not changed (i.e. pelagic zooplankton mainly consist of those reported above in both historic L240 sampling and in samples collected for the BOREAL pilot study). This is based on samples collected through IISD-ELA's Long-Term Ecological Research (LTER) database (unpublished data, M. Paterson).

On Day 11, overall zooplankton densities declined with dilbit exposure (**Figure 4.1**). Species diversity (based on two diversity metrics; Gini-Simpson and Shannon Diversity) and evenness were greatest in the low exposure tank, and least in the high-oil tank (**Table 4.1**). *H. glacialis* was the dominant species in all samples except for the high tank, where its population density was $0.117 \cdot L^{-1}$ (5.7% of sample) relative to densities of 4.84 (33.4% of sample) and $1.90 \cdot L^{-1}$ (20.2% of sample) in control and low treatments, respectively. *B. longirostris* was the dominant taxa within the high tank, representing 54% of crustacean zooplankton at a density of $1.12 \cdot L^{-1}$.

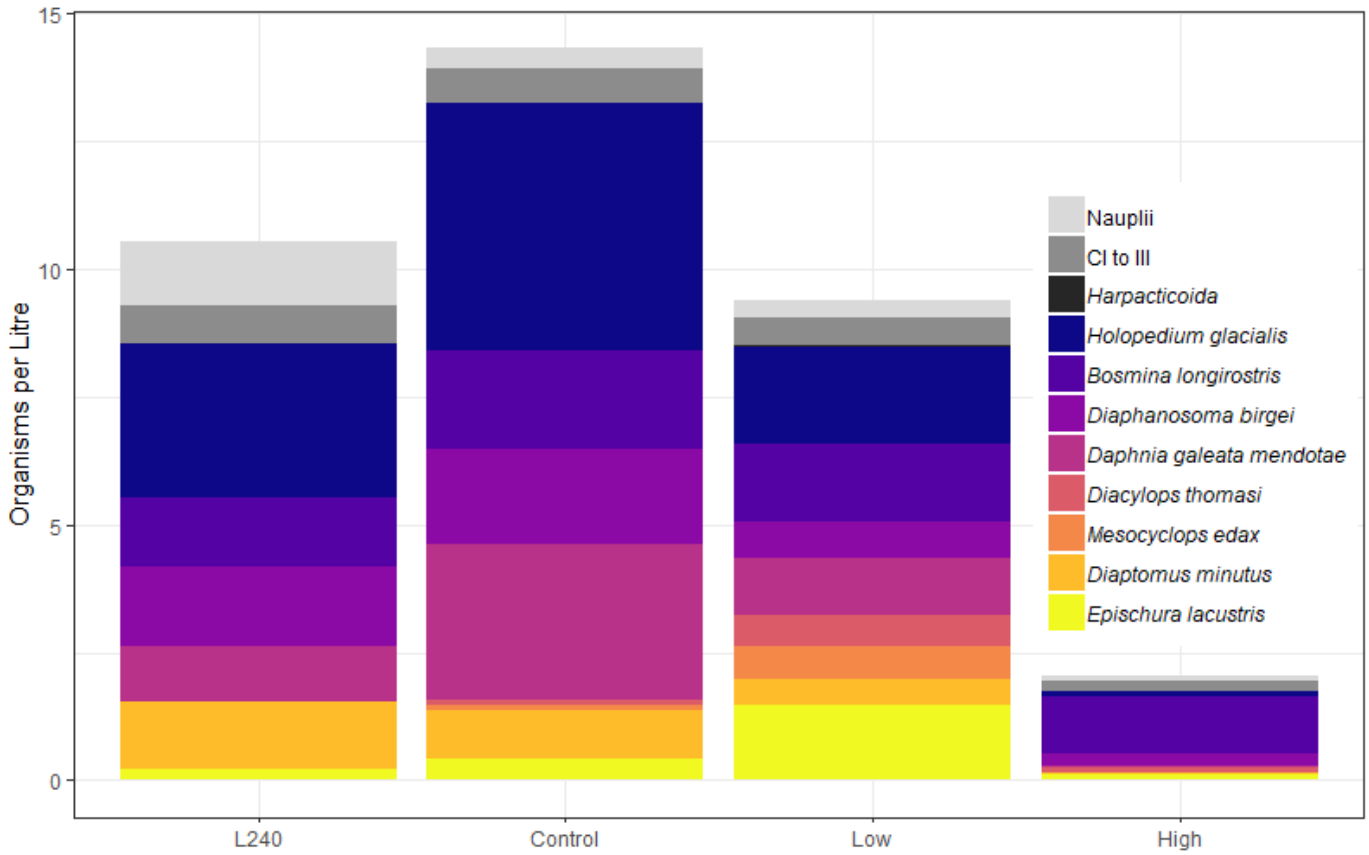

Figure 4.1: Community density (number of organisms per litre) for several crustacean zooplankton taxa collected post-treatment (Day 11) in control, low, and high microcosm tanks. The zooplankton data from Lake 240 used to amend microcosms pre-treatment (Day -1) is provided for comparison.

Table 4.1: Diversity metrics, including Simpson's index, Shannon Wiener diversity index, evenness, and community density (number of organisms per litre) for Control, Low, and High tanks at Day 11. Lake 240 data (Day -1) from the zooplankton addition sample are provided for baseline comparison.

Diversity Index	L240	Control	Low	High
Species Richness	6	8	8	8
Shannon Diversity, N_1	5.03	5.32	7.19	3.62
Gini-Simpson, N_2	4.49	4.39	6.58	2.30
Evenness (E_{21})	5.67	5.05	5.74	4.55
Community Density (no. per L)	10.53	14.51	9.40	2.05

4.2 In-Lake Limnocorral Study

A total of nine limnocorrals (seven treated, two controls) were monitored in this study; however, the near-control (NC) has been excluded from analysis in this thesis. Prior to the addition of dilbit, four white suckers (*Catostomus commersonii*) were observed in this limnocorral. Attempts to remove them were not successful, with only one captured. As white sucker are benthivores and given the substantially high relative density of white sucker within the limnocorral (as compared with historical Lake 260 white sucker data with white sucker biomass was reported between 20 and 55 kg/ha in Lake 260 between 1999 and 2005 (Kidd et al, 2014)), the consequences of predation and a top-down trophic cascade within this limnocorral would severely hinder its ability to serve as an effective control. See **Appendix Figure B.1** for a comparison in total zooplankton abundance with the NC included.

4.2.1 Environmental Data

Environmental parameters were evaluated weekly to biweekly throughout the study duration. Nutrients and other water chemistry parameters varied with the introduction of oil into the limnocorrals. DO was markedly lower following oil addition (Day 1) to the end of the study (Day 76) in the higher treatment, with the 180-L limnocorral having lowest DO saturation among all limnocorrals at almost every time point assessed. DO reached a minimum in this limnocorral between Day 27 and Day 34 and remained relatively low for the duration of the study (**Figure 4.2**). There is a treatment-dependent response in which DO decreases with increasing volume of oil added. pH also showed no treatment-based difference (**Figure 4.3**). Total dissolved phosphorous showed no differences

based on treatment applied, although total dissolved nitrogen was higher in the 180-L limnocorral prior to and following oil addition (**Figure 4.4**; **Figure 4.5**). Dissolved organic carbon was substantially more elevated in the 180-L limnocorral, with this rise occurring directly following oil addition (**Figure 4.6**). The 82-L and 42-L limnocorrals also showed elevated levels of DOC at the end of the study relative to the lower treatments and control, exceeding 550 µM DOC. DIC was also evaluated in the 180-L limnocorral immediately post-spill but was comparable with DIC concentrations in the FC and other treatments at the end of the study (**Figure 4.7**).

Chlorophyll *a* also showed changes attributed to volume of oil added (**Figure 4.8**). Chl *a* peaked in the 180-L limnocorral on Day 13, with a poorly significant ($p = 0.031$) response observed. Following this spike in chl *a*, values returned to near pre-spill concentrations.

Principal component analysis (PCA) pre-spill (Day -3; June 18th, 2018) and post-spill (Day 69; August 28th, 2018) for selected environmental variables are included in **Figure 4.9**. Some parameters showed a strong (usually non-significant) correlation among each other (**Appendix Figure B.2** and **Appendix Figure B.3**). Six principal components were required to explain 95% of the variance pre-spill, with five principal components required for the post-spill environmental variables. Pre-spill, PC1 and PC2 explained 33.7% and 28.3% of the variance, respectively. Post-spill, PC1 and PC2 explained 34.9% and 26.7% of the variance, respectively. Initially, there was little distinction among limnocorrals (approximately 18 days following the establishment of the limnocorrals) based on the PCAs. Conductivity and TDS were the main contributors

to PC1, pre-spill. 69 days post-spill, the main contributor to PC1 was TDN, agreeing with the TDN data below. There is, also, no clear distinction of physical and chemical parameters within the limnocorrals attributed to volume of oil added based on the PCA.

Temperatures within the limnocorrals peaked in mid-July and began falling in early August and into September (**Figure 4.10**). Average daily temperature changes ranged between 1 and 3˚C at the time of oil addition (18th June to 4th July; **Figure 4.11**). The FC showed elevated temperatures relative to the other limnocorrals and may be attributed to its position in Lake 260, less sheltered than the other limnocorrals. This contrasts with the 180-L limnocorral, which was furthest from the FC limnocorral and nearest the shore, with the lowest temperatures reported throughout the study, both pre- and post-spill. Light intensity also reflects these differences, with light intensity appearing to be elevated in the FC for the study duration (**Figure 4.12**). Average light intensity from Day -2 (18th June) to Day 14 (4th July) is also higher in the FC, and lowest in the 180-L and 1.5-L limnocorrals, with the latter two limnocorrals positioned closest to the shore (**Figure 4.13**).

Rainfall from 20th June, 2018 (Day 0) to 4th July, 2018 (Day 14) is reported in **Figure 4.14.** Rainfall for the entire study duration is reported in **Appendix Figure B.4**. Major rainfall events occurred on the 27th and 29th of June and were the first reports of rainfall following the addition of oil to limnocorrals.

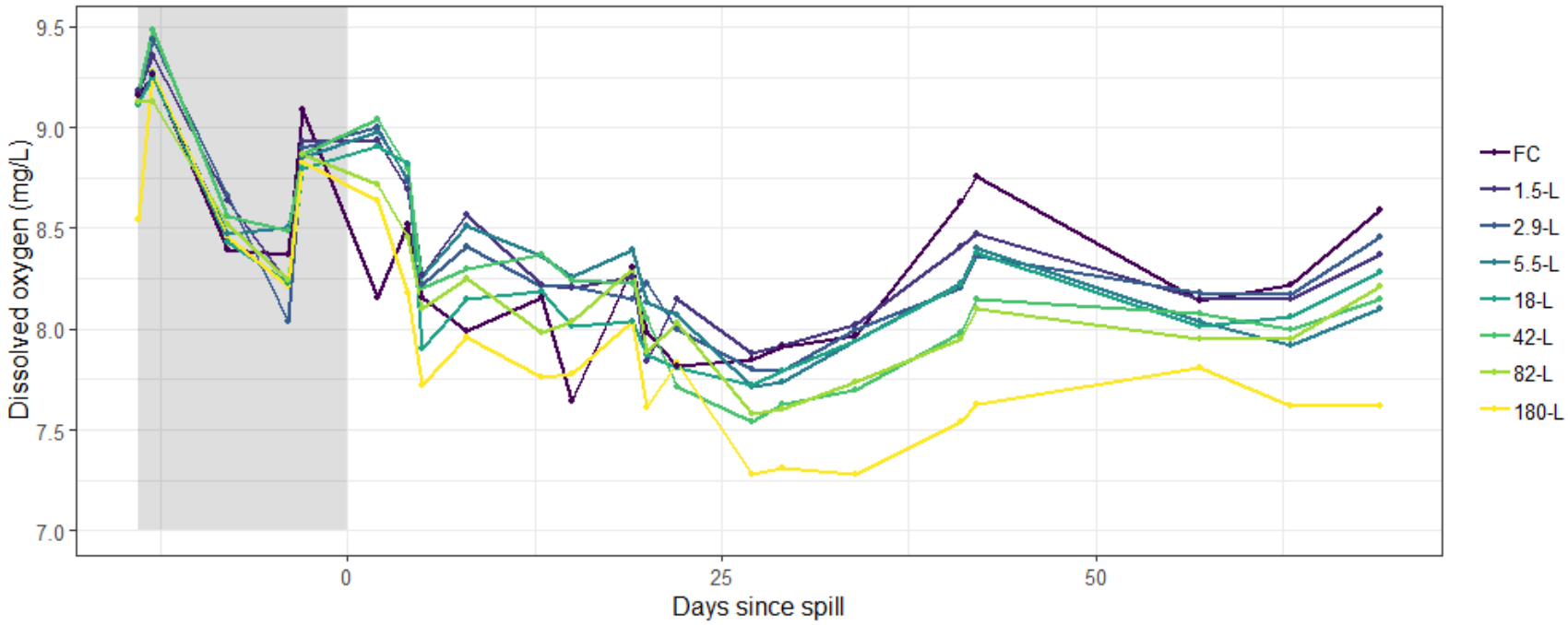

Figure 4.2: Dissolved oxygen (DO) concentrations within eight limnocorrals treated with varying volumes of dilbit. Shaded region represents the time before dilbit application. DO was monitored from Day -14 (6th June, 2018) to Day 69 (28th August, 2018).

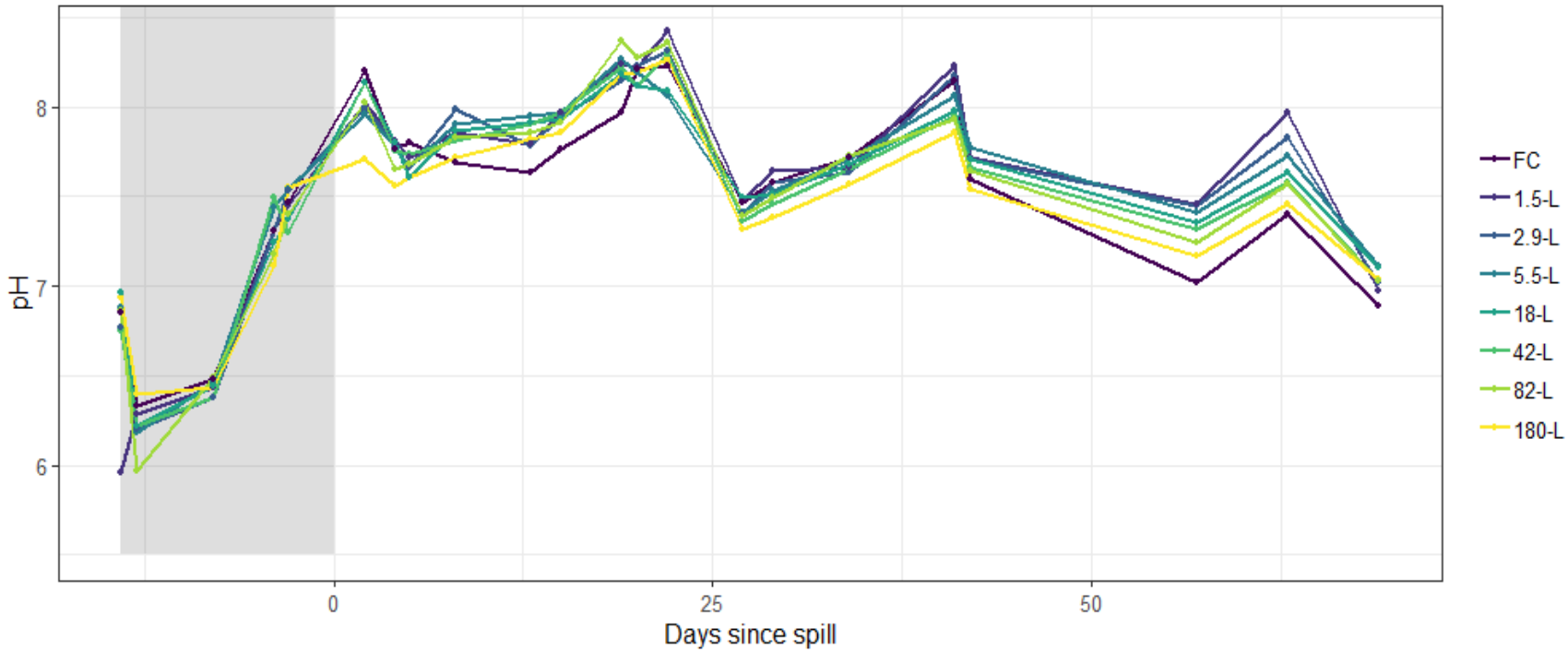

Figure 4.3: pH values within eight limnocorrals treated with varying volumes of dilbit. Shaded region represents the time before dilbit application. pH was monitored from Day -14 (6th June, 2018) to Day 69 (28th August, 2018).

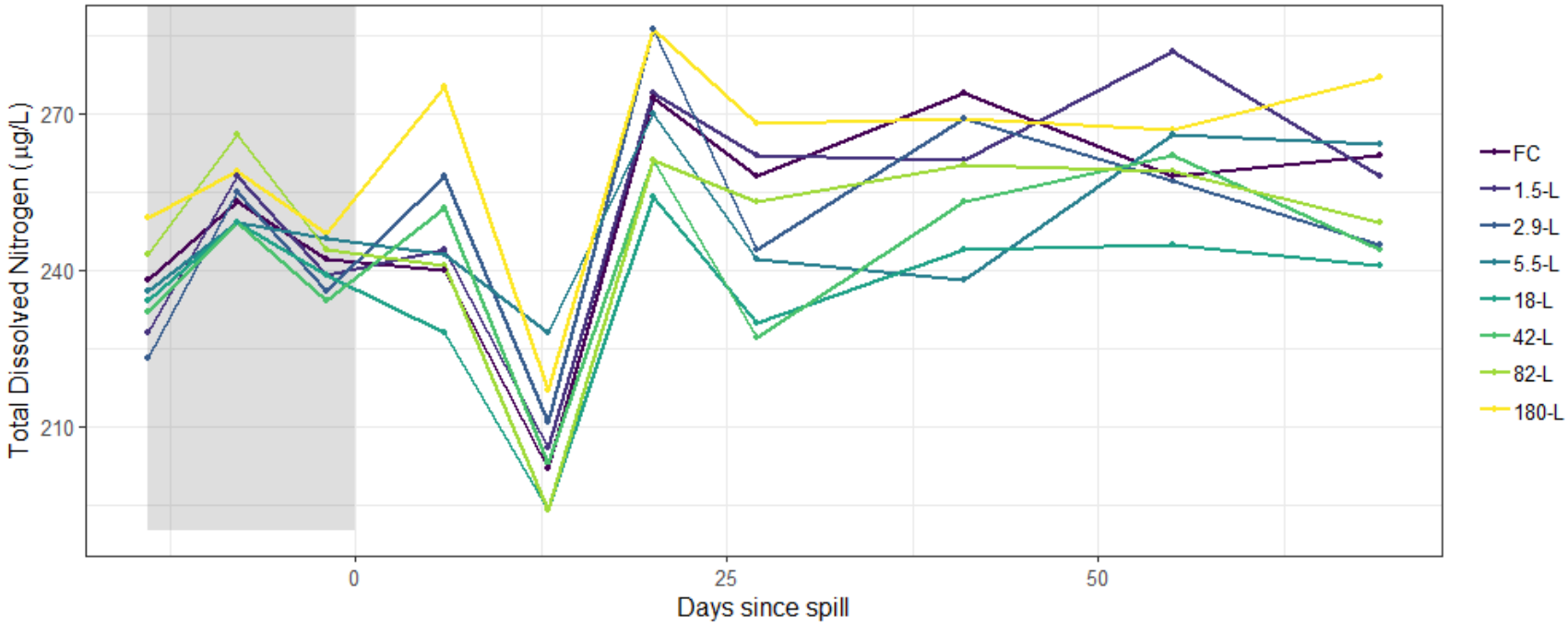

Figure 4.4: Total dissolved nitrogen (TDN) within eight limnocorrals treated with varying volumes of dilbit. Shaded region represents the time before dilbit application. TDN was monitored from Day -14 (6th June, 2018) to Day 69 (28th August, 2018).

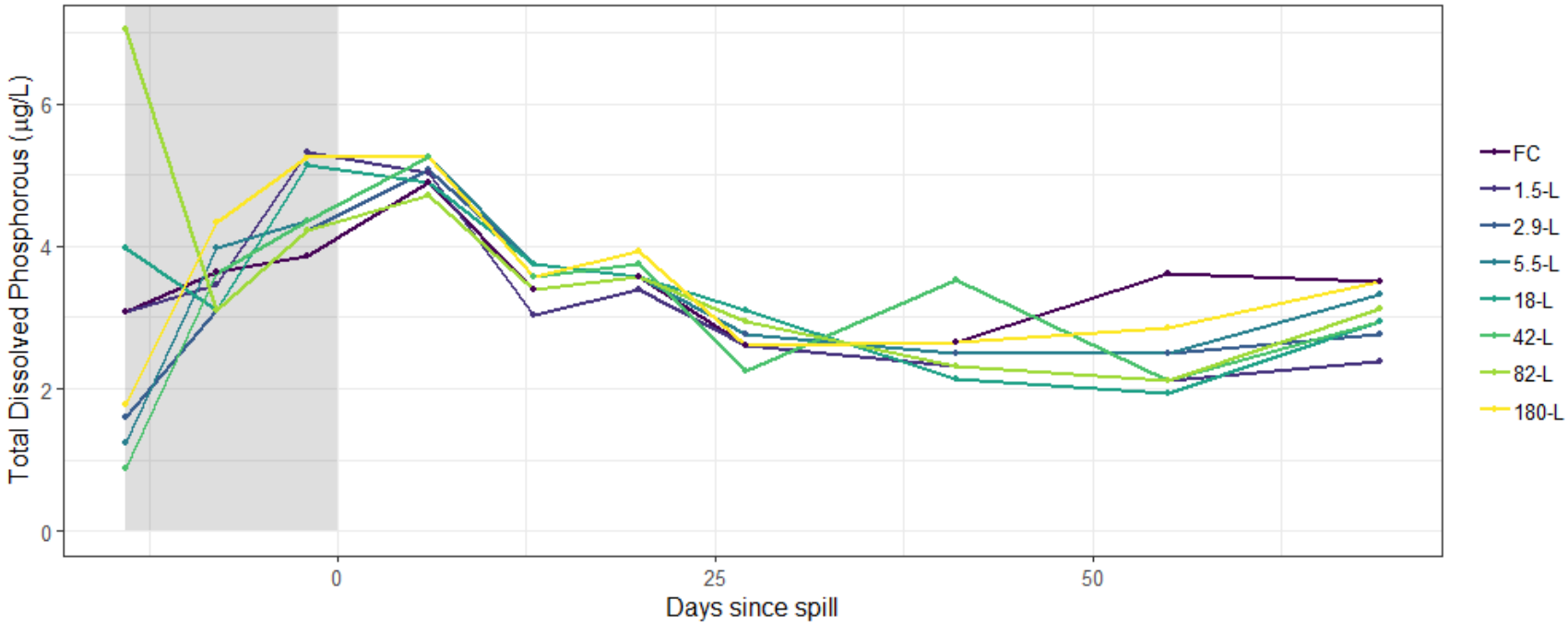

Figure 4.5: Total dissolved phosphorous (TDP) within eight limnocorrals treated with varying volumes of dilbit. Shaded region represents the time before dilbit application. TDP was monitored from Day -14 (6th June, 2018) to Day 69 (28th August, 2018).

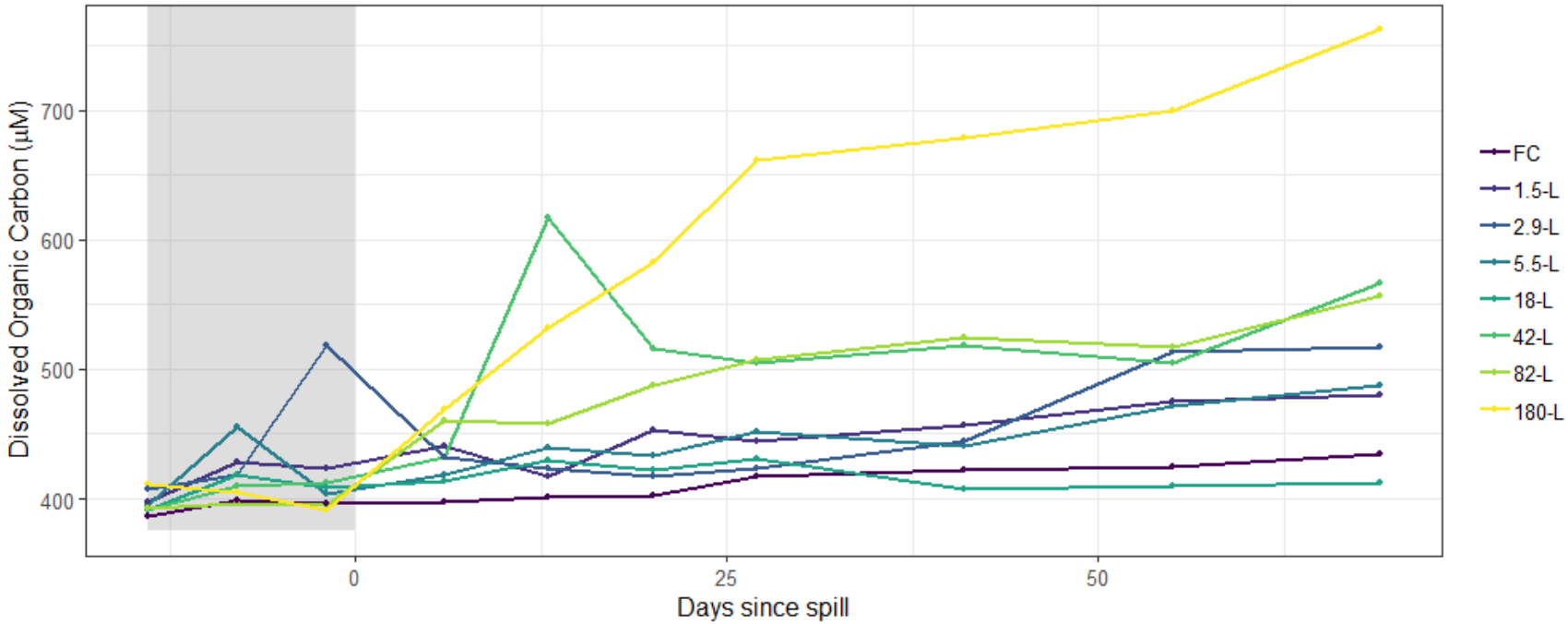

Figure 4.6: Dissolved organic carbon (DOC) content within eight limnocorrals treated with varying volumes of dilbit. Shaded region represents the time before dilbit application. DOC was monitored from Day -14 (6th June, 2018) to Day 69 (28th August, 2018).

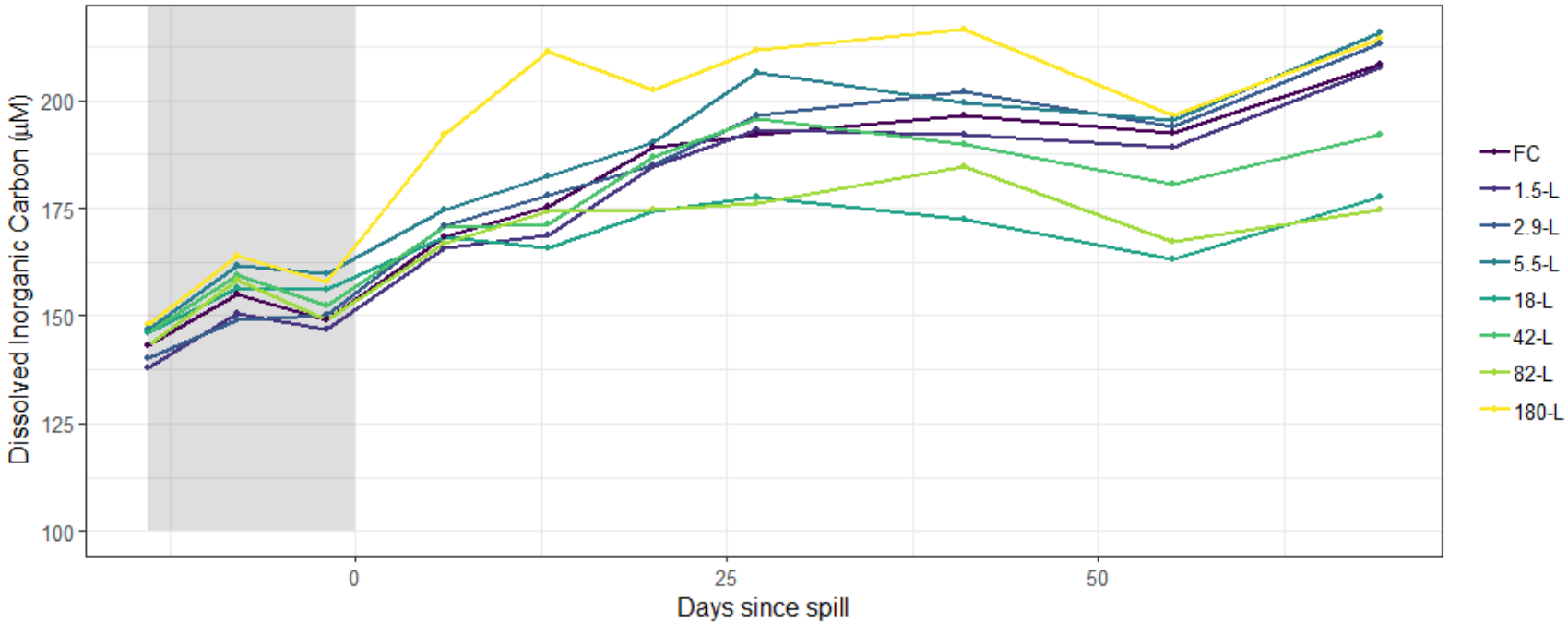

Figure 4.7: Dissolved inorganic carbon (DIC) content within eight limnocorrals treated with varying volumes of dilbit. Shaded region represents the time before dilbit application. DIC was monitored from Day -14 (6th June, 2018) to Day 69 (28th August, 2018).

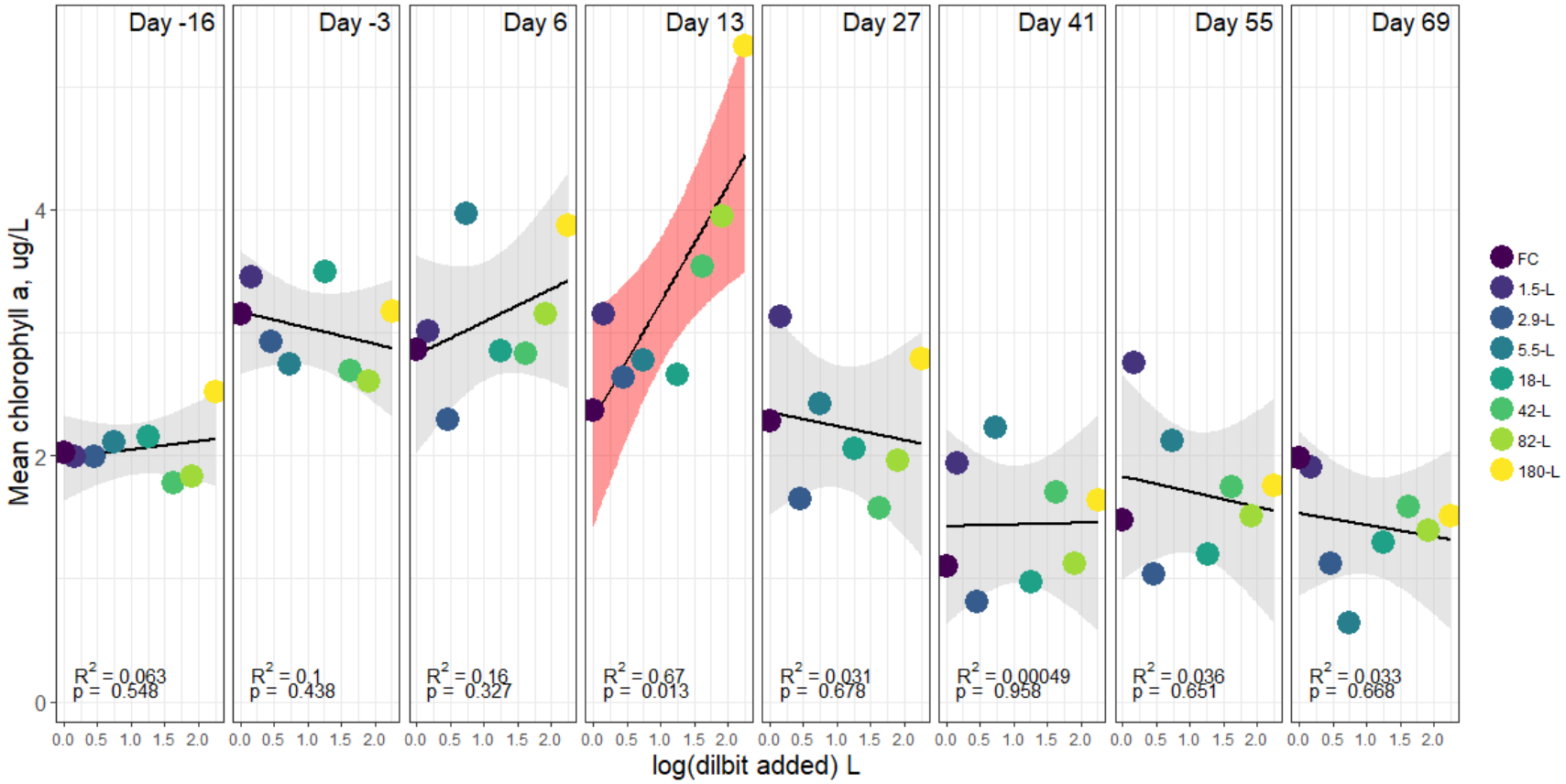

Figure 4.8: Chlorophyll *a* values linearly regressed against volume of dilbit added, log-transformed, at eight time points throughout the 90-day study. Chlorophyll data was collected from Day -14 (6th June, 2018) to Day 76 (4th September, 2018). Black lines represent the linear model, with shaded regions indicating the confidence intervals associated with the linear model (95%). Significance (α = 0.10) is observed on Day 13 (p = 0.013) with a coefficient of determination of 0.67 (highlighted in red). Data collected and analyzed by J. Cederwall.

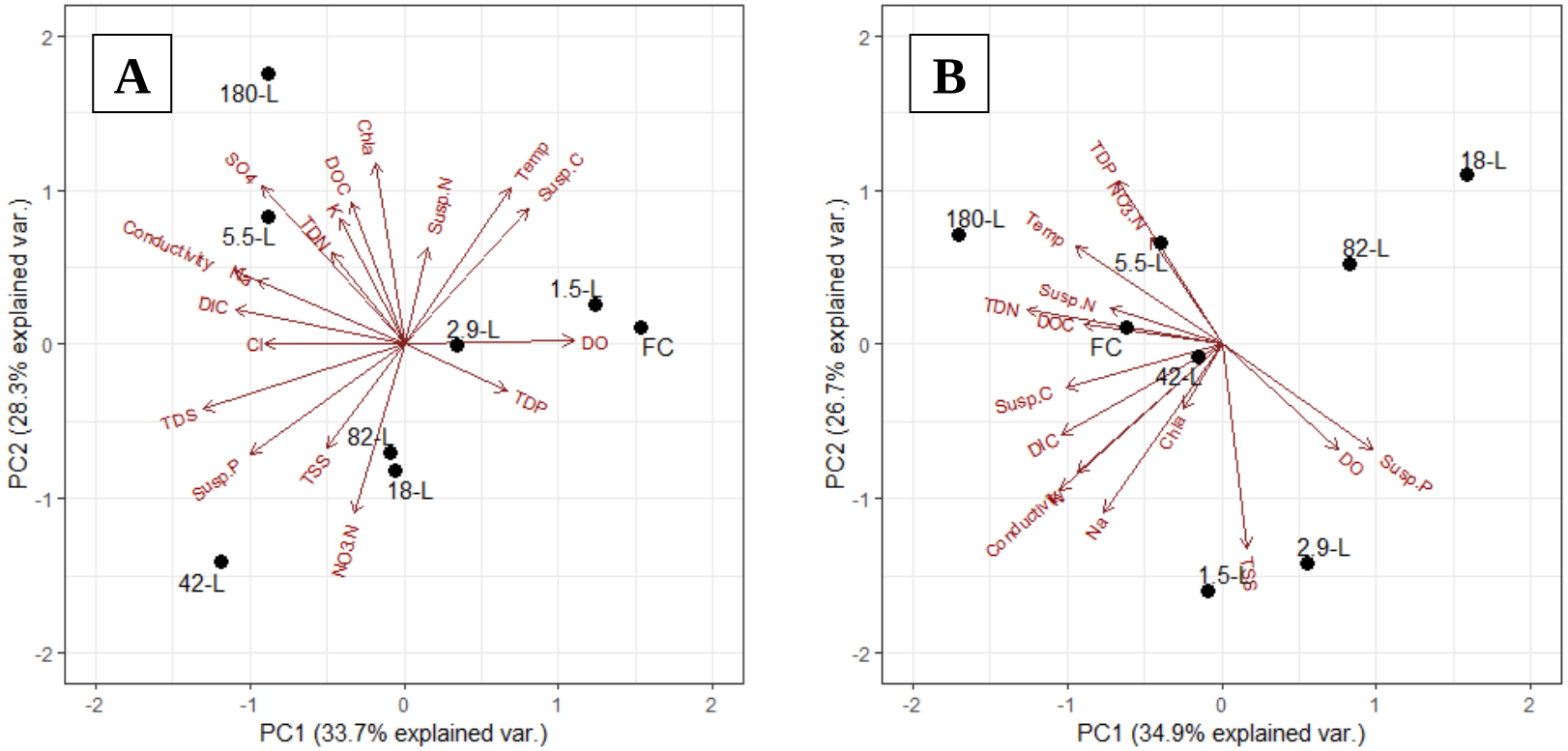

Figure 4.9: Principal component analysis (PCA) biplot for (A) pre-spill (Day -3; 18th June, 2018) and (B) post-spill (Day 69; 28th August, 2018) nutrient and water chemistry parameters assessed for the eight limnocorrals in the BOREAL 2018 study. These parameters include: chlorophyll a (Chla), anions (Cl^-, SO_4^{2-}), cations (K^+, Na^+), conductivity, dissolved inorganic carbon (DIC), dissolved oxygen (DO), dissolved organic carbon (DOC), nitrate (NO_3^-), suspended carbon (Susp.C), suspended nitrogen (Susp.N), suspended phosphorus (Susp.P), total dissolved nitrogen (TDN), total dissolved phosphorus (TDP), total dissolved solids (TDS), temperature (Temp), and total suspended solids (TSS).

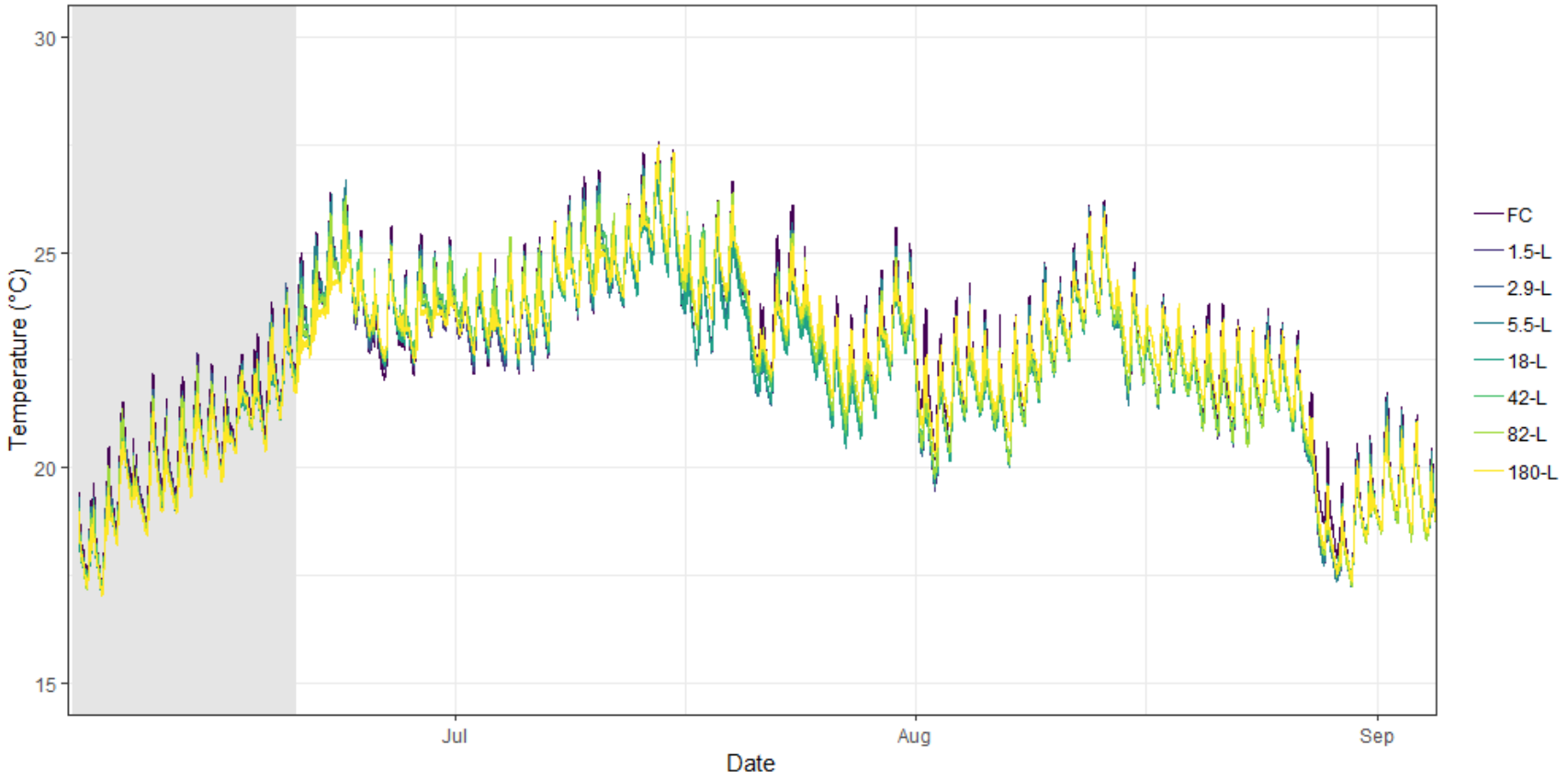

Figure 4.10: Temperature from Day -15 (5th June, 2018) to Day 76 (4th September, 2018) reported within each limnocorral via HOBO loggers positioned mid-water column. Shaded area represents the time pre-spill Day -15 to Day 0 (20th June, 2018).

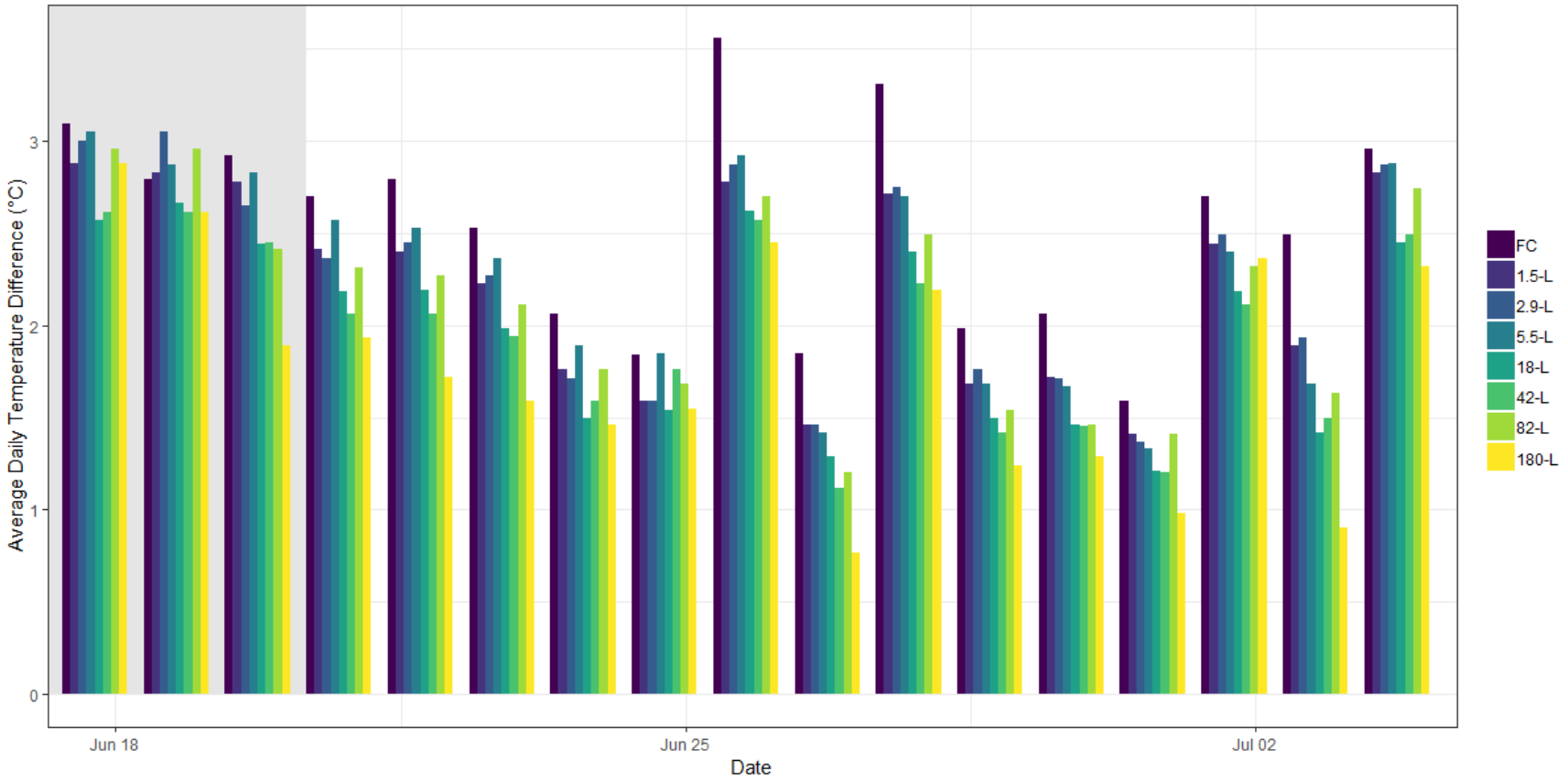

Figure 4.11: Daily temperature ranges within the mid-water column of each limnocorral between Day -2 (18th June, 2018) and Day 14 (4th July, 2018). Shaded area indicates Day -2 to Day 0 (20th June, 2018).

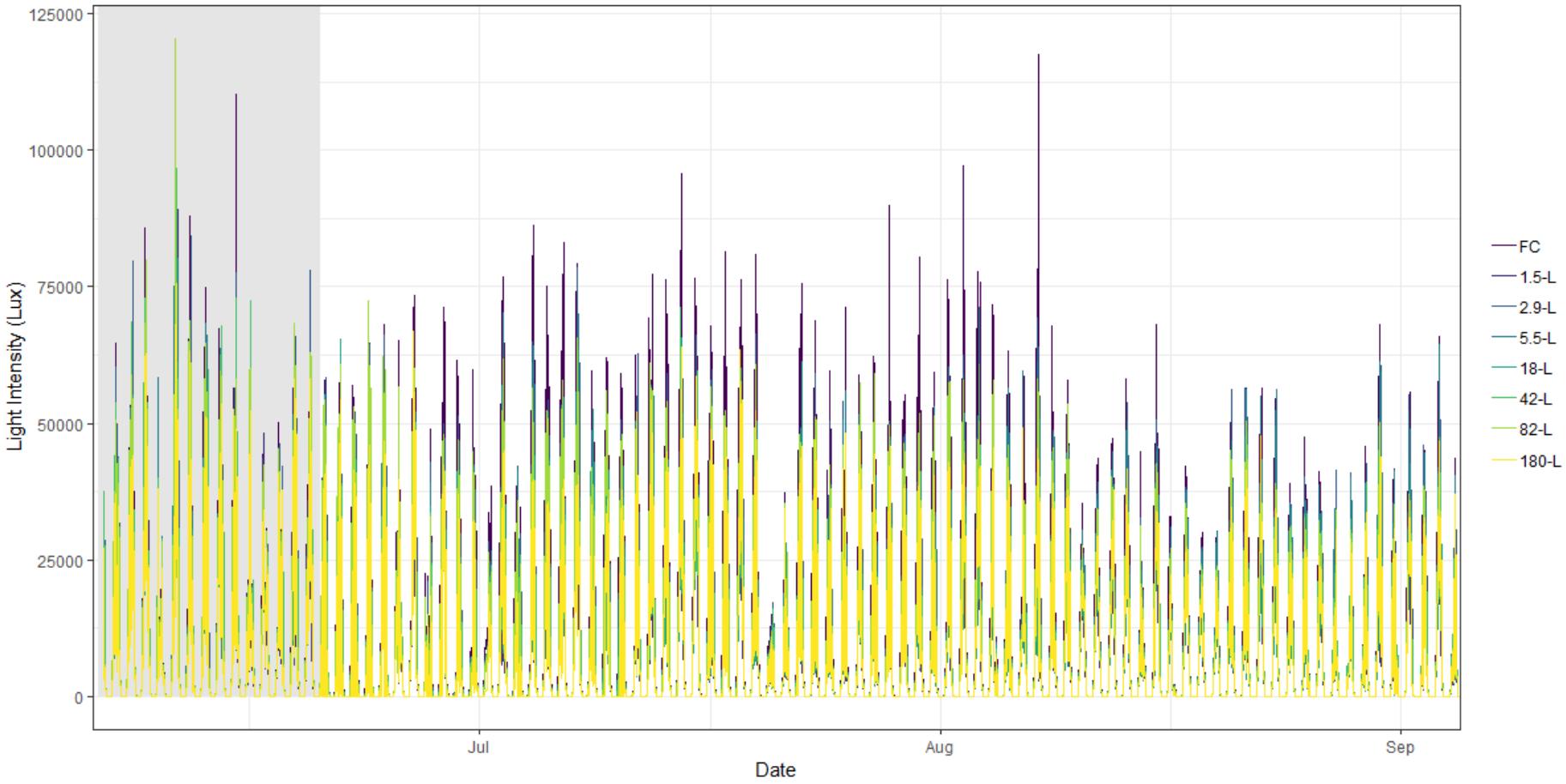

Figure 4.12: Light intensity (lux) from Day -15 (5th June, 2018) to Day 76 (4th September, 2018) reported within each limnocorral via HOBO loggers positioned mid-water column. Shaded area represents the time pre-spill Day -15 to Day 0 (20th June, 2018).

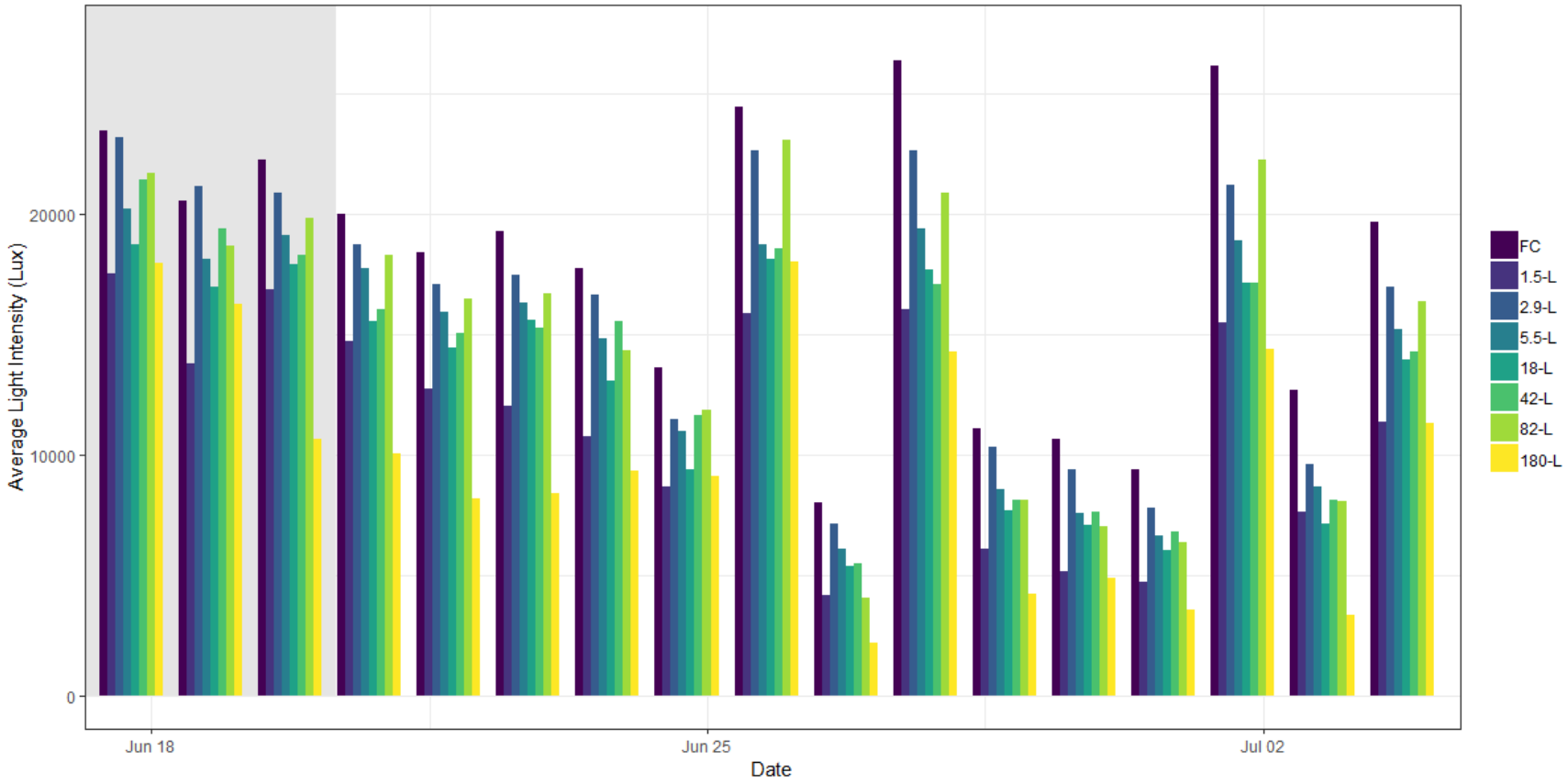

Figure 4.13: Average light intensity (lux per 24 hours) within the mid-water column of each limnocorral between Day -2 (18th June, 2018) and Day 14 (4th July, 2018). Shaded area indicates Day -2 to Day 0 (20th June, 2018).

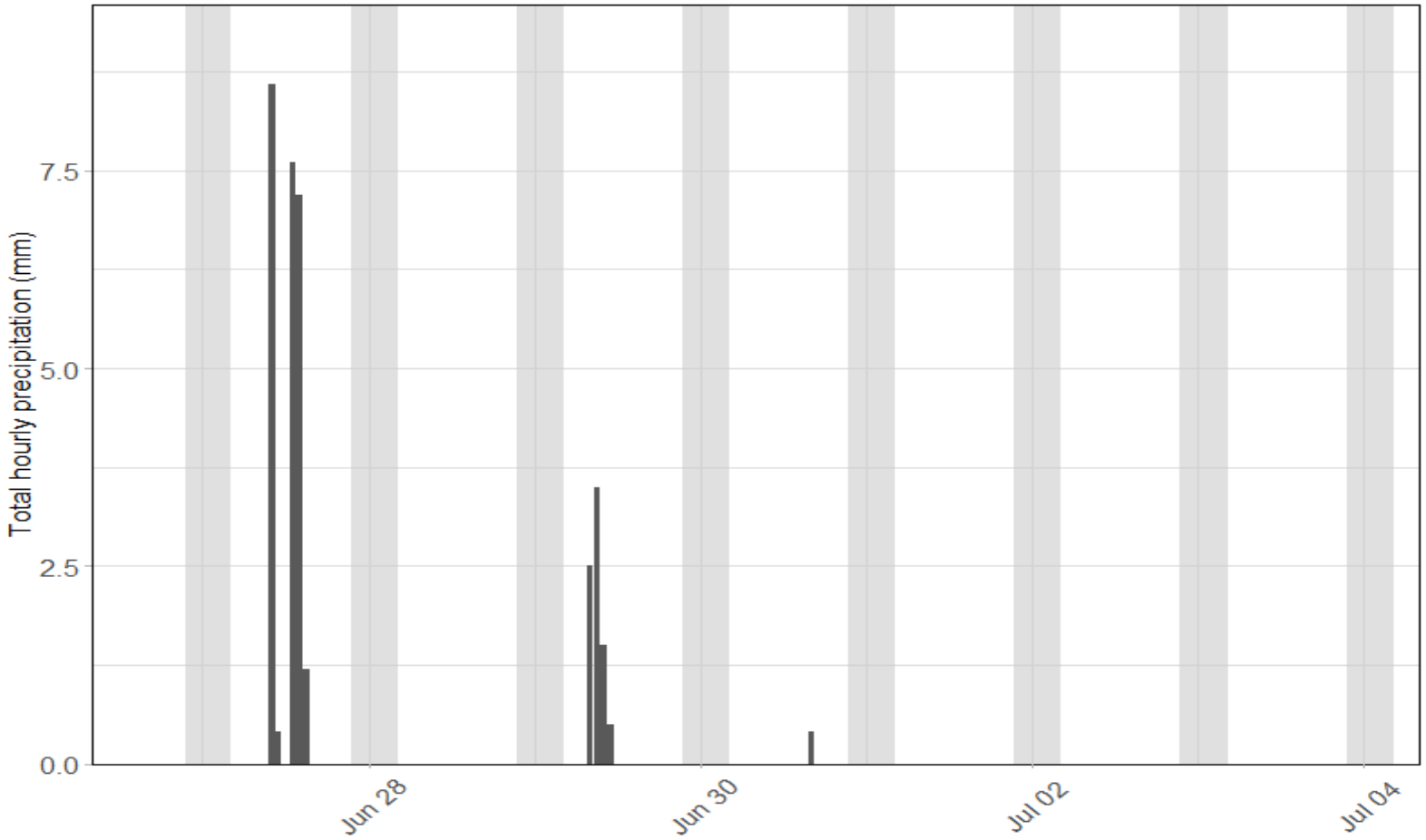

Figure 4.14: Total rainfall reported on Lake 260 via a weather station set up near the limnocorral site. Rainfall reported here is from 27th June, 2018 to 4th July, 2018. No rainfall was detected between Day 0 (20th June, 2018) and Day 6 (27th June, 2018).

4.2.2 Oil Chemistry

Oil components were assessed as part of a mass-balance to understand the fate and behaviour of spilled diluted bitumen under natural conditions (Objective #1 for the BOREAL project). The results for oil chemistry are not included in detail here but provide a general overview of PAH and BTEX presence within the water column. Sediment-based oil characterisation is not included here and will be evaluated in coming publications/theses from the BOREAL project.

Total PAHs within the water column (**Figure 4.15**) increased between oil addition and Day 15 sampling of PAHs before plateauing beyond Day 20 and eventually decreasing. However, there was an increase at the end of the study (Day 70) in PAH concentration in the 180-L limnocorral. BTEX components also increased post-spill in all treatments, peaking at 24 hours before falling to pre-spill levels in most limnocorrals (**Figure 4.16**). BTEX continued to decrease beyond 96 hours post-spill in the two highest treatments (180-L and 82-L) and had not yet reached pre-spill concentrations. BTEX totals peaked at 107.71 ug/L in the 180-L limnocorral at 24 hours post-spill and did not exceed 5 ug/L in the three lowest oil treatments (1.5-L, 2.9-L, and 5.5-L). TPAC spikes also occur following rainfall events on Day 7 and Day 9 (indicated in **Figure 4.14** and outlined in context of PAH concentrations in **Figure 4.15**) prior to biological sampling that occurred on Day 13 (dotted line in **Figure 4.15**).

TPACs were observed to have a greater affinity for the sediments with higher partitioning rates observed in TPAC mass (poster by Stoyanovich et al, 2019b at: SETAC North America 40th Annual Meeting). This may be attributed to the loss of

alkanes and increases in diluted bitumen density. Densities in the two highest dilbit treatments (180-L and 82-L) exceeded that of water (~ 1.00 g/mL) around Day 20, with dilbit density in the former limnocorral reaching 1.007 g/mL near the end of the study (presentation by Stoyanovich et al, 2019c at: SETAC North America 40th Annual Meeting).

Submergence of the oil occurred in the lower dilbit treatments between Day 12 and Day 19 (1.5-L limnocorral) and between Day 14 and Day 28 (18-L limnocorral). Submergence occurred later in the higher treatments, between Day 19 and Day 36 in the 42-L treatment and between Day 31 and Day 70 (end of study) in the 180-L treatment. Overall, submergence was observed within the first month of the dilbit spills in all dilbit treatments but there was a treatment-related effect in how long submergence took to begin (i.e. time at which oil was first observed on the sediments was directly related to increasing oil volume). A direct correlation between spill volume and initial submergence was significant ($R^2 = 0.76$, $p = 0.01$) (Stoyanovich et al, 2019c). Submerged was first characterised by the presence of neutrally buoyant tar balls (tar balls floating within the water column), followed by observation of tar balls on the sediment surface, and then the presence of larger tar mats later in the study (conglomerates of tar balls and larger masses that sunk from the surface; mostly present in the higher dilbit treatments) (observations noted in Stoyanovich et al, 2019c). In the higher dilbit treatments, tar mats would cover a great proportion of the sediment surface, likely exceeding 30-40% of the sediments surface in the 180-L limnocorral. This was markedly reduced in the lower dilbit treatments, although exact quantification of tar mat coverage was not determined.

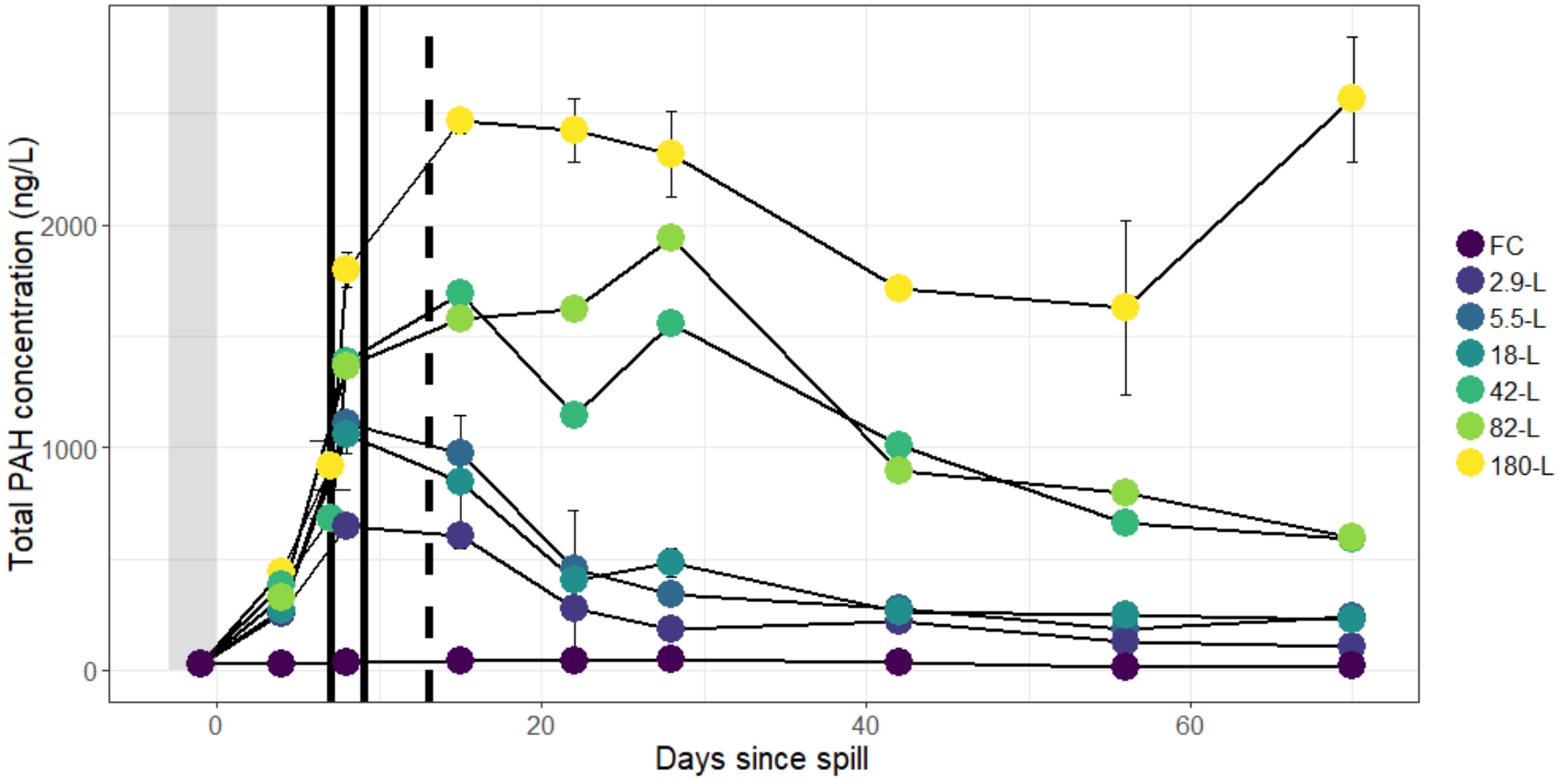

Figure 4.15: Total polycyclic aromatic hydrocarbon (TPAH) concentrations (ng/L) from Day -1 (19th June 2018) to Day 70 (29th August, 2018). Solid vertical black lines indicate days on which the first reported instance of rainfall occurred post-spill (Day 7, 27th June; Day 9, 29th June). Dotted vertical black line indicates the Day 13 sampling event for biota (chlorophyll a, zooplankton, emergence; 3rd July 2018). Error bars represent +/- standard error based on triplicate TPAH samples taken; triplicate samples were taken for the 180-L and 18-L limnocorrals.

*No data available for the 1.5-L limnocorral.
**Data collected and analyzed by S. Stoyanovich and Environment and Climate Change Canada.

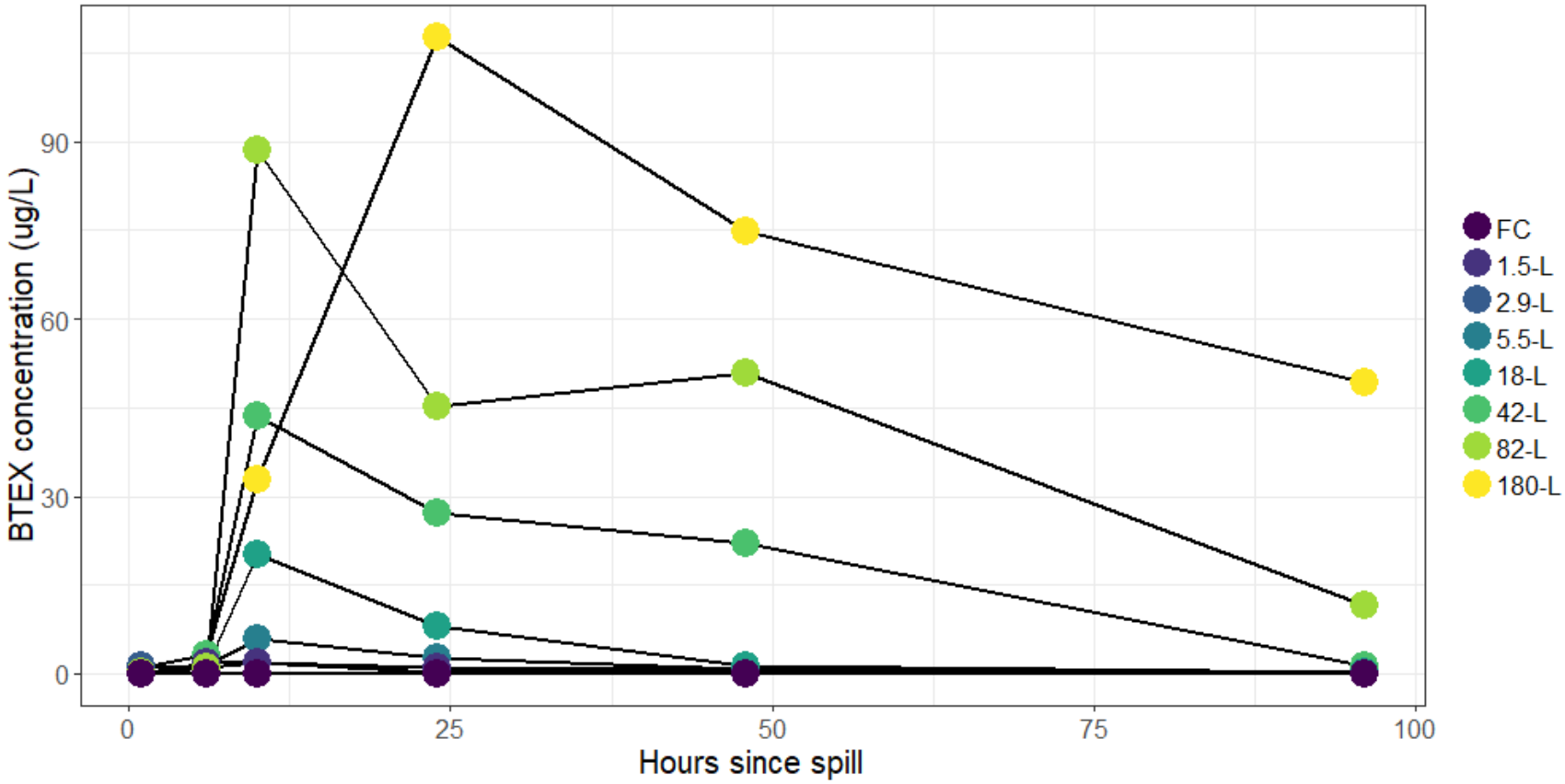

Figure 4.16: Total BTEX (benzene, toluene, ethylbenzene, xylenes) concentration (ug/L) reported in the limnocorrals from 1 hour post-spill (Day 0; 20th June, 2018) to 96 hours post-spill (Day 4; 24th June, 2018).

**Data collected and analyzed by S. Stoyanovich and Environment and Climate Change Canada.

4.2.3 Fish Communities

Fish were added to limnocorrals on Day 21 (July 11th) and included seven adult female and seven adult male finescale dace (*Phoxinus neogaeus*). Upon retrieving fish between Day 58 and Day 72, numerous juvenile fish were found within the limnocorrals that exceeded the numbers added on Day 21. They were assumed to be present at the time the limnocorrals were installed (Day -21 to Day -14). Fish captured within each limnocorral were recorded in **Table 4.2** and include other species such as fathead minnow (*Pimephales promelus*) and pearl dace (*Margariscus margarita*). There is high variability in fish capture numbers, ranging from 0 fish collected (82-L and 180-L limnocorrals) to 124 (5.5-L limnocorral). There is also considerable variability in fish size (based on fork length, body length, total length, and weight) with fish in the 5.5-L limnocorral (n = 124) being smaller relative to other limnocorrals with fewer collected fish.

Table 4.2: Length and weight measurements for fishes (added finescale dace and by-catch consisting of other finescale dace, pearl dace, fathead minnows, and unknown) and total fish numbers collected from the BOREAL limnocorrals between 17th August (Day 58) and 31st August, 2018 (Day 72) using a catch per unit effort of 1 minnow trap per 24 hour period.

Limnocorral	Fork Length (mm), +/- SD	Body Length (mm), +/-SD	Total Length (mm), +/- SD	Body Weight (g), +/- SD	n, Total	n, FSD	n, FHM	n, PD
FC	49 +/- 6.2	45 +/- 6.0	54 +/- 5.9	1.3 +/- 0.4	4	0	3	1
1.5-L	35 +/- 8.7	31 +/- 7.6	38 +/- 8.6	0.5 +/- 0.4	104	13	61	1
2.9-L	52 +/- 8.9	46 +/- 8.3	55 +/- 8.9	1.3 +/- 0.6	21	14	2	3
5.5-L	29 +/- 8.9	26 +/- 7.9	31 +/- 8.9	0.3 +/- 0.5	124	31	57	3
18-L	46 +/- 13	42 +/- 12	49 +/- 13	1.2 +/- 1.0	24	1	20	3
42-L	46 +/- 2.5 §	46 +/- 3.9 §	49 +/- 2.5	1.1 +/- 0.2	7	3	0	3

§Fork and body lengths for the 42-L limnocorral are based on n = 3 (data not available for four of the fish).
*Data collected by L. Timlick; data not available for limnocorrals 82-L and 180-L as no fish were captured.

4.2.4 Zooplankton

4.2.4.1 Zooplankton Community Characterisation

The zooplankton community was monitored at 14 time points throughout the BOREAL 2018 study and was assessed for changes in composition and relative abundance among limnocorrals. The zooplankton community in Lake 260 (**Table 4.3**) was dominated by calanoid copepods (*D. minutus* and *D. sicilis*) and the cladocerans *H. glacialis, B.* cf. *longirostris,* and *D. mendotae*. This community offers diversity in size and feeding habits, and therefore could provide indication of species-specific and functional group responses.

The zooplankton community within the limnocorrals at the start of the study (Day -14) was dominated by *D. minutus*, *E. lacustris*, *D. thomasi*, and *M. edax* (**Table 4.3**). Additionally, this table outlines how the limnocorrals varied on Day -14 from Lake 260 (outside of the limnocorrals). Densities are lower in the limnocorrals for most taxa, although there is a large range for some taxa (e.g. *K. taurocephala* and *D. minutus*). Juvenile calanoid copepodids dominated most limnocorrals apart from the 18-L limnocorral, which was dominated by calanoid nauplii (**Table 4.4**). Species richness across limnocorrals ranged from 10 to 14 taxa, with inverse Simpson diversity varying from 1.6 to 3.7.

Table 4.3: Zooplankton species densities in Lake 260 at the IISD-Experimental Lakes Area on Day -14 (6th June, 2018). Data for L260 proper were collected as part of the Long-Term Ecological Research (LTER) program at IISD-ELA and were collected on 11th June, 2018 ł. Limnocorral densities on Day -14 are reported as limnocorral averages +/- one SD, with ranges in parentheses. Data for copepods only includes those in which a positive species identification was possible (Copepodid stage IV or later). "n" indicates the number of limnocorrals in which the taxa was observed.

Species	L260 Density (organisms L^{-1})	Limnocorral Densities on Day -14 (organisms L^{-1})
Cladocera		
Bosmina cf. *longirostris*	0.36 ł	0.40 +/- 0.19 (0.12 – 0.63), n = 8
Daphnia mendotae	1.9 ł	0.033, n = 1
Daphnia pulicaria	0.060 ł	0.12, n = 1
Diaphnosoma birgei	0.060 ł	0.017, 0.033, n = 2
Holopedium glacialis	2.2 ł	0.050 +/- 0.036 (0.017 – 0.10), n = 4
Calanoida		
Epischura lacustris	0.14 ł	1.4 +/- 1.5 (0.25 – 5.0), n = 8
Diaptomus minutus	4.8 ł	18 +/- 13 (2.8 – 27), n = 8
Diaptomus sicilis	3.2 ‡	0.039 +/- 0.019 (0.017 – 0.050), n = 3
Cyclopoida		
Diacyclops thomasi	0.30 ł	2.9 +/- 2.7 (0.32 – 9.0), n = 8
Mesocyclops edax	0.060 ł	0.83 +/- 0.76 (0.067 – 2.5), n = 8
*Tropocyclops extensus**	0.060 ł	0.075 +/- 0.050 (0.033 – 0.13), n = 4
Rotifera		
Kellicottia longispina	3.6 ł	(0.42 +/- 0.35 (0.071 – 0.96), n = 8
Keratella cochlearis	10 ł	(0.49 +/- 0.48 (0.036 – 1.4), n = 8
Keratella taurocephala	0.60 ł	(4.5 +/- 4.1 (0.70 – 11), n = 8
Lecane sp.	Not observed	0.33 +/- 0.26 (0.16 – 0.63), n = 3
Polyarthra vulgaris	7.7 ł	0.39 +/- 0.51 (0.050 – 1.3), n = 5
Trichocerca cylindrica	0.89 ‖	0.16, n = 1
Other		
Chaoborus spp.	0.012 §	0.017, n = 2

‡Collected only on the 9th July, 2018
§Collected only on the 30th July, 2018
‖Collected only on the 13th August, 2018
Note: *Macrothrix sp.* was found in several limnocorrals beyond Day -14 but has not been reported in L260 as part of the LTER monitoring program
*Formerly *Tropocyclops prasinus mexicanus* (Dussart and Fernando, 1990)

Table 4.4: Diversity metrics and abundance data for zooplankton communities determined pre-spill (Day -14) for nine limnocorrals, seven of which were treated with varying volumes of diluted bitumen.

Limnocorral	Dominant Taxa	Dominant Taxa Abundance (org L^{-1})	Total Zooplankton Abundance (org L^{-1})	Richness	Inverse Simpson Index
NC	Calanoid Copepodids I – III	39.9	115.8	10	5.28
FC	Calanoid Copepodids I – III	45.7	136.9	10	1.86
1.5-L	Calanoid Copepodids I – III	30.0	77.4	10	3.53
2.9-L	Calanoid Copepodids I – III	29.7	76.3	14	1.57
5.5-L	Calanoid Copepodids I – III	21.7	92.3	14	2.41
18-L	Calanoid Nauplii IV – VI	2.0	15.1	11	3.73
42-L	Calanoid Copepodids I – III	2.9	42.2	10	2.66
82-L	*D. minutus* C IV – V	21.8	66.3	12	1.96
180-L	Calanoid Copepodids I – III	29.5	111.3	12	3.17

4.2.4.2 Zooplankton Community Response to Dilbit Additions

The zooplankton communities within the limnocorrals shifted throughout the study duration. Pre-spill, total zooplankton densities ranged from 15 (18-L) to 137 (FC) organisms per litre. By Day 76, densities were substantially lower in all enclosures, with a range of 0.25 (5.5-L) to 10 (18-L) organisms per litre (**Figure 4.17**). Biomass measures (**Figure 4.18**) indicated a large drop in total zooplankton biomass beyond Day 13. However, this decrease in biomass in the lower treatments and the control relative to the higher treatments occurred at different times. The FC sees a decrease in biomass occurring between Day -14 and Day 6, whereas this drop occurs in the 180-L limnocorral between Day 13 and Day 27.

Zooplankton abundance in all limnocorrals declined immediately post-spill and then returned to pre-spill densities on Day 6 in some limnocorrals (**Figure 4.17**). There was then a second, sustained, loss in abundance on Day 13. Given the loss in zooplankton within the FC limnocorral, and lack of observed recovery in abundance among all limnocorrals, a principal response curve (PRC) was generated to discern impacts relative to the control (**Figure 4.19**). This PRC used total abundance of each taxa within each limnocorral as the metric in generating a response curve.

The PRC demonstrates a community response across all limnocorrals, relative to the control, directly following dilbit addition (Days 2 to 6) – this is observed in the drop in the canonical coefficient (C_{dt}) used to quantify the response, relative to the control. Species responses are outlined in **Figure 4.20**. When contrasting species scores with the PRC,

when both values (C_{dt} and b_k) are negative, this indicates that species increased in the corresponding limnocorral relative to the control. Scores and coefficients with opposite signs indicate a decrease of that species in the corresponding limnocorral. The initial change in C_{dt} between Day 0 and Day 13 indicates an increase *K. taurocephala* and *D. minutus* and juvenile calanoids in most limnocorrals relative to the control. Most other taxa showed no strong response. On Day 55, the opposite response is observed (curves peak at $C_{dt} \approx 0.15$). On this day, the FC total abundance peaks at 16.8 organisms L^{-1} (relative to 2.5 and 4.7 organisms L^{-1} in the prior and following weeks, respectively) and likely explains the reported response observed on Day 55. Species responses will be further explored in the next section.

Redundancy analysis (RDA) of zooplankton data pre- (Day -3) and post-spill (Day 69) also provide no strong association between environmental data and zooplankton community composition (**Figure 4.21**; **Figure 4.23**). Due to issues with overparameterization when including several environmental variables, only a few variables were included in the RDAs; these included chlorophyll a (Chla), pH, dissolved oxygen (DO), temperature (Temp), and dissolved organic carbon (DOC). Permutation tests on the RDA outputs were significant post-spill Day 69 for the first RDA axis (p-value = 0.058 [α = 0.10]; F statistic = 17.509). In contrast with the Day -3 and Day 69 RDAs, **Figure 4.22** identifies a positive correlation along the first RDA axis between chlorophyll *a* (Chla) and the highest treatment (180-L) on Day 13. The RDA was significant for the first RDA axis (p = 0.085 [α = 0.10]; F statistic = 17.723) on Day 13. This relationship agrees with the univariate assessments described by **Figure 4.8** and

discussed above. The 180-L limnocorral was also negatively correlated with dissolved oxygen (DO), as above. No other treatment-based response was apparent in the RDA.

Table 4.5 shows community composition and densities of zooplankton collected in L260 proper at the end of the study (Day 76). The lake community was greater in richness and relative densities of organisms compared to the limnocorrals and dominated by *D. minutus* and *K. cochlearis*. This contrasts with the limnocorrals (including the Far Control), where mainly rotifera were present at the end of the study. Additionally, L260 community composition did not vary greatly from the beginning of the summer season (June 11th, **Table 4.3**).

A decrease in species richness was observed from an average of 11.6 (+/- 1.7, n = 8) to 6.5 (+/- 2.3, n = 8) (**Figure 4.24**). However, zooplankton diversity, measured by the inverse Simpson index, varied minimally over the study duration with an average diversity index on Day -14 of 2.6 (+/- 0.8, n = 8) to 2.2 (+/- 0.9, n = 8) on Day 76 (**Figure 4.25**). There was no concentration-response in diversity. The clearest response (i.e. a decrease in diversity) attributed to an increase in oil volume was observed on Day 13 following linear regression of oil volume on diversity ($p = 0.080$, $R^2 = 0.43$), with the 1.5-L limnocorral being a clear outlier. This relationship reversed on Day 27, showing an increase in diversity with added oil volume ($p = 0.022$, $R^2 = 0.61$). No concentration-response was observed for species richness (**Figure 4.24**).

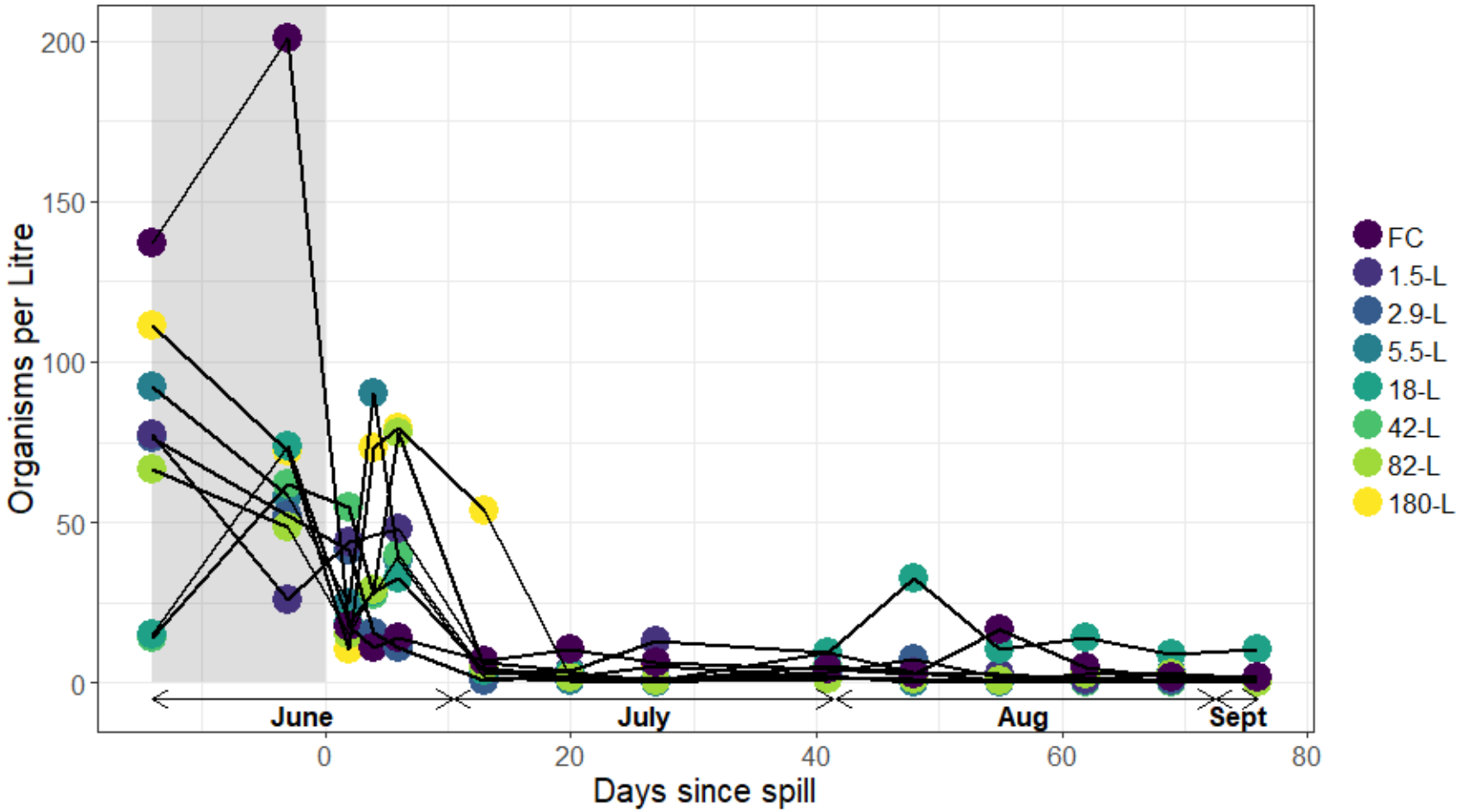

Figure 4.17: Total zooplankton abundance over time within eight limnocorrals treated with varying volumes of dilbit. Shaded region represents the time before dilbit application. Zooplankton were monitored for 90 days, from Day -14 (6th June, 2018) to Day 76 (4th September, 2018).

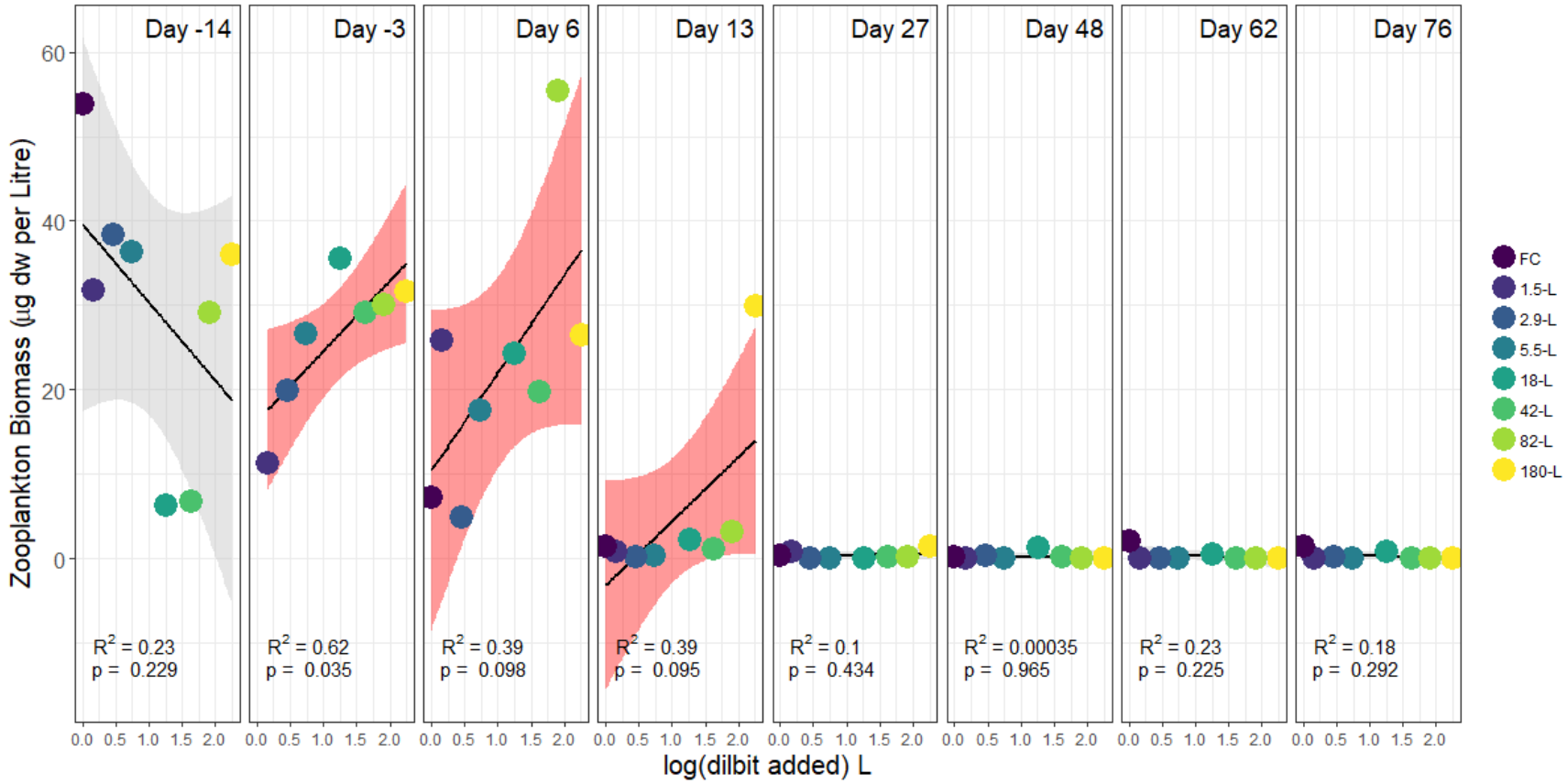

Figure 4.18: Zooplankton biomass, determined using length-weight regression estimation, linearly regressed against volume of dilbit added, log-transformed, at eight time points throughout the 90-day study. Zooplankton were monitored from Day -14 (6th June, 2018) to Day 76 (4th September, 2018). Black lines represent the linear model, with shaded regions indicating the confidence intervals associated with the linear model (95%). Significance (α = 0.10) is observed on Day -3 (p = 0.035), Day 6 (p = 0.098) and Day 13 (p = 0.095), with coefficient of determinations of 0.62, 0.39, and 0.39 (highlighted in red).

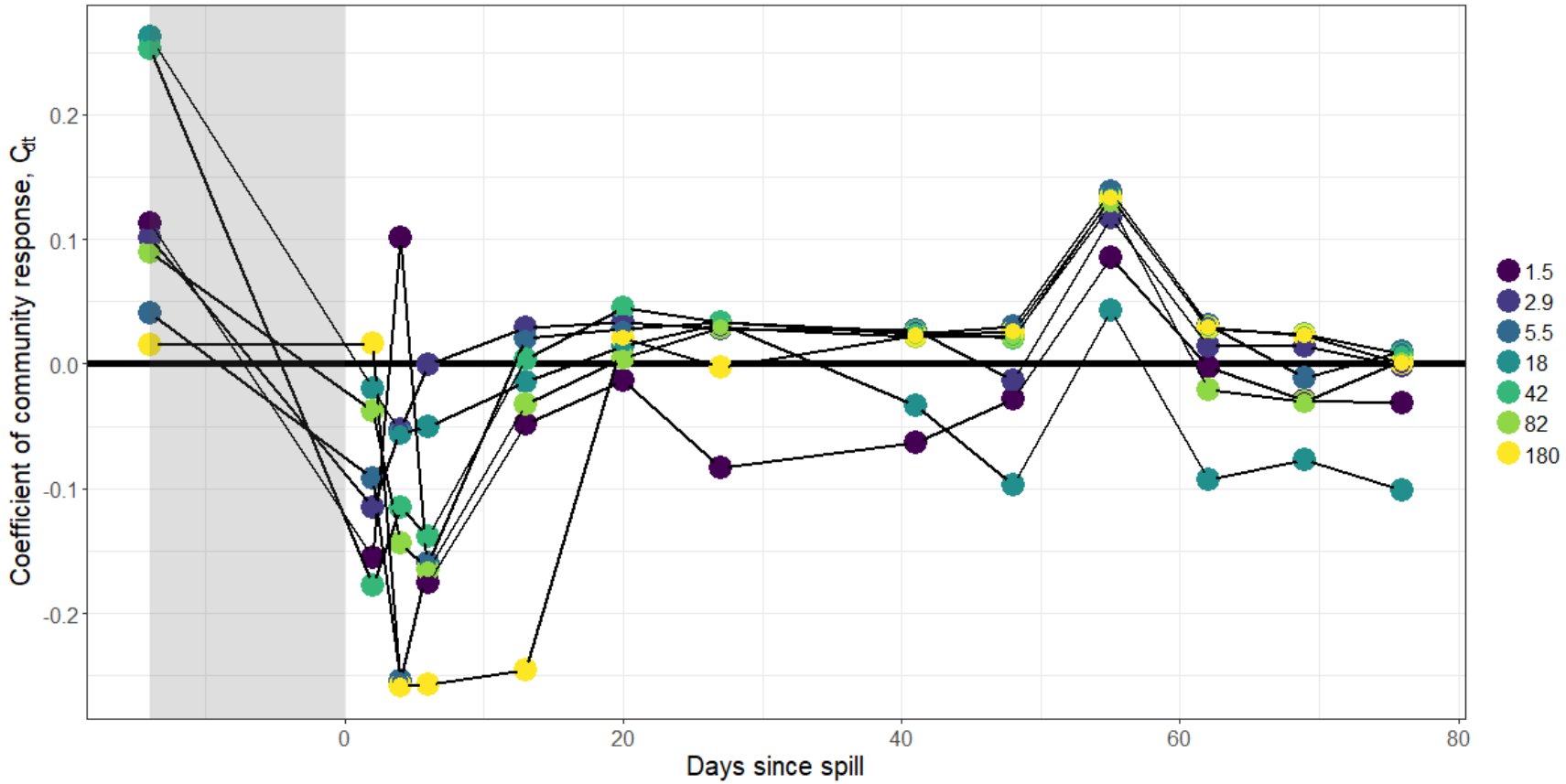

Figure 4.19: Principal Response Curve (PRC) axis 1 generated using total zooplankton abundance (organisms L^{-1}) observed within each limnocorral in Lake 260 following the addition of 7 different volumes of dilbit (1.5 L to 180 L) and the associated community response (C_{dt}) over time. The black horizontal line (C_{dt} = 0) represents the FC and is used as the basis in determining response coefficients for each treatment (T_{dtk}) relative to the control. T_{dtk} is determined as the product of the coefficient of the community response and the species scores (b_k). Conditional variance (i.e. time) accounts for 63.9% of variance and constrained variance (i.e. treatment, interacting with time) accounts for 36.1% of the total variance.

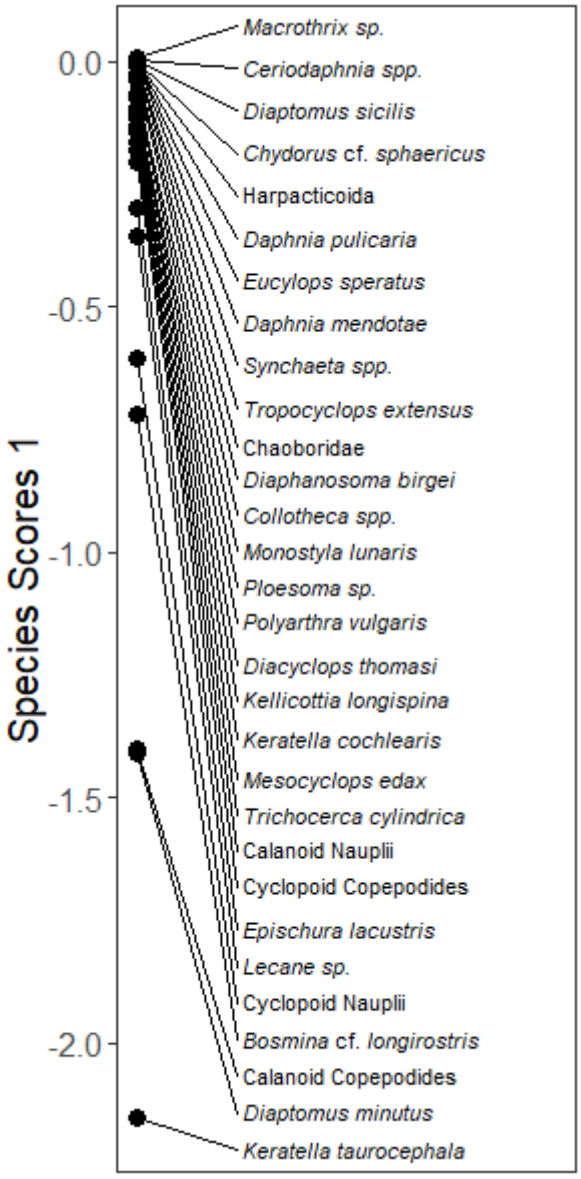

Figure 4.20: Species scores (b_k) associated with the first (47.29% of constrained variance) axis of the Principle Response Curve developed in **Figure 4.19** in the assessment of response to seven different volumes of dilbit (1.5 L to 180 L). Scores were used in development of the principle response curve's community response (C_{dt}) from the study start date (Day -14, 6th June, 2018) to Day 76 (4th September, 2018).

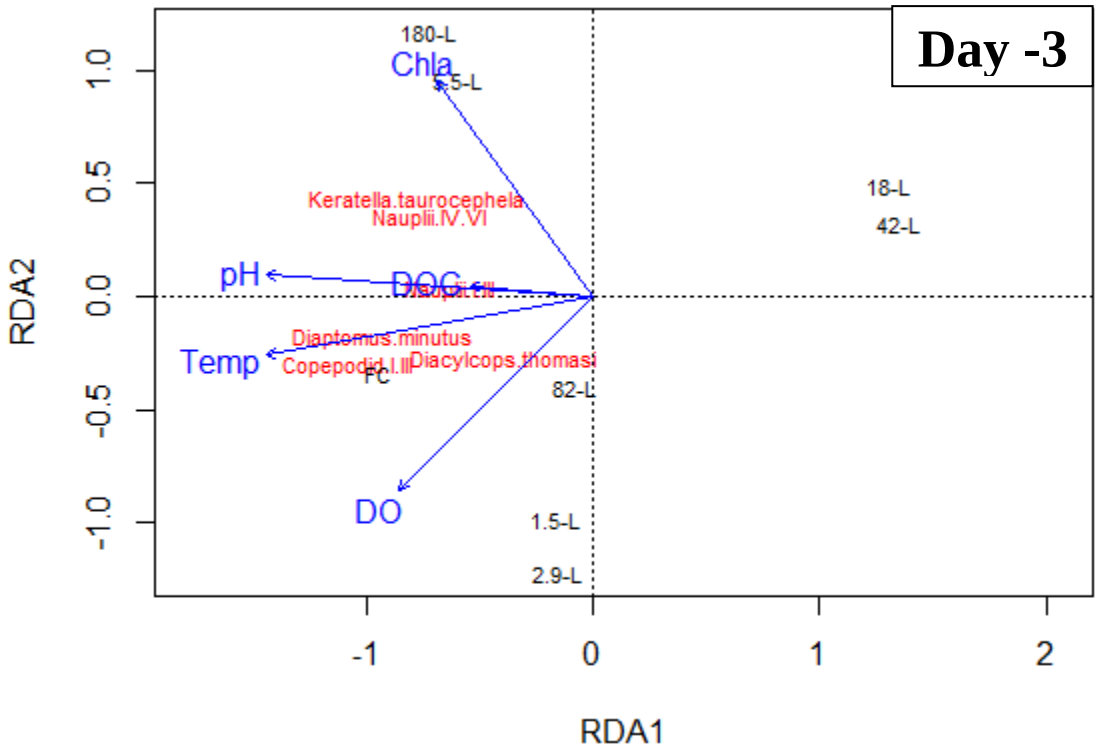

Figure 4.21: Redundancy analysis (RDA) triplot for pre-spill (Day -3; 18th June, 2018) zooplankton data and five environmental parameters including temperature (Temp), dissolved organic carbon (DOC), pH, chlorophyll *a* (Chla), and dissolved oxygen (DO). RDA1 explained 84.09% of total constrained variance (87.55%) and RDA2 explains an additional 11.54%. RDA1 is not significant at α = 0.10 (p = 0.121).

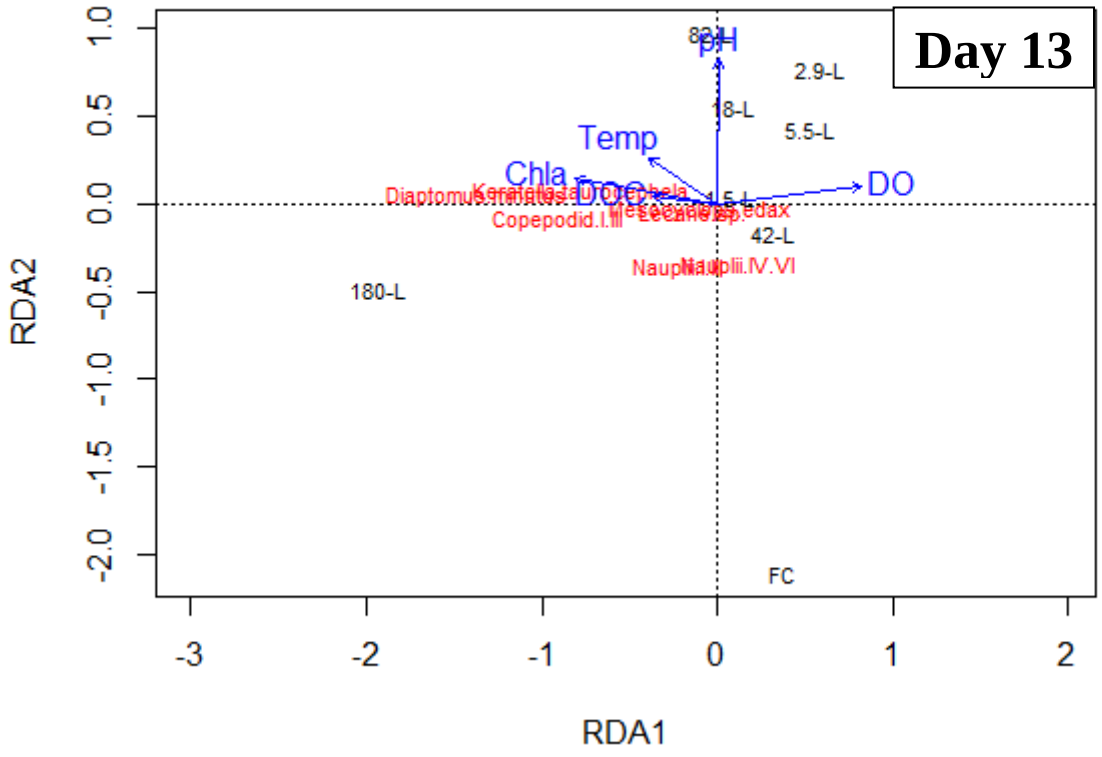

Figure 4.22: Redundancy analysis (RDA) triplot for post-spill (Day 13; 3rd July, 2018) zooplankton data and five environmental parameters including temperature (Temp), dissolved organic carbon (DOC), pH, chlorophyll *a* (Chla), and dissolved oxygen (DO). RDA1 explains 77.39% of total constrained variance (91.27%) and RDA2 explains an additional 6.31%. RDA1 is significant at α = 0.10 (p = 0.085).

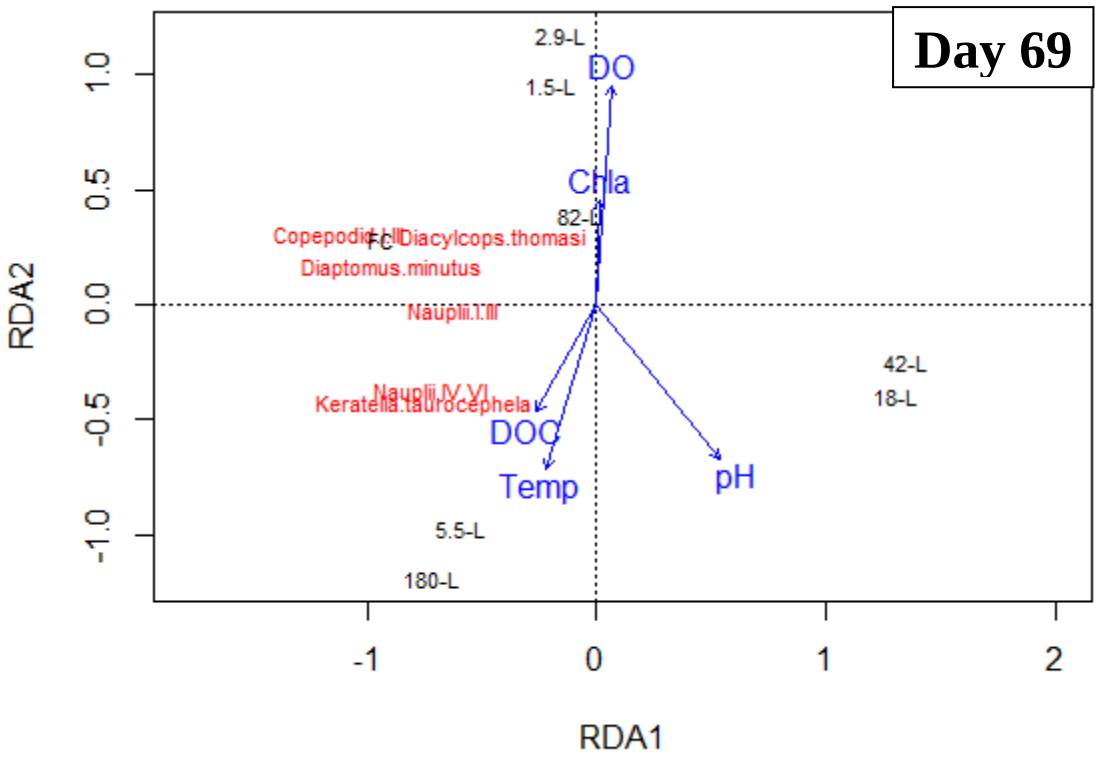

Figure 4.23: Redundancy analysis (RDA) triplot for post-spill (Day 69; 28th August, 2018) zooplankton data and five environmental parameters including temperature (Temp), dissolved organic carbon (DOC), pH, chlorophyll *a* (Chla), and dissolved oxygen (DO). RDA1 explains 81.17% of total constrained variance (91.52%) and RDA2 explains an additional 11.55%. RDA1 is significant at α = 0.10 (p = 0.058).

Table 4.5: Species densities and community composition on 27th August, 2018 for Lake 260 proper and on 28th August, 2018 (Day 69) for the limnocorrals.

Species	L260 Density (organisms L^{-1})	Limnocorral Densities on Day 69 (organisms L^{-1})
Cladocera		
Bosmina cf. *longirostris*	0.79	0.020 +/- 0.014 (0.0080 – 0.042), n = 5
Daphnia mendotae	0.60	Not observed
Daphnia pulicaria	0.079	Not observed
Diaphnosoma birgei	1.3	Not observed
Holopedium glacialis	0.36	0.017, n = 1
Calanoida		
Epischura lacustris	0.12	0.0080, 0.15, n = 2
Diaptomus minutus	7.0	0.15 +/- 0.21 (0.0080 – 0.39), n = 3
Diaptomus sicilis	1.1	Not observed
Cyclopoida		
Diacyclops thomasi	Not observed	0.0080, 0.17, n = 2
Mesocyclops edax	0.44	0.23 +/- 0.30 (0.041 – 0.68), n = 4
*Tropocyclops extensus**	0.16	0.061 +/- 0.044 (0.0080 – 0.11), n = 5
Rotifera		
Kellicottia longispina	Not observed	0.0080, 0.065, n = 2
Keratella cochlearis	12	Not observed
Keratella taurocephala	1.8	2.0 +/- 2.4 (0.017 – 6.5), n = 6
Lecane sp.	Not observed	0.11 +/- 0.085 (0.0080 – 0.23), n = 8
Polyarthra vulgaris	9.5	Not observed
Trichocerca cylindrica	1.8	0.0080, n = 1
Other		
Chaoborus sp.	0.0060	0.027 +/- 0.012 (0.017 – 0.050), n = 7

*Formerly *Tropocyclops prasinus mexicanus*

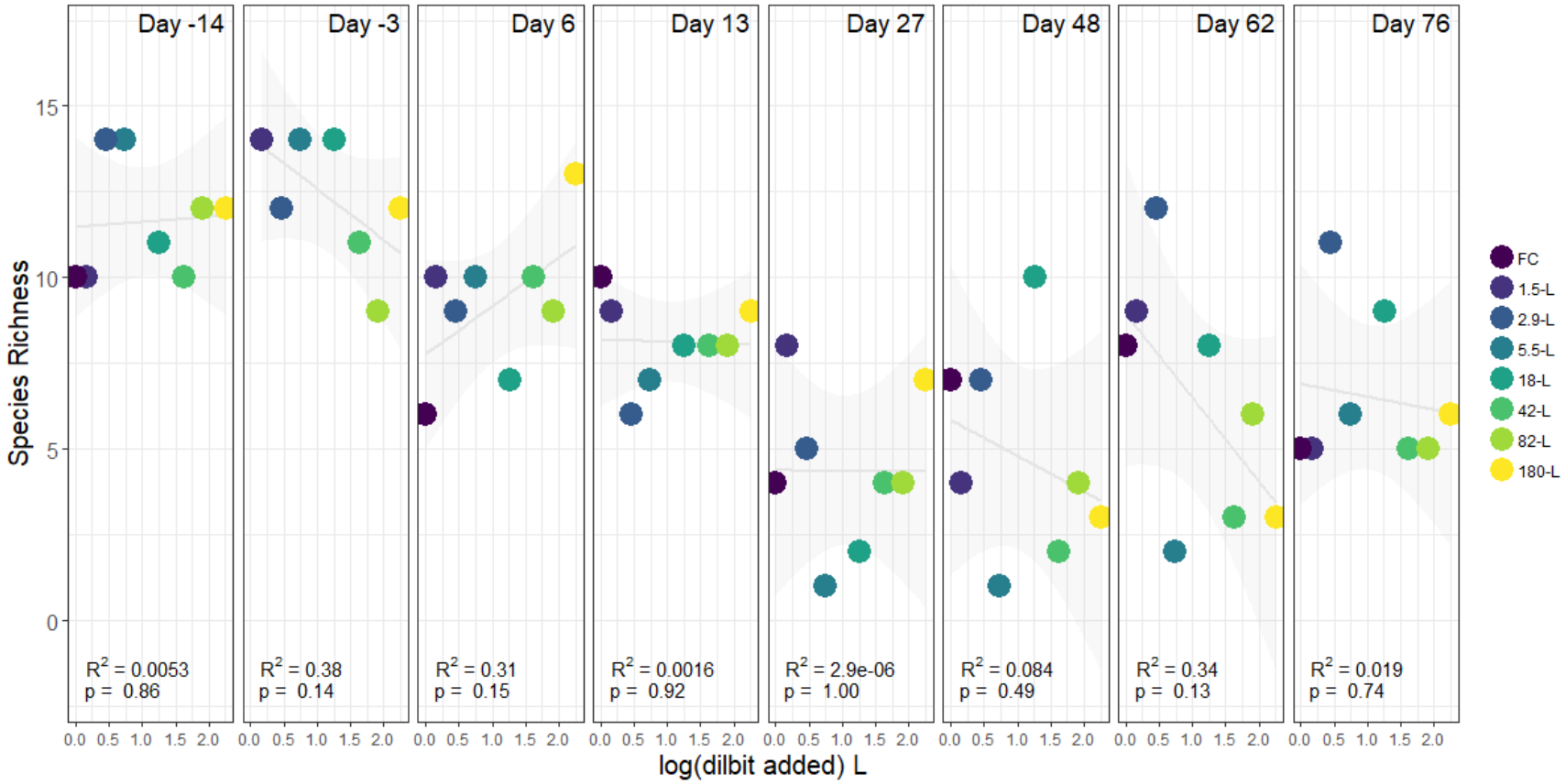

Figure 4.24: Zooplankton species richness linearly regressed against volume of dilbit added, log-transformed, at eight time points throughout the 90-day study. Richness was determined based on the lowest taxonomic classification available for a given taxa. Nauplii and other unidentifiable organisms were excluded from richness counts. Black lines represent the linear model, with shaded regions indicating the confidence intervals associated with model fit (95%). No statistical significance (α = 0.10) was observed across all time points.

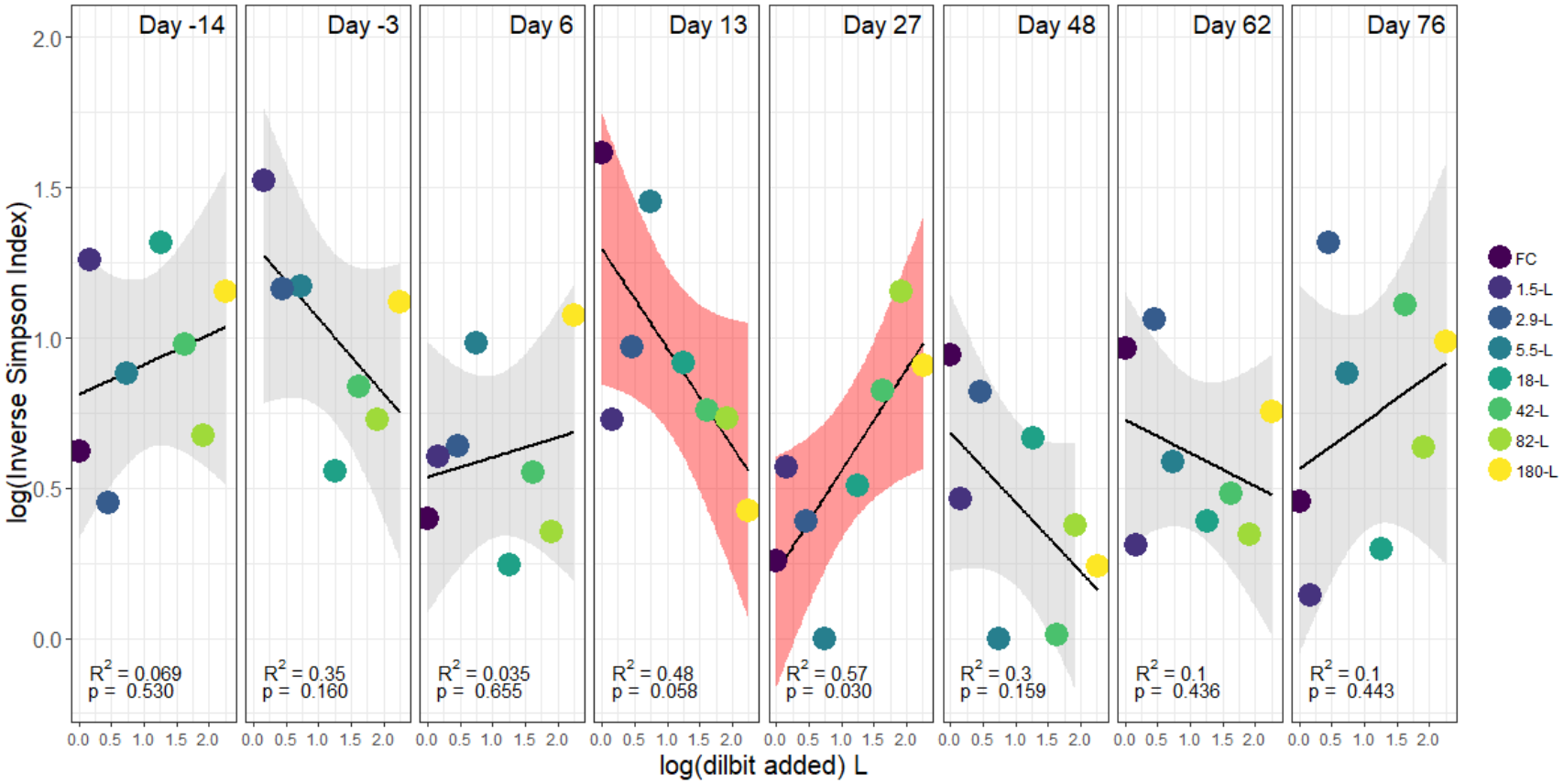

Figure 4.25: Zooplankton diversity, measured by the inverse Simpson index, linearly regressed against volume of dilbit added, log-transformed, at eight time points throughout the 90-day study. Black lines represent the linear model, with shaded regions indicating the confidence intervals associated with the linear model (95%). Significance ($\alpha = 0.10$) was observed on Day 13 ($p = 0.06$) and Day 27 ($p = 0.03$), with coefficient of determination of 0.48 and 0.57, respectively (highlighted in red).

4.2.4.3 Zooplankton Species Response

All limnocorrals exhibited a shift from cyclopoid copepods (mostly juvenile) to small rotifer taxa (including *Keratella taurocephala* and *Lecane sp.*). **Figure 4.26** outlines the dominant taxa within each limnocorral and the relative abundance of those taxa at six time points throughout the study. Limnocorrals were dominated by juvenile calanoid copepods pre-spill, followed by an increase in older calanoid copepods (copepodid IV and later). A major shift in dominant taxa within the limnocorrals – characterized by rotifer dominance – was observed beyond Day 41. The first instance of rotifer dominance was present 6 days post-spill in the 180-L limnocorral. Minimal differences can be discerned at the end of the monitoring period among the limnocorrals. Each were dominated by the genus *Keratella* or *Lecane sp.* (both are small rotifer taxa), except for the Far Control (FC), which was dominated by *Mesocyclops edax* copepodids.

There was an overall decline in copepod abundance, independent of treatment, beyond Day 13 of the study (**Figure 4.27**). Prior to this, there was proliferation of Cladocera (mainly *B. longirostris*) within the 5.5-L (peaks at 28.6 org L^{-1}) and 180-L (peaks at 11.3 org L^{-1}) limnocorrals between Days 4 and 6, and a smaller increase in copepod abundance. Conversely, the 82-L limnocorral observed no change in *B. longirostris* abundance but did see a large increase in calanoid and cyclopoid copepods on Day 6 (59.4 org L^{-1} and 11.2 org L^{-1}, respectively). The other limnocorrals, including the control saw smaller changes; regardless of the community composition changes immediately

post-spill, copepod and cladoceran abundance dropped substantially beyond Day 13 and did not recover in these limnocorrals.

Rotifers were dominant in all limnocorrals after Day 41 with total rotifer relative abundance ranging from 70.6% (FC) to 99.5% (2.9-L and 42-L). There was no clear direct impact of oil on rotifer abundance directly following the dilbit addition, but there was a substantial increase in rotifer absolute abundance in the 180-L limnocorral (peaking at 32.5 org L^{-1}). A similar increase in rotifer abundance was observed in the 18-L limnocorral starting on Day 41 and peaked on Day 48 (31.2 org L^{-1}), with rotifer abundance remaining around Day 41 levels for the remaining study duration. This change did not correspond with any increase or decrease in other zooplankton taxa.

Juvenile copepods (nauplii and copepodids) accounted for most copepods found within the limnocorrals. Pre-spill (Day -14), juveniles accounted for between 88 and 97% of all copepods – this range was between 74% and 100% on Day 76 (4 of the 8 limnocorrals had no adults present). Nauplii represented a smaller portion (as copepodids were more dominant) at the start of the study (**Figure 4.28**). No dominance between calanoids and cyclopoids was apparent, and juvenile copepods were a mix between these two groups. Juvenile dominance within the limnocorrals changed drastically over the duration of the study and may be attributed to the volume of oil added to each limnocorral. On Day 13, a strong significant negative relationship was observed regarding nauplii representation within limnocorrals, associated with increasing oil volume ($p = 0.0048$, $R^2 = 0.76$). However, the higher treatments showed little change in copepod age composition immediately post-spill – this correlation is likely attributed to the increase in nauplii

composition in the lower treatments and the control immediately post-spill. There was a general decrease in zooplankton biomass and zooplankton abundance, so the increase in nauplii proportions with decreasing dilbit volume may indicate a loss in larger and older zooplankton. Over time, a shift in age composition was observed following a concentration-dependent response. This was indicated by the gradual decrease in copepodid and adult representation within limnocorrals (**Figure 4.28**) and increase in proportion of nauplii with increasing oil volume (**Figure 4.29**) on Days 62 and 76 ($p = 0.0917$ and $p = 0.0959$, respectively). Comparatively, copepodid stages 4 and 5 were significantly higher in limnocorrals treated with more oil ($p = 0.00078$, $R^2 = 0.87$) on Day 13. Although nauplii abundance dropped, copepodid and adult abundance was affected to a greater extent, thereby increasing nauplii proportions within the limnocorrals (increased relative abundance of nauplii).

Figure 4.30 provides species-level details corresponding to the PRC developed in **Figure 4.19**. The first axis of the PRC accounts for 47.29% of the constrained (treatment effects) variance with the second axis providing an additional 16.16% of the explanation. The species scores biplot outlines which species played the greatest role in changes observed in community composition and abundance within the limnocorrals. Taxa that are not grouped near zero (+/- 0.1) show some affinity towards the responses observed in the PRC. The first axis (observing differences from left to right) correspond with the species scores in **Figure 4.20**, detailing the species affinity for the responses attributed to the first PRC axis. This figure adds the second axis (differences along the vertical axis; top to bottom), allowing increased resolution in understanding individual taxon roles in shaping community composition post-spill. *D. minutus* adults and

copepodids (note that "*D. minutus"* refers to adults and IV to V copepodids, whereas "Calanoid Copepodids*"* refers to CI to C3 – i.e. copepodids that were not identified to species) appear to contribute the most to the changes observed in **Figure 4.19**. For *D. minutus* and other Calanoid Copepodids, this corresponds with the univariate assessments of species-level changes above – that is, *D. minutus* and copepodids gradually decrease and this change appears more dependent on treatment in the final two weeks of the study. Additionally, *K. taurocephala* appears to also contribute substantially to the PRC and can likely be attributed to its dominance within the limnocorrals beyond Day 41 (**Figure 4.26**).

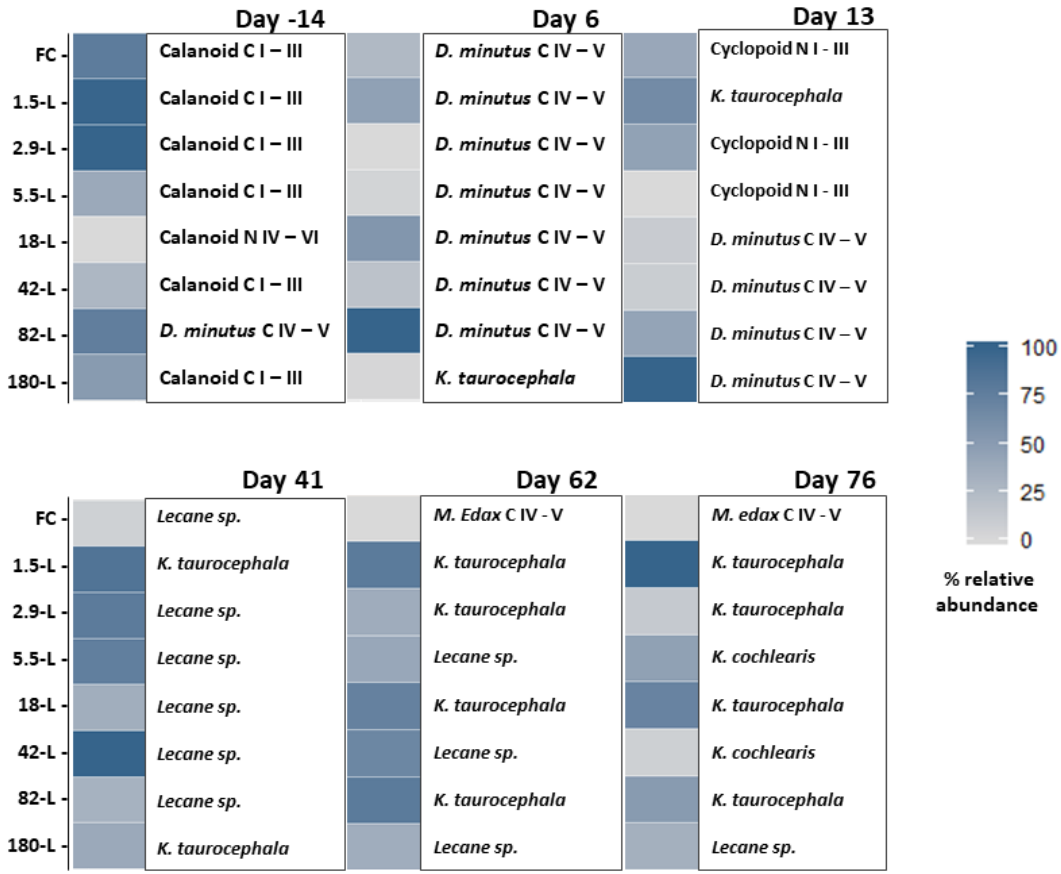

Figure 4.26: Dominant taxa and their % relative abundance within eight limnocorrals in Lake 260. Dominant taxa consist of the most abundant taxa, proportionally, within a given limnocorral and at each sampling day included here. Dominant taxa were evaluated at six time points from the start of the study (6th June, Day -14) to the end of the study (4th September, Day 76).

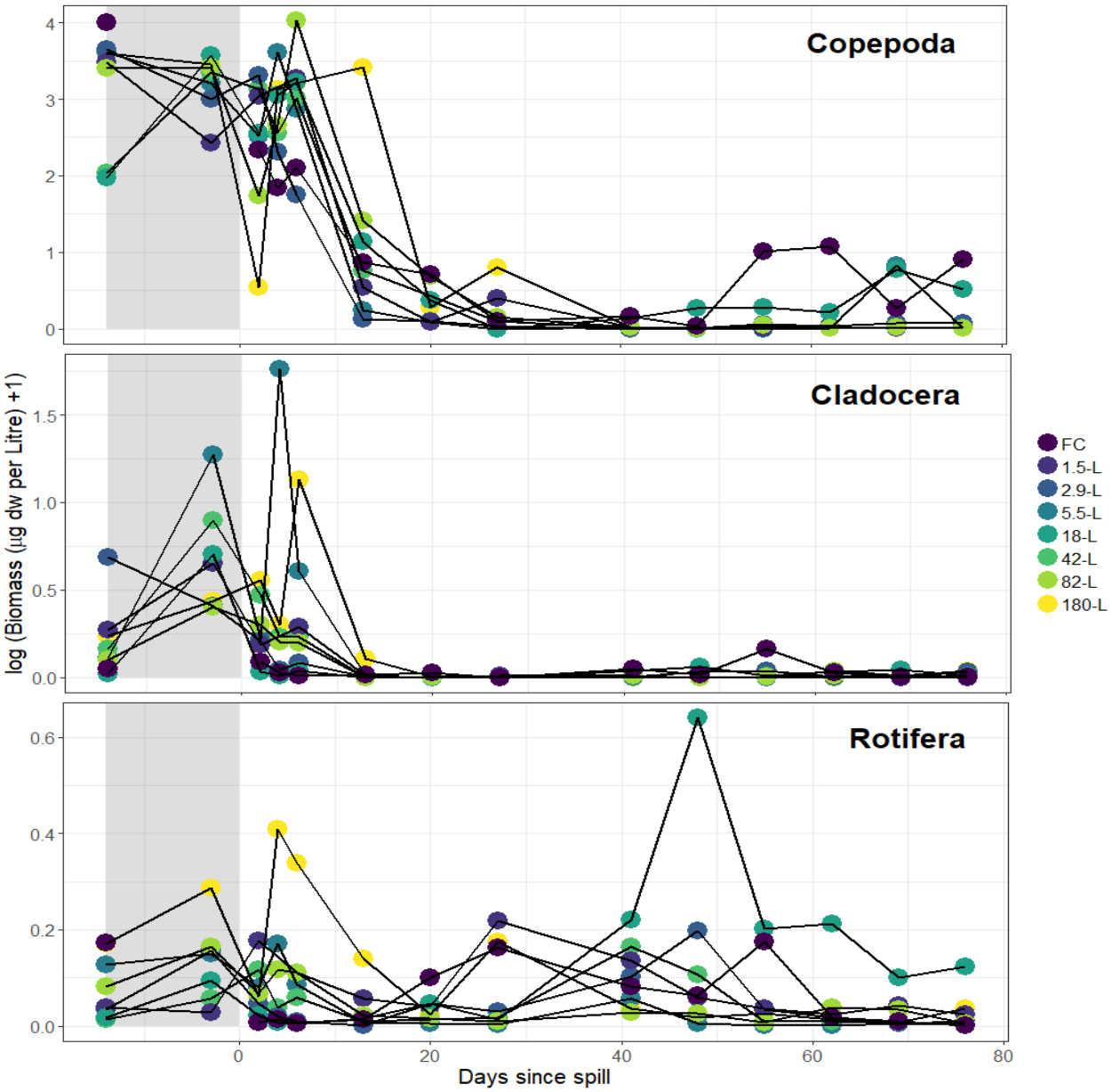

Figure 4.27: Estimated biomass (ug dry weight per litre) log transformed (+1) for three taxonomic groups of zooplankton (Copepoda, Cladocera, and Rotifera) over time within eight limnocorrals treated with varying volumes of dilbit. Shaded region represents the time before (Day -14 to Day -1; 6th June to 19th June, 2018) dilbit application. Copepods include all juveniles (nauplii and copepodids) and adults.

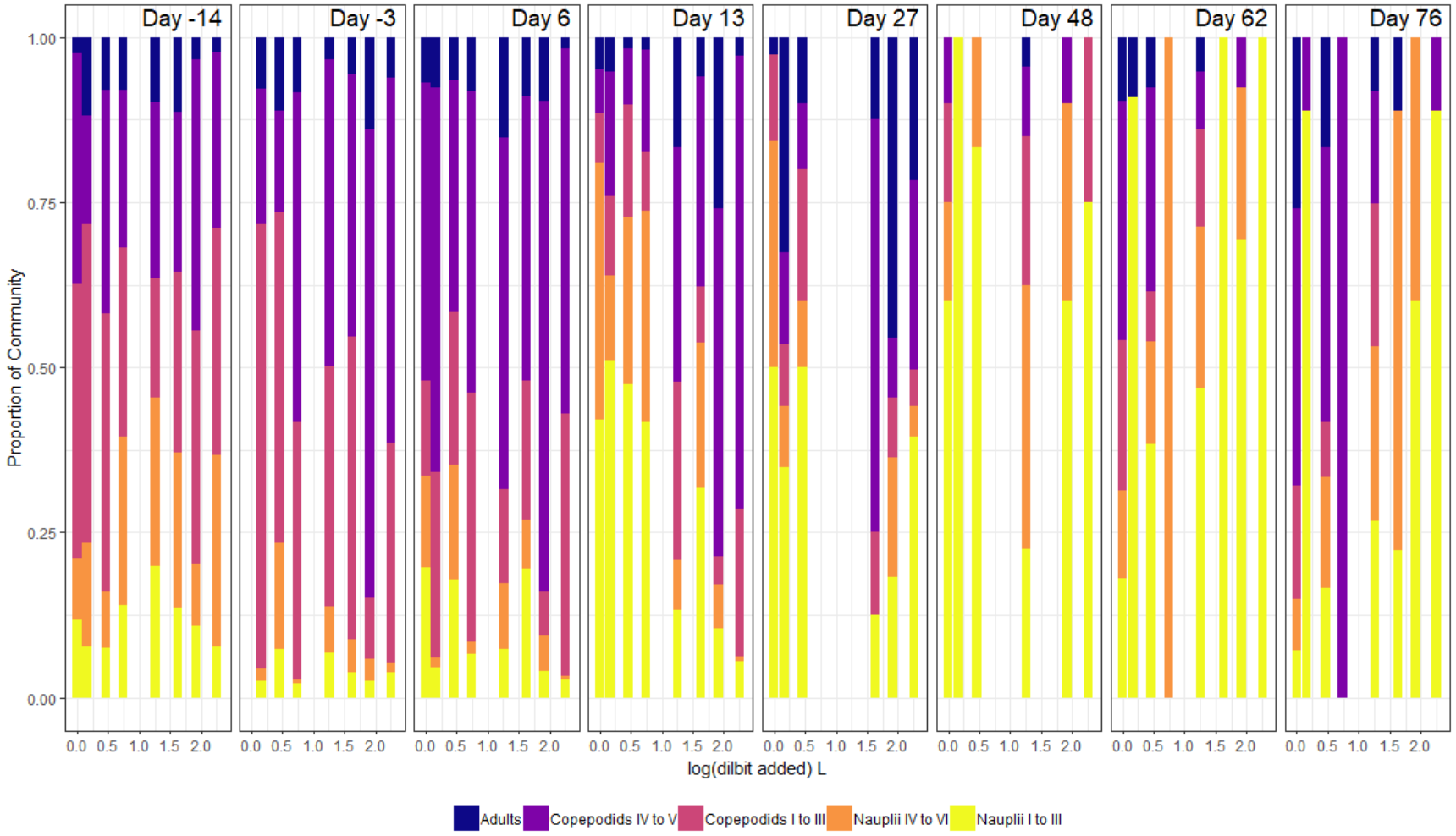

Figure 4.28: Changes in copepod life stage composition at eight time points throughout the 90-day study (6th June to 4th September, 2018). Copepod life stage was evaluated based on five categories: adults (sexually mature, C VI), copepodid stages IV to V, copepodid stages I to III, naupliar stages IV to VI, and naupliar stages I to III and evaluated over time as proportion of the total copepod community within each limnocorral.

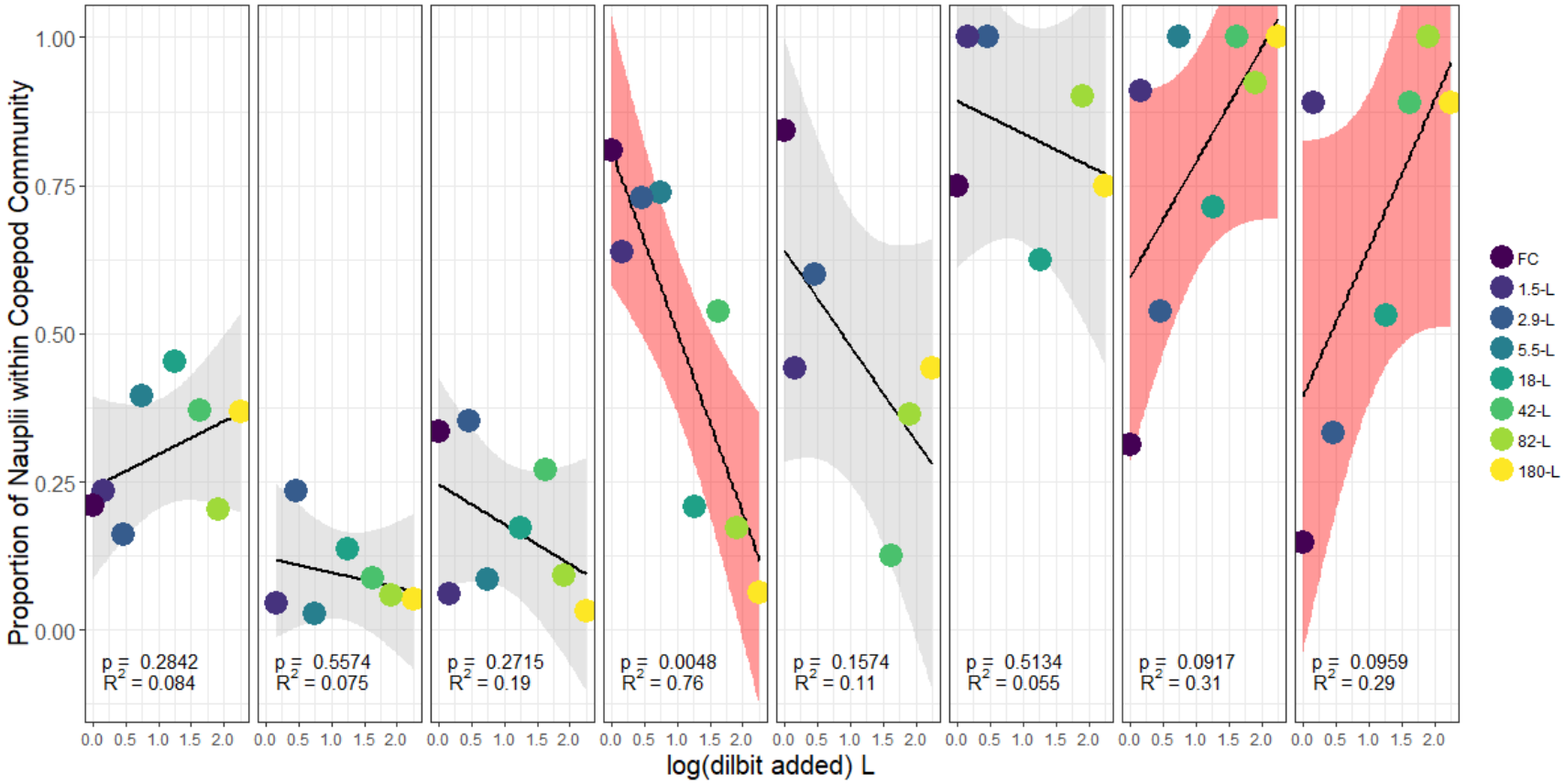

Figure 4.29: Nauplii proportional composition linearly regressed against volume of dilbit added, log-transformed, at eight time points (Day -14 to Day 76) throughout the 90-day study (6th June to 4th September, 2018). Black lines represent the linear model, with shaded regions indicating the confidence intervals associated with model fit (95%). Significance (α = 0.10) is observed on Day 13 (p = 0.0048), Day 62 (p = 0.0917), and Day 76 (p = 0.0959), with coefficient of determinations of 0.76, 0.31 and 0.29, respectively (highlighted in red).

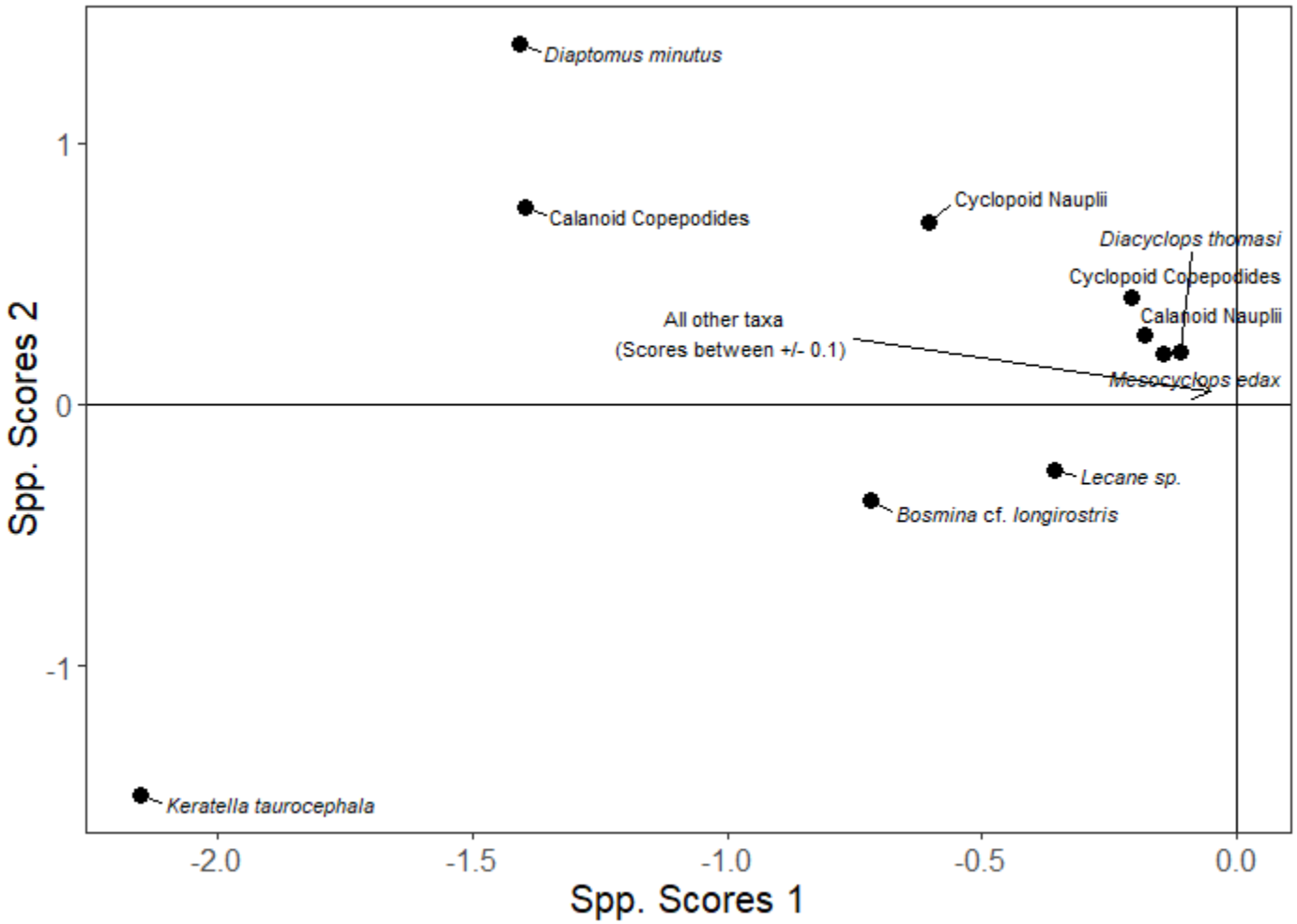

Figure 4.30: Species scores (b_k) associated with the first (47.29% of constrained variance) and second (16.16% of constrained variance) axes of the Principle Response Curve developed in **Figure 4.19** in the assessment of response to seven different volumes of dilbit (1.5 L to 180 L). Scores were used in development of the principle response curve's community response (C_{dt}) from the study start date (Day -14, 6th June, 2018) to Day 76 (4th September, 2018). Taxa with scores between +/- 0.1 were grouped together and are not distinguished here.

4.2.5 Benthic Invertebrates

4.2.5.1 Benthic Invertebrate Community Characterisation

Benthic invertebrate data from outside of the enclosures were collected in the summer of 2017 (**Table 4.6**). This served to provide an idea of the benthic community before limnocorrals were installed and dilbit was added. The community was dominated by larval *Chironomidae* (non-biting midges), *Ceratopogonidae* (sand flies/biting midges), *Leptoceridae* (caddisfly), and *Hyalella sp.,* with other taxa present at low densities. *Chironomidae, Ceratopogonidae*, and L*eptoceridae* (three of the dominant insect taxa in Lake 260) all have a benthic larval stage and emerge through the water column at their adult life stage. This is important for the emergence data as most organisms living in the benthos could be captured by emergence traps, as reported below.

As no benthic sampling was conducted following installation of the limnocorrals and prior to oil addition, emergence data must be relied upon in understanding pre-spill benthic communities specific to the limnocorrals. A total of three reference sites were included in this study to understand the impact that the limnocorrals may have had on community composition and species densities (**Table 4.7**). Pre-spill, the FC site had the lowest emergence (69.33 organisms m^2) with the 5.5-L limnocorral having the highest (376.9 organisms m^2). The main emerging organisms observed include *Ceratopogonidae* and *Chironomidae* Diptera families as observed in the L260 surveys (by J. Tonin) that were conducted in 2017.

Table 4.6: Invertebrate densities around the BOREAL site in Lake 260 between 31st August and 7th September, 2017. The diversity in each area was calculated from averaging three separate Ekman grabs at three different locations around the BOREAL site. The Ekman sampler that was used had a surface area of 0.0231 m^2.

Order – Family	L260 Density (organisms m^{-2})
Diptera - Chironomidae	6602 +/- 2332
Diptera - Ceratopogonidae	216.3 +/- 86.57
Trichoptera - Leptoceridae	149.1 +/- 54.62
Trichoptera - Hydroptilidae	43.28
Amphipoda - Hyalellidae	269.3 +/- 120.1
Odonata - Gomphidae	43.28
Odonata - Libellulidae	43.28
Odonata - Coenagrionidae	43.28
Ephemeroptera - Caenidae	57.71 +/- 24.99
Gastropoda - Amnicolidae	64.92 +/- 30.61
Gastropoda - Planoibidae	86.57 +/- 61.21
Oligochaeta	57.71 +/- 12.49
Hydrachnidia - Hydrachnidae	93.79 +/- 69.57
Veneroida - Sphaeriidae	64.92 +/- 30.61
Mean Density (organisms m^2)	7397
Mean Number of Taxa per Sample	6.89

Table 4.7: Pre-spill (Day -3) densities of emerging insects within limnocorrals in L260. Insects were collected using an emergence trap and densities listed below were calculated by dividing sample counts by the trap surface area (0.5625 m^{-2}). Traps were deployed for a 1-week period (Day -10 to Day -3). Dash indicates that the taxa was not observed.

Taxa	Density (organisms per m^{-2} wk^{-1})										
	FC	1.5-L	2.9-L	5.5-L	18-L	42-L	82-L	180-L	A	B	C
Diptera - Ceratopogonidae	51.6	37.3	55.1	4.89	23.1	48.0	17.8	14.2	1.78	7.11	3.56
Diptera - Chironomidae	16.0	254	288	377	180	155	226	196	309	242	304
Diptera - Dolichopodidae	-	-	-	-	-	-	-	-	1.78	-	-
Diptera – Simulidae	-	-	-	-	-	-	1.78	-	-	-	-
Ephemeroptera - Isonychiidae	-	-	-	-	-	-	-	-	1.78	3.556	-
Hydrachnidia	-	-	-	-	-	-	1.78	-	-	-	-
Hymenoptera - Ichneumonidae	-	-	-	-	1.78	-	-	-	-	-	-
Hymenoptera - Scelionidae	1.78	-	-	-	-	-	-	-	1.78	-	-
Plecoptera - Nemouridae	-	-	-	-	-	-	-	-	-	-	3.56
Thysanoptera	-	-	-	-	-	-	1.78	-	-	-	-
Trichoptera - Dipseudopsidae	-	1.78	-	-	5.33	1.78	-	3.56	-	3.56	3.56
Trichoptera - Leptoceridae	-	-	-	-	-	-	-	1.78	1.78	-	-
Trichoptera - Hydroptilidae	-	-	-	-	-	1.78	-	1.78	-	-	
Total Density (organisms m^2)	69.3	293	343	377	210	206	249	217	316	256	315
Richness	3	3	2	2	4	4	5	5	6	4	4

4.2.5.2 Emergence Trends

Immediately following oil addition, impacts on total emergence were observed in an oil volume-dependent manner ($p = 0.001$, $R^2 = 0.861$) (**Figure 4.31**). Emergence at the in-lake reference sites was similar to the limnocorrals until Day 11, after which they had greater emergence occurring. The proportion of insects that emerged pre-spill (n = 1 week) accounted for 89% and 74% of the total emergence in the two highest treatments (180-L and 82-L), respectively. This ranges from 11% to 59% in the lower treatments and pre-spill emergence accounted for 5.6% of total emergence in the FC limnocorral. This corresponded with emergence rates of 217 m^{-2} $week^{-1}$ pre-spill in the highest treatment to 0 to 4 organisms m^{-2} $week^{-1}$ following oil addition (**Table 4.8**). In contrast, emergence ranged from 228 to 795 organisms m^{-2} $week^{-1}$ in the FC for the first two weeks post-spill. Over the entire post-spill period, there was a strong negative relation between emergence rates and added oil volume (**Figure 4.31**; $R^2 = 0.86$; $p = 0.001$). The p-value listed is based on a linear regression of treatment (log-transformed oil volume) and total number of organisms for the FC and all treated limnocorrals post-spill and represents a significant negative relationship between emergence and oil volume. On Day 11 (the first post-spill time point), a distinct difference in number of organisms was observed among all limnocorrals and the reference sites (**Figure 4.32**).

The first PRC axis (representing treatment effects) accounted for 98.7% of the constrained variance (**Figure 4.33**). Conditional variance (i.e. time) accounts for 54.2% of the total variance and constrained variance (i.e. treatment, interacting with time) accounts for an additional 45.9% of the total variance. Near the end of the study, this

volume-dependent response continued to be observed (**Figure 4.33 - A**), although to a lesser degree than before. Given their high relative abundance within the emergence samples, changes in Chironomid emergence contributed the most to the observed changes, followed by Ceratopogonidae and Leptoceridae (**Figure 4.34**). Due to the dominance of Chironomids within the limnocorrals, no further statistical analysis was conducted – counts of other taxa were too low to discern other treatment-related, species-specific impacts.

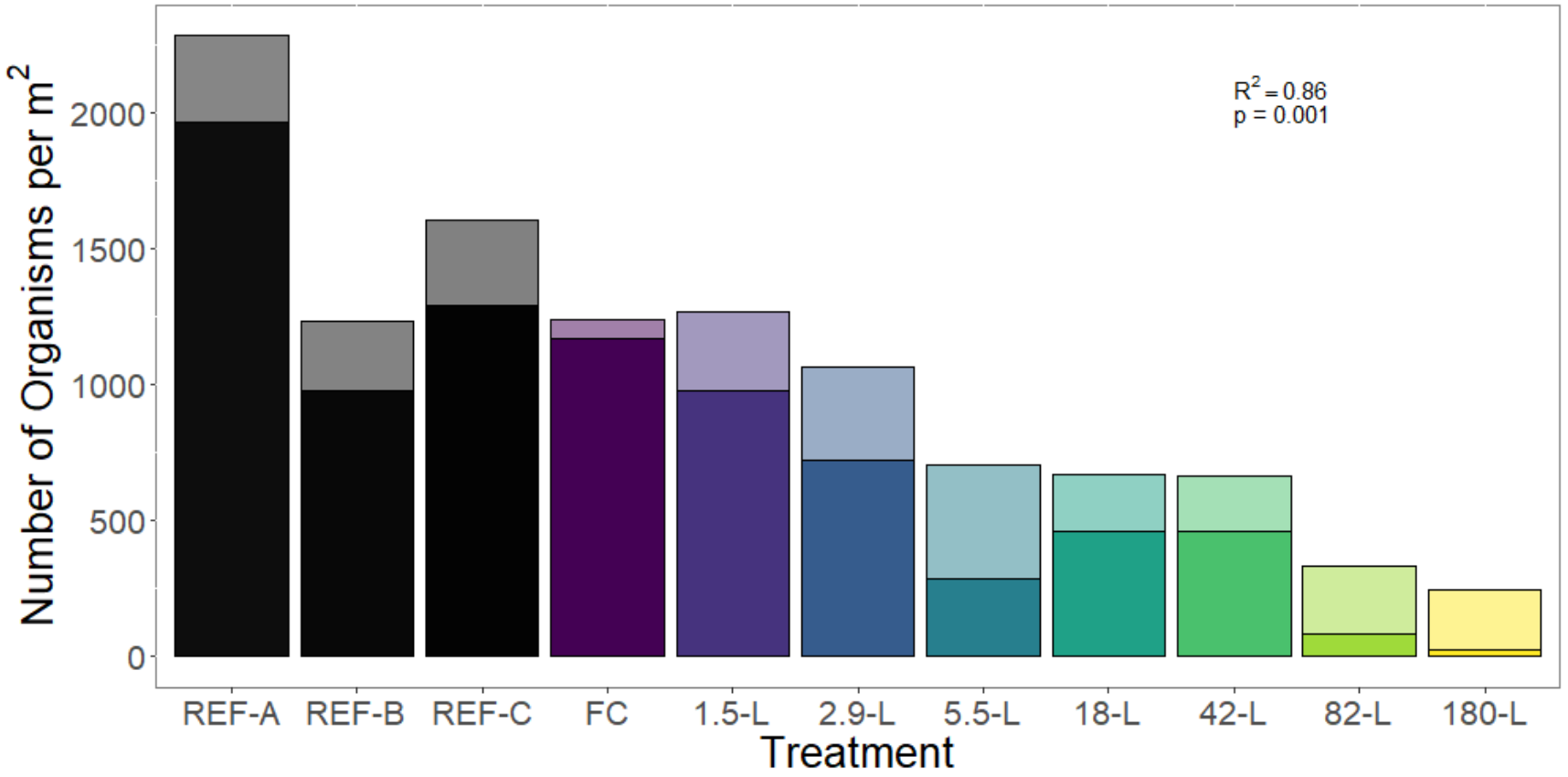

Figure 4.31: Total emergence (0.5625 m^2 emergence traps) from eight limnocorrals and three reference sites in Lake 260 from June 2018 to August 2018. Shaded regions represent the portion of the total emergence that occurred pre-spill (from Day -10 to Day -3, n = 1 week), whereas the darker regions represent the portion of the total emergence that occurred following addition of dilbit (n = 9 weeks). R^2 and the p-value correspond to summary statistics for the regression output of total organisms emerging post-spill relative to oil volume.
*REF-B only has n=3 samples; trap was damaged following Day 25 and following samples were not viable.

Table 4.8: Emergence rates (0.5625 m^2 emergence traps) from eight limnocorrals in Lake 260 from June 2018 to August 2018. Traps were deployed for 1-week periods and dates listed account for the one week prior to sample collected (e.g. emergence rates on Day 19 include emergence between Day 11 and Day 19).

Limnocorral	Emergence Rate (organisms per m^{-2} wk^{-1})									
	Day -3	11	19	25	32	40	47	53	61	68
FC	69.3	795	228	64.0	17.8	17.8	16.0	8.89	12.4	14.2
1.5-L	293	489	176	172	53.3	19.6	19.6	17.8	16.0	12.4
2.9-L	343	254	252	112	42.7	19.6	24.9	3.56	12.4	3.56
5.5-L	418	97.8	40.9	37.3	35.6	30.2	23.1	16.0	1.78	5.33
18-L	210	233	103	30.2	37.3	16.0	14.2	7.11	12.4	7.11
42-L	206	343	67.6	21.3	1.78	12.4	7.11	3.56	0	1.78
82-L	249	24.9	19.6	19.6	5.33	0	5.33	3.56	0	7.11
180-L	217	14.2	0	3.56	1.78	3.56	0	1.78	1.78	0

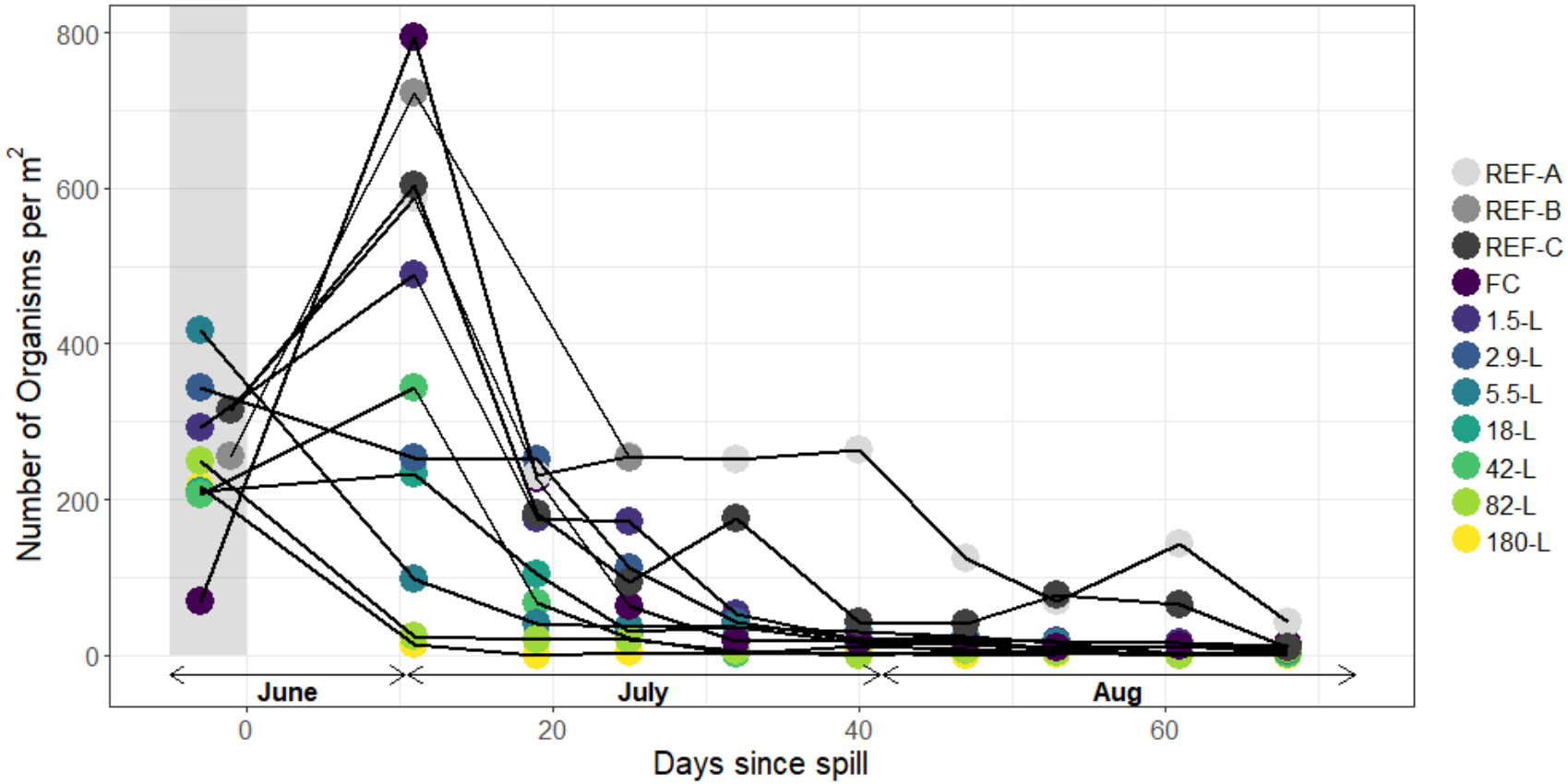

Figure 4.32: Temporal changes in total emergence across seven treated limnocorrals, the Far Control (FC) limnocorral, and three reference sites in L260 from the 6th June, 2018 (Day -14) to the 28th August, 2018 (Day 68). The shaded region represents the time pre-spill (prior to Day 0).

*REF-B only has n=3 samples; trap was damaged following Day 25 and following samples were not viable.

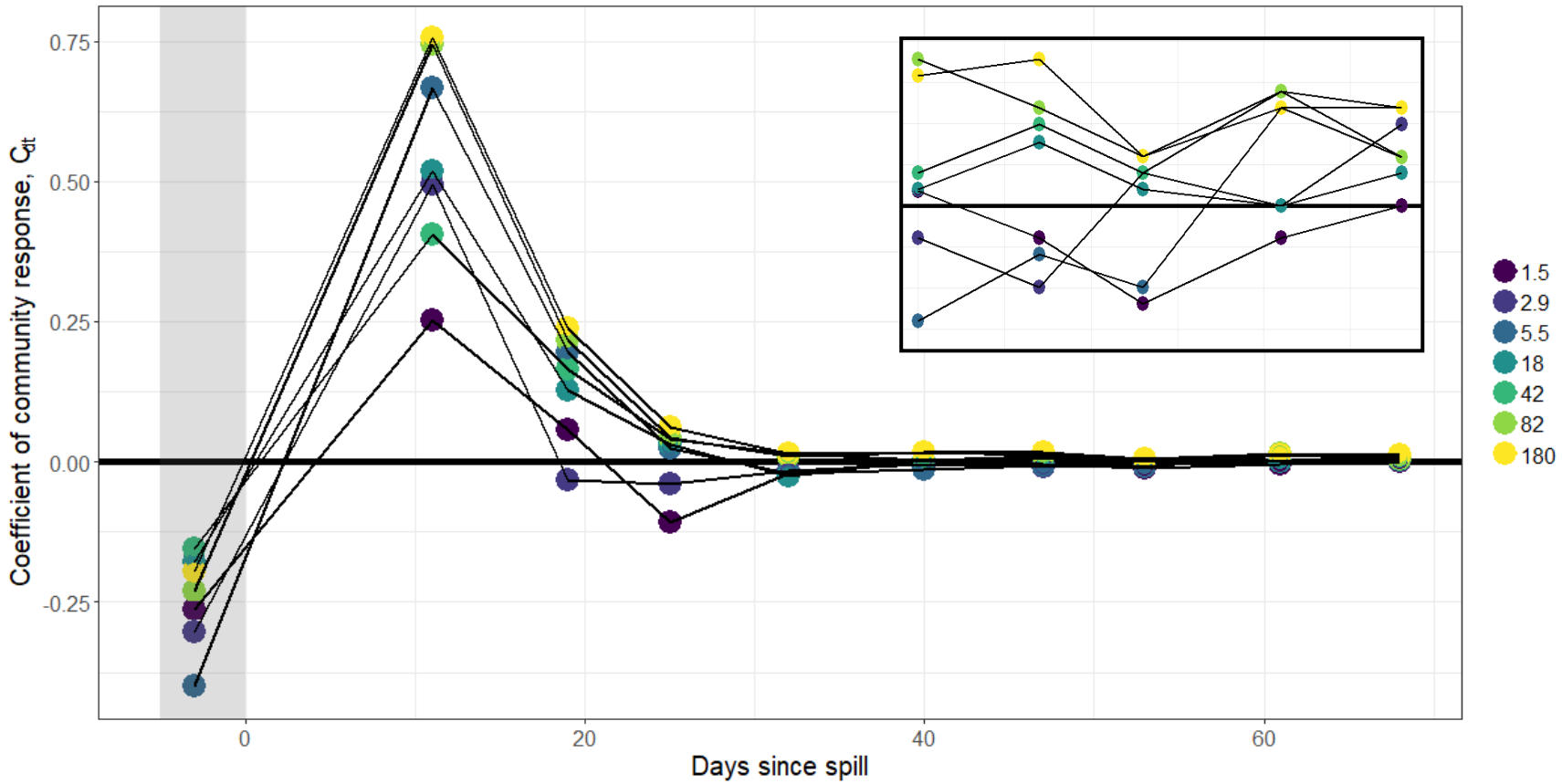

Figure 4.33: Principal response curve (PRC) axis 1 (98.7% of constrained variance) based on total emergence (organisms m^{-2}) observed within each limnocorral in Lake 260 following the addition of 7 different volumes of dilbit (1.5 L to 180 L) and the associated community response (C_{dt}) over time (6th June to 28th August, 2018); Inset shows expanded view of PRC axis 1 between Days 40 and 68. The black horizontal line (C_{dt} = 0) represents the FC and is used as the basis in determining response coefficients for each treatment (T_{dtk}) relative to the control. Conditional variance (i.e. time) accounts for 54.2% of the total variance and constrained variance (i.e. treatment, interacting with time) accounts for an additional 45.9% of the total variance.

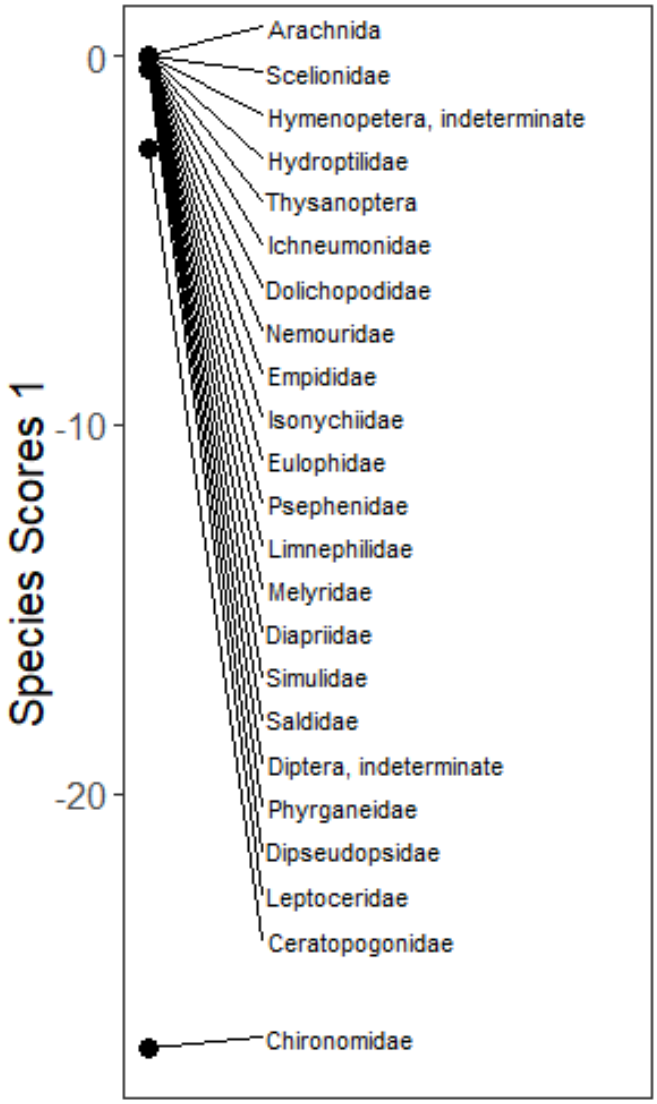

Figure 4.34: Species scores (bk) associated with the first (98.7% of variance) axis of the Principle Response Curve developed in **Figure 4.33** in the assessment of response to seven different volumes of dilbit (1.5 L to 180 L). Scores were used in development of the principle response curve's community response (Cdt) from the study start date (Day -14, 6th June, 2018) to Day 68 (28th August, 2018).

4.2.5.3 Benthic Invertebrate Communities Post-Spill

Benthic invertebrate communities across the limnocorrals showed no discernible change attributable to increasing oil volume (**Figure 4.35**). Of note was the lack of organisms found within the 2.9-L limnocorral, although there were organisms present within this limnocorral based on emergence data. Replicate sampling within the 1.5-L limnocorral indicated a coefficient of variation (CV) of 40.7% of the average total invertebrate abundance. Species richness and diversity (

Figure 4.36) also had no statistically significant relationship with added oil volume ($p = 0.471$ and $p = 0.758$, respectively).

Non-metric multidimensional scaling (nMDS) was used to compute species and "site" scores using data for four taxonomic groups Chironomidae, Trichoptera, Ephemeroptera, and Other (**Figure 4.37**). A stress value of 0.029 and an R^2 of 0.99 indicated a strong ordination following scaling using 1,000 nMDS iterations. Ephemeroptera were only found within the reference sites, 42-L, 1.5-L, and the FC indicated by their position in the ordination. Total Chironomidae, however, show no distinction among treatments and appear diffuse throughout the ordination. A few taxa only appeared in certain limnocorrals (*Paratanytarsus sp.* and *Clinotanypus sp.* in 180-L; *Tribelos sp., Zavrelia sp., Limnophyes sp.* in 18-L; *Macropelopia sp.* in 1.5-L; *Natarsia sp.* in FC), as demonstrated by their positions in the ordination. Beyond these poorly abundant taxa, dominant taxa within the limnocorrals showed no trend in proportions within the limnocorrals based on treatment (**Figure 4.38**).

Chironomidae were the most abundant benthic invertebrate group in all limnocorrals, as was also observed for emergence data. *Cladotanytarus sp.* (Chironominae), *Djalmabatista sp*. and *Procladius sp*. (Tanypodinae) were the most abundant genera (**Figure 4.38**). No clear trend based on treatment was observed for any of these groups.

Spongillidae (freshwater sponges) were found in high densities within all limnocorrals. No relationship with added oil volume was observed (**Figure 4.39**; $R^2 = 0.108$, $p = 0.471$).

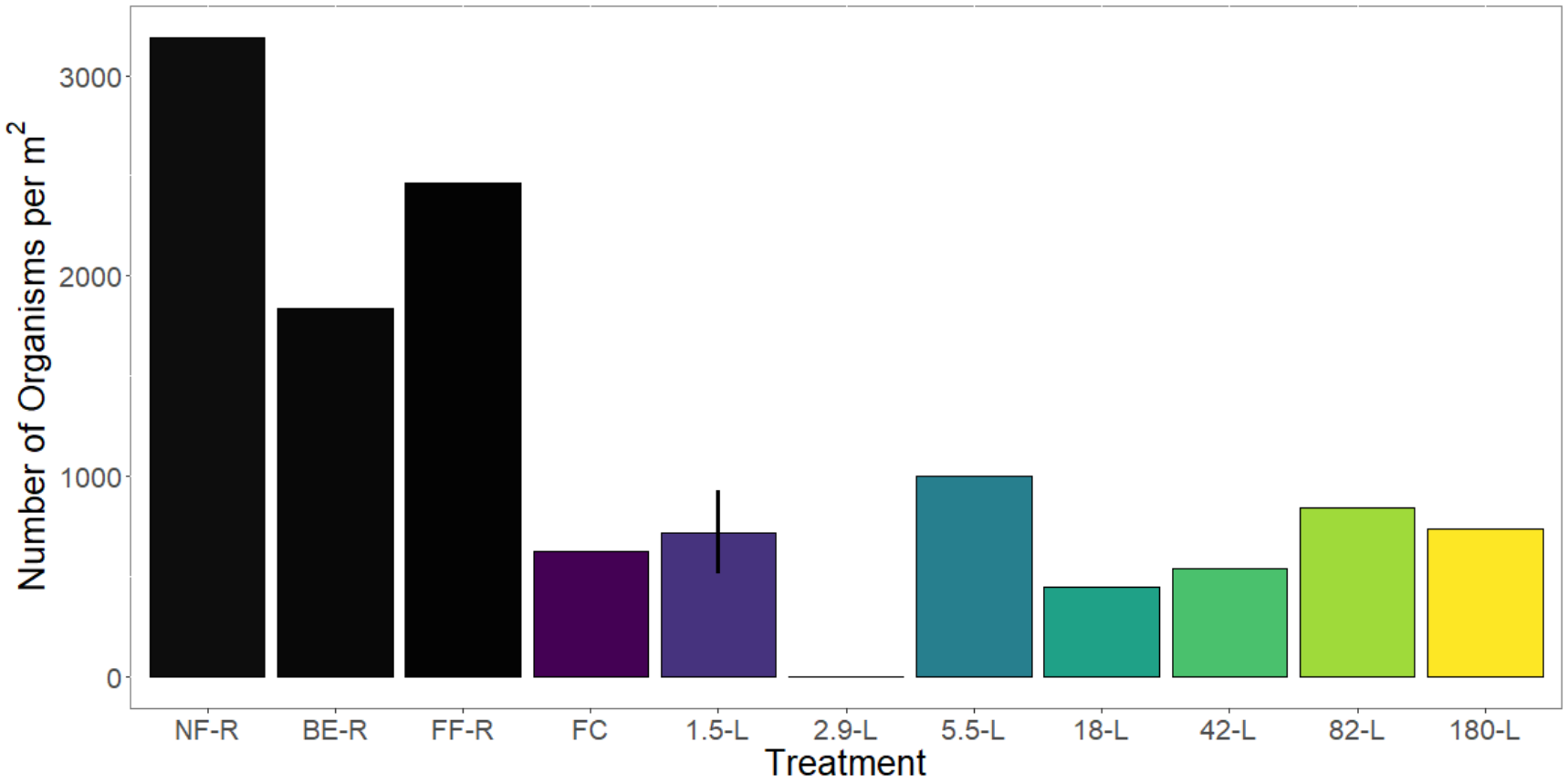

Figure 4.35: Total benthic invertebrate abundance from eight limnocorrals and three reference sites in Lake 260 on the 4th and 5th of September, 2018 (Days 76 and 77 post-spill). The standard error of replicate samples collected within the 1.5-L enclosure is indicated with a vertical bar.

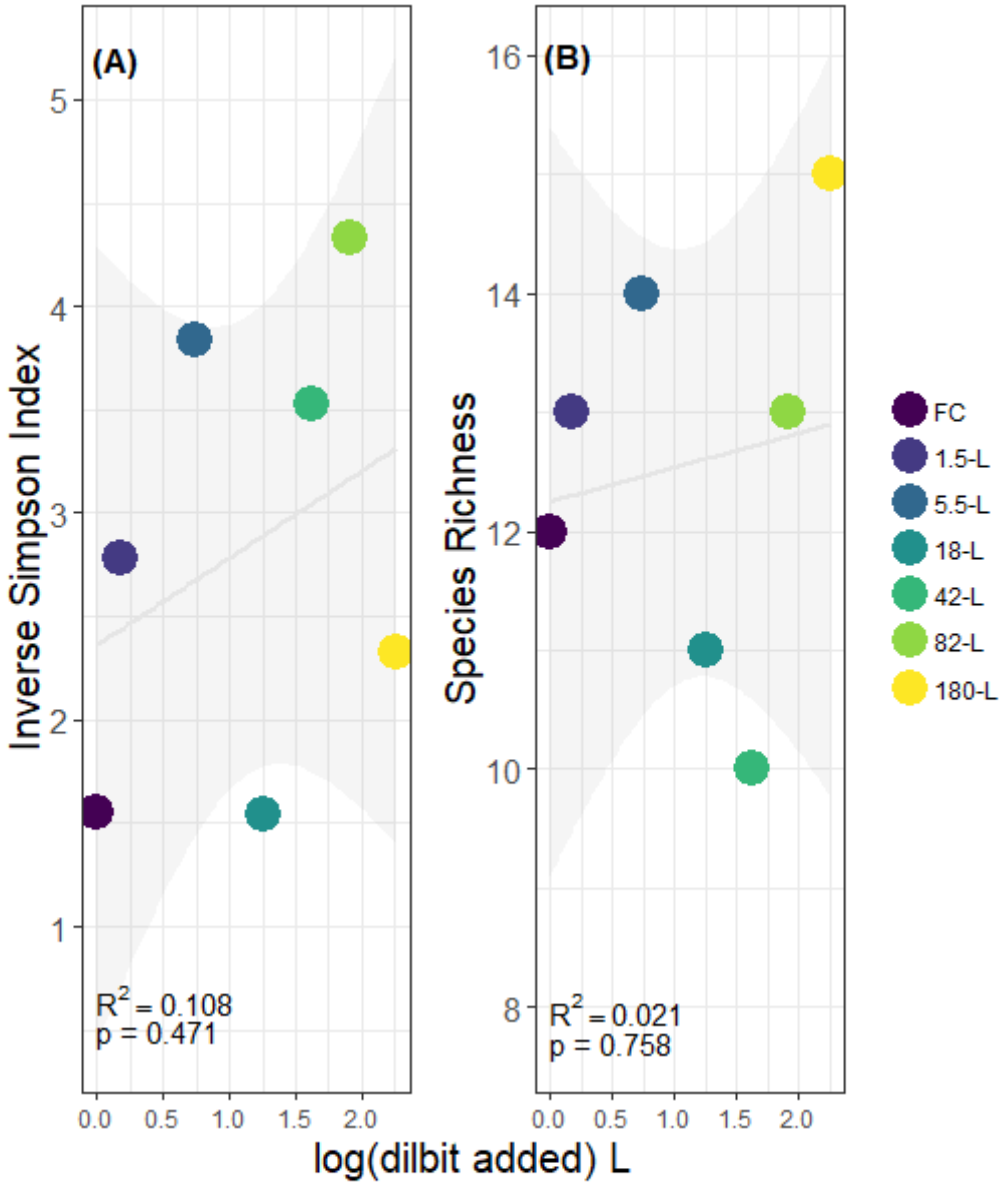

Figure 4.36: (A) Inverse Simpson Index and (B) taxa richness linearly regressed against volume of dilbit added, log-transformed, at the end of the 90-day study (4th and 5th September, 2018). Lines represent the linear model, with shaded regions indicating the confidence intervals associated with model fit (95%). No significance was reported.

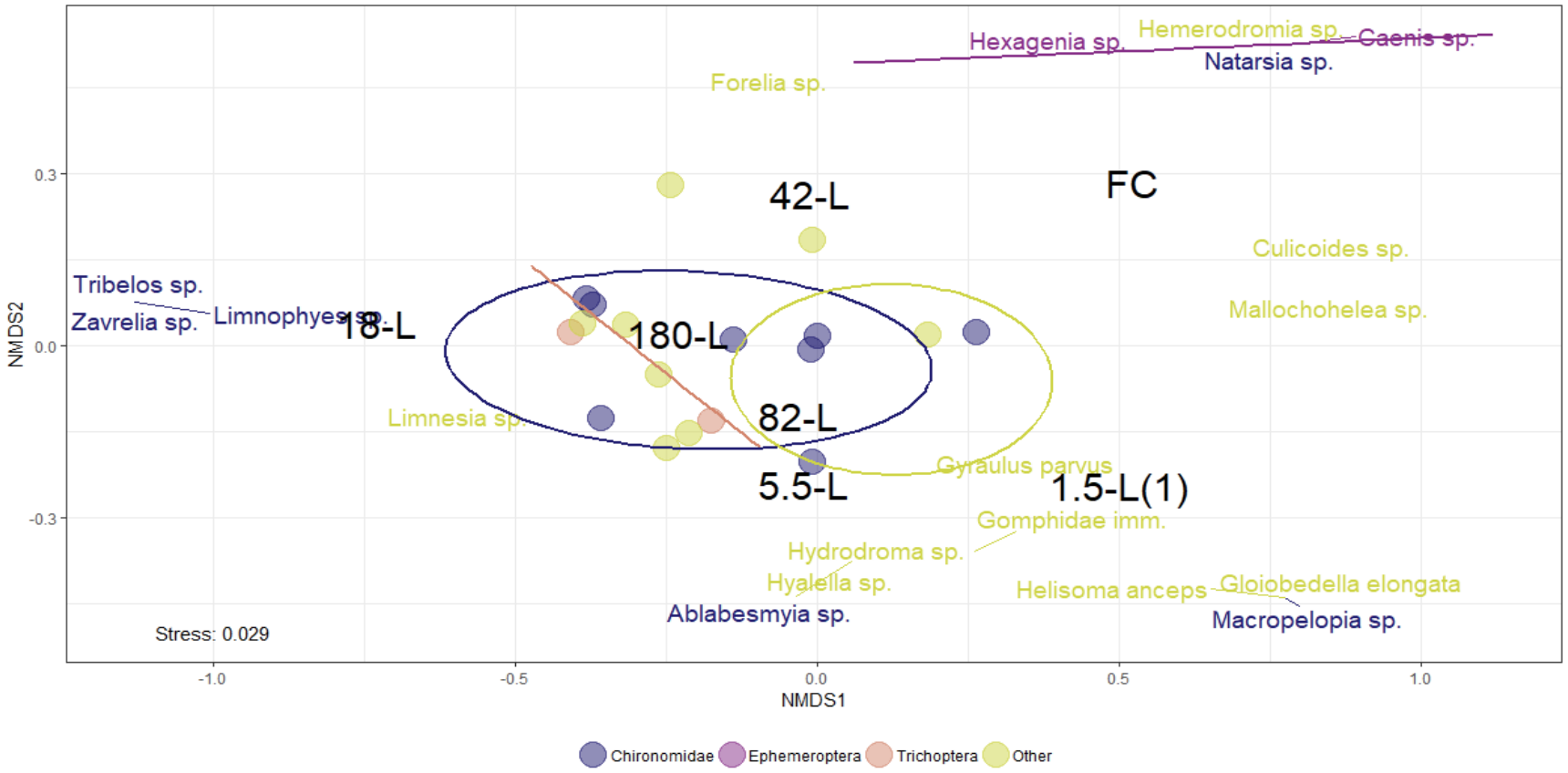

Figure 4.37: Non-metric multidimensional scaling (NMDS) for benthic invertebrate taxa collected on the 4th and 5th of September, 2018 (Day 76 and 77 of study) across seven limnocorrals (2.9-L limnocorral excluded due to absence of organisms) using Bray Curtis dissimilarity index. Stress value of 0.029 indicates a strong fit of the limnocorrals and taxa – this corresponds with a strong linear fit (R^2 = 0.99) based on observed dissimilarity of ordination distances. Ellipses (the lines are narrow ellipses) represent the mean species scores for each group and associated 95% confidence intervals. Species scores beyond +/- 0.5 (NMDS1) or +/- 0.3 (NMDS2) are indicated with the taxa name – others are indicated by a point.

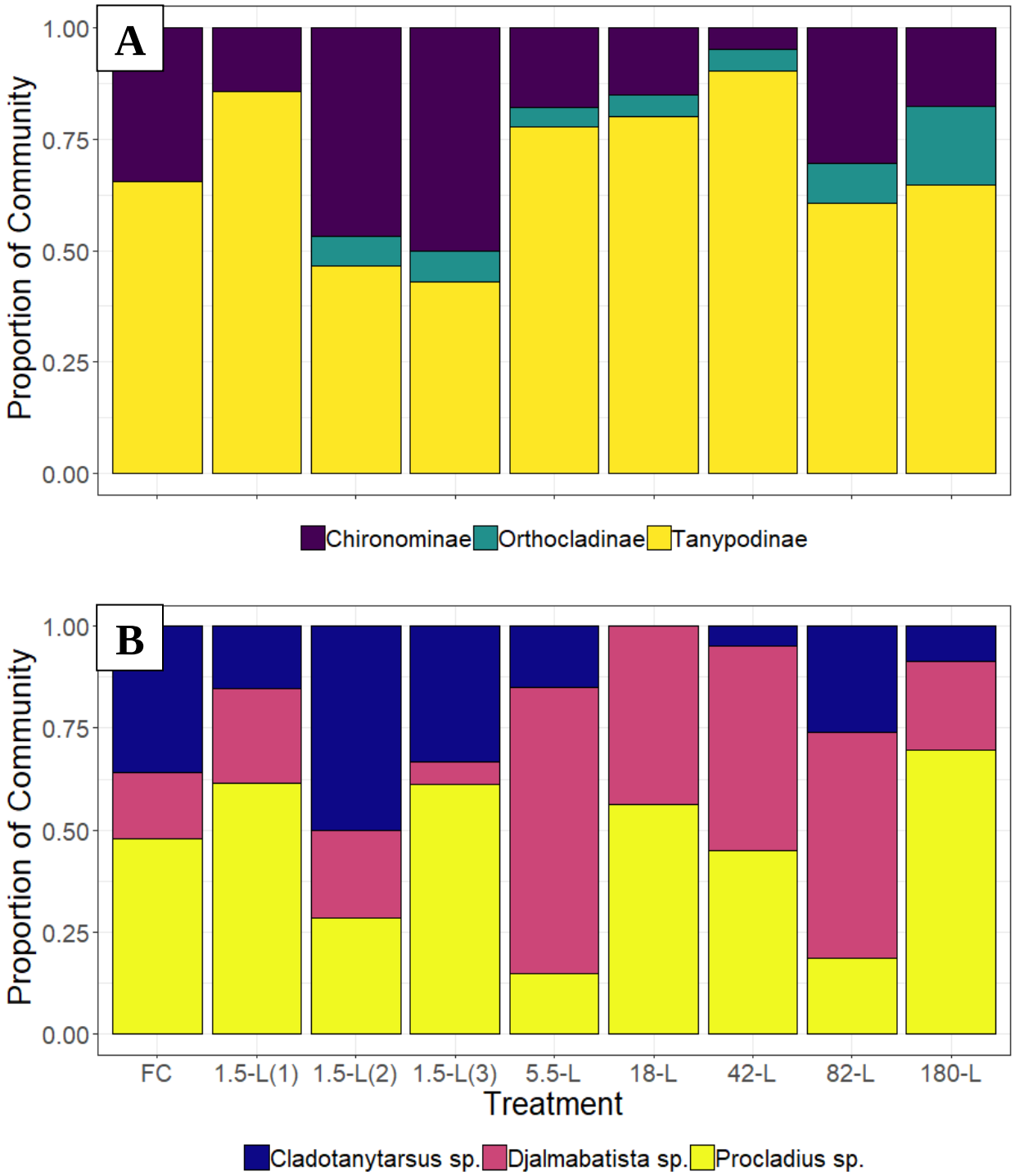

Figure 4.38: Chironomid community composition based on (A) Chironomidae subfamily and (B) the dominant genus' within Chironominae and Tanypodinae. Three subsamples were collected on Day 76 and 77 (4th and 5th of September, 2018, respectively) and pooled to form a composite sample for each limnocorral. Three 1.5-L triplicates were collected and are included to show within-limnocorral heterogeneity.

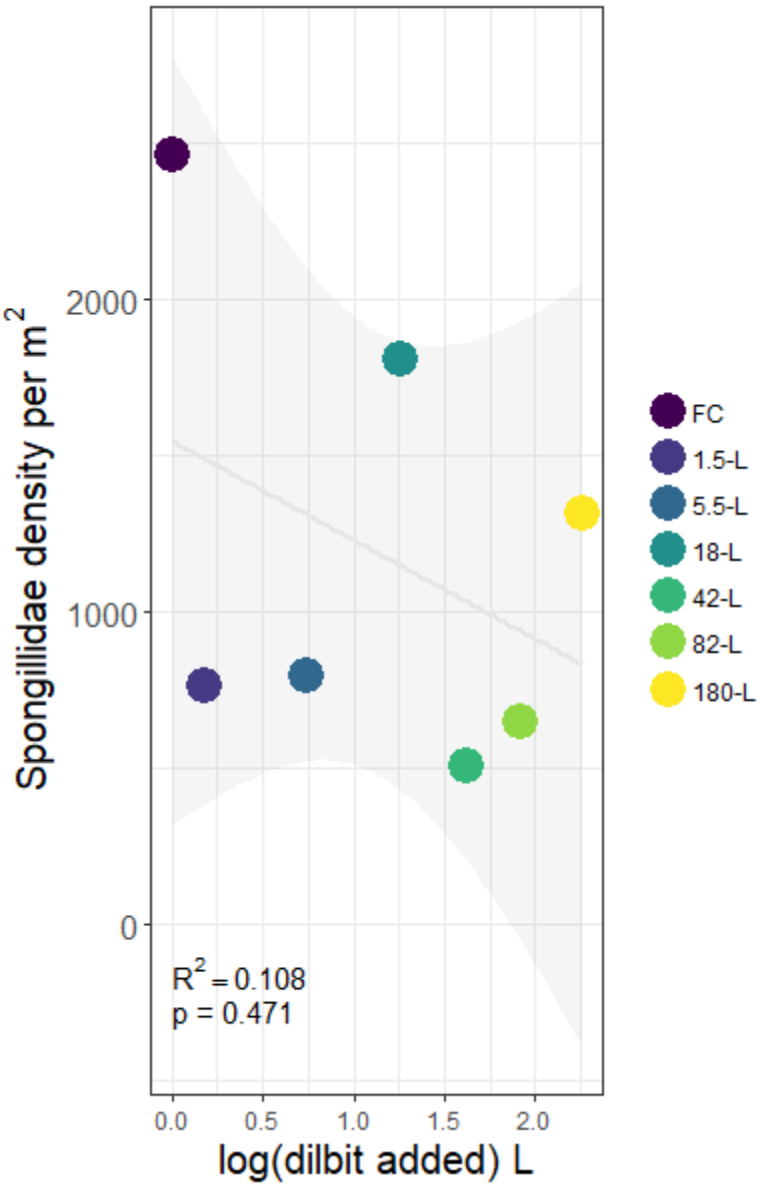

Figure 4.39: Spongillidae (Phylum: Porifera, Order: Spongillida) densities within limnocorrals linearly regressed against volume of dilbit added, log-transformed, collected on the 4th and 5th of September, 2018 (Day 76 and 77, respectively). Lines represent the linear model, with shaded regions indicating the confidence intervals associated with model fit (95%). No statistical significance (α = 0.10) was observed.

4.2.6 Chitobiase

Chitobiase was evaluated at six time points throughout the BOREAL 2018 study, including Days -4, 8, 22, 29, and 42. Out of logistical considerations, zooplankton and other invertebrate sampling did not align exactly with the sampling of chitobiase; however, samples were taken within 48 hours (usually 24 hours) of zooplankton sampling and there were no significant rainfall events between invertebrate and chitobiase sampling that may have influenced dilution or mixing .

All standard curves developed for the analysis of chitobiase samples were within acceptable QA/QC parameters (outlined in **A.4 Chitobiase Data**): no coefficient of variation (CV) exceeded 5% of the mean (based on calculated mean fluorescence of the standards) and the calculated concentration of MUF ranged from 36.4 to 41.6 nM (within 5 nM of the QA/QC standard of 40 nM of MUF). Limit of quantitation ranged from 21.8 nM (July 12th; Day 22) to 55.6 nM (June 17th; Day -4) and the method detection limit (i.e. lowest value that can be detected by the fluorescence assay) ranged from 6.9 nM (July 18th; Day 22) to 17.5 nM (June 17th; Day -4).

Chitobiase production as generated using an 8-hour degradation curve was not statistically significantly related to the volume of oil added to each limnocorral on any day (**Figure 4.40**). Day 22 and Day 29 show the strongest relationships between these two parameters; however, they present conflicting results – Day 22 shows an inverse relationship ($p = 0.092$, $R^2 = 0.4$) relative to an increase in CPR with increasing oil on Day 29. Several samples were found to be below the detection limit for the chitobiase assay based on chitobiase standing activity (i.e. chitobiase present within the water

column following zero hours of degradation) (**Table 4.9**). It may be that invertebrate biomass was too low within the limnocorrals to make use of this assay beyond a relative comparison among limnocorrals.

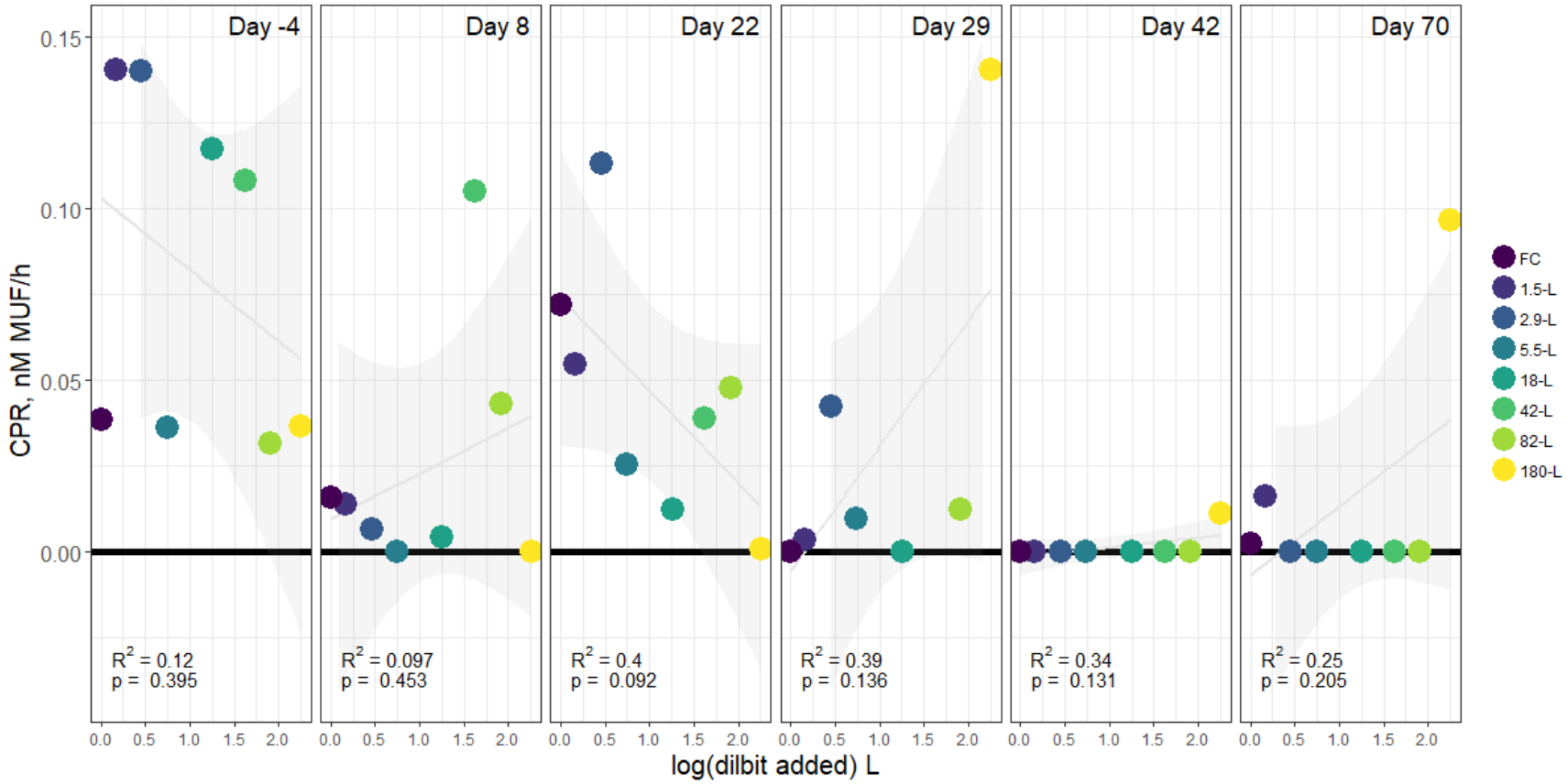

Figure 4.40: Chitobiase production rate (CPR) – measured as nM 4-methylumbelliferyl acetate (MUF) produced per hour– linearly regressed against volume of dilbit added, log-transformed. Chitobiase was measured at the "middle" sampling port of each limnocorral at six time points and MUF concentration determined using a 7-point standard curve generated from known MUF solutions. Values below 0.0 were changed to 0.0, indicating non-detects or no chitobiase production, and may be attributed to the high variability and low sample detection within the limnocorrals. Lines represent the linear model, with shaded regions indicating the confidence intervals associated with model fit (95%). No significance was reported ($\alpha = 0.05$).

Table 4.9: Standing chitobiase activity (nM 4-methylumbelliferyl acetate [MUF] h^{-1}) within each limnocorral at 5 time points (n = 4, mean +/- SD).

Day	Chitobiase Activity (nm MUF per hour)									
	Limnocorral								MDL	LOQ
	FC	1.5-L	2.9-L	5.5-L	18-L	42-L	82-L	180-L		
-4	27.9 +/- 1.5	< MDL	< MDL	< MDL	< MDL	< MDL	20.9 +/- 0.6	22.4 +/- 0.5	17.5	55.6
8	< MDL	11.2 +/- 1.2	< MDL	< MDL	16.7 +/- 1.0	< MDL	< MDL	10.4 +/- 2.2	9.9	31.6
22	11.1 +/- 0.5	< MDL	< MDL	7.3 +/- 0.9	7.9 +/- 0.9	< MDL	9.0 +/- 0.3	< MDL	6.9	21.8
29	< MDL	9.5 +/- 0.5	15.5 +/- 1.1	< MDL	< MDL	< MDL	< MDL	11.7 +/- 1.1	8.3	26.3
42	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	12.4	39.5

MDL= method detection limit
LOQ = limit of quantitation

4.3 Pleuston Bioassays

Water striders (Family: Gerridae) were evaluated to understand impacts of surface oil sheens on water strider survival. Striders were assessed for impairment and immobility over 48 to 96 hours following addition of fresh Cold Lake Winter Blend to (a) 48.1-cm x 26.7-cm x 7.2-cm aluminum test containers and (b) 2.5-m diameter land-based tanks. Rationale for this study stemmed from observations made in the BOREAL limnocorrals following addition of dilbit on 20th June, 2018. In early July, water striders began to proliferate in Lake 260. Although no quantitative assessment of densities was made, it appeared that densities were substantial.

A comparison over several weeks between limnocorrals treated with oil, limnocorrals without oil (FC and NC), and the open lake, indicated substantial differences in presence and density of water striders. No water striders were observed within the limnocorrals treated with oil (indiscriminate of oil volume) prior to August 2018. However, water striders were observed directly outside of these limnocorrals and within the control limnocorrals. As water striders are capable of some degree of vertical movement (i.e. can jump) and were present in similar densities in the controls, this ruled out limnocorral exclusion of water strider communities. To attempt to quantify these observations, counts were performed in mid-August. Between the 14th August and 22nd August, water striders were observed in the FC, NC, 1.5-L, 2.9-L, 5.5-L, and 18-L limnocorrals. Counts in the oiled treatments never exceeded more than 8 striders, except in the lowest treatment (1.5-L) where 3-16 striders were normally observed on a given day. In contrast, counts within the controls often ranged between 8 and 30 striders

on a given day. These data were confounded by high winds in mid-August, limiting observational capability. As such, two assays were conducted to quantify impacts of surface oil to water striders in a closed and controlled environment.

4.3.1 Part 1 – Pilot-Scale Water Strider Semi-Field Bioassay

Striders saw immediate, sustained impacts following the addition of fresh CLB-W (**Figure 4.41**). Except for the control, striders were immobilized in all containers within 96 hours, with 50% immobility being observed within 24 hours of treatment. Impairment in striders occurred immediately across all oiled treatments (i.e. at first observation, 15 minutes, all striders were impaired in some way). The control (0-uL) saw no change in water strider activity or behaviour for the study duration.

4.3.2 Part 2 – Large-Scale Water Strider Semi-Field Bioassay

The initial study was repeated using larger tanks to provide greater room for the striders and to allow a reduction in the oil:surface area ratio in the lower treatments. This would provide for the possibility of observing this ratio and treatments over a greater range.

Much like the prior assay, striders saw an immediate and sustained reduction in mobility and impairment following oil addition; however, this impairment was not observed in the control (0-uL) and lowest treatment (5.7-uL), likely attributed to the increase in test vessel size and oil dilution. Impairment was observed upon first observation (15 minutes) in the other four treatments, with immobility exceeding 50% within 4 hours in the highest treatment, and 8 hours in the other three treatments (**Figure 4.42**). Surface sheens were visible immediately after addition in all treatments but disappeared in the

5.7-µL treatment within 15 minutes and within 1 hour in the 17-uL treatment. Sheen coverage reached 100% in the three highest treatments at 4 hours and was present for the rest of the study.

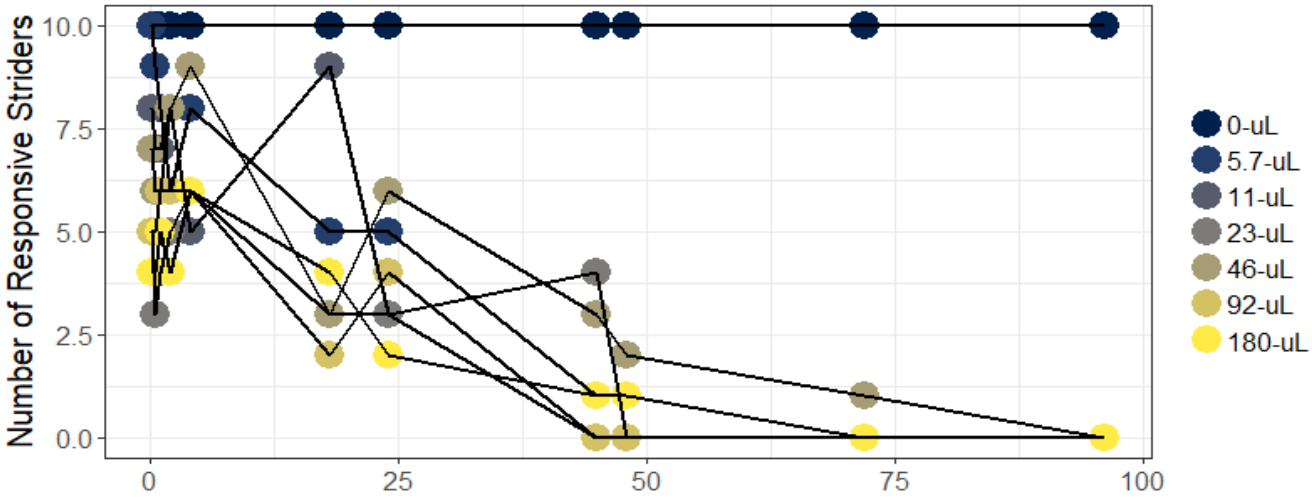

Figure 4.41: Water strider response following the addition of six volumes of oil to small aluminum test vessels. Striders were monitored for 96 hours and evaluated based on responsiveness (i.e. are they immobile).

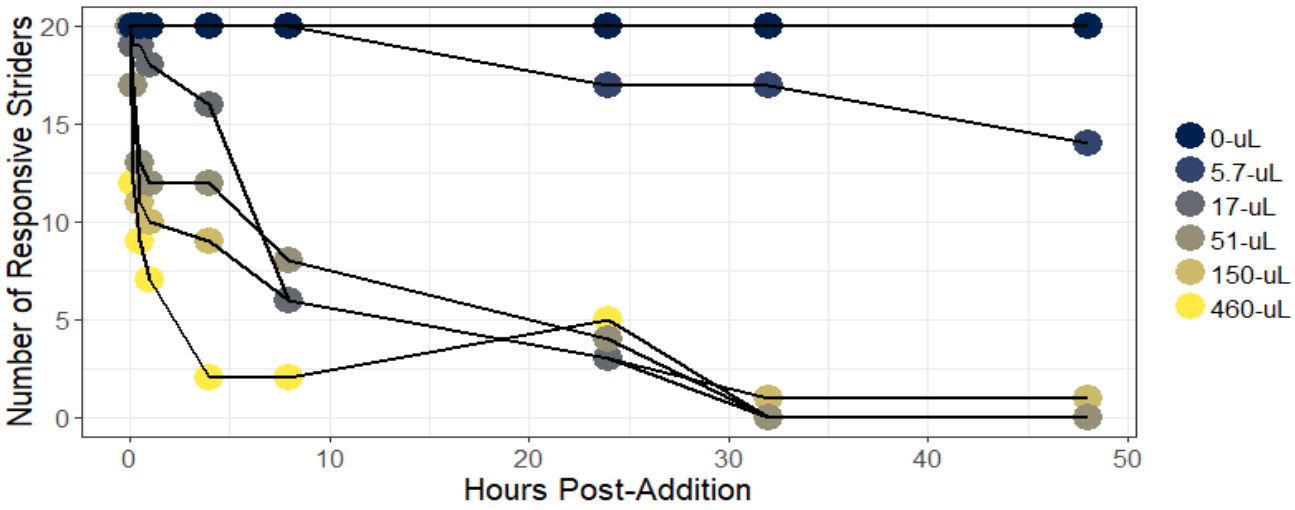

Figure 4.42: Water strider response following the addition of five volumes of oil to large, 2.5-m diameter, tanks. Striders were monitored for 48 hours and evaluated based on responsiveness (i.e. are they immobile).

Chapter 5
Discussion

5.1 Pilot-Scale Mesocosm Study

The pilot-scale study in 2017 assessed how fresh diluted bitumen would behave when added to freshwater in a small-scale modelled boreal environment. Amended zooplankton communities were monitored at the end of the 11-day study and evaluated for community composition and abundance changes. These communities were impacted based on oil volume added and the corresponding trophic-level interactions are reported in Cederwall et al (2019). The pilot-scale study only focused on Hypothesis 1-A (impacts of oil on zooplankton community composition and abundance) and to inform the study of this hypothesis in the in-lake limnocorral study.

Zooplankton abundance was reduced with increasing dilbit exposure, with a marked reduction in the high-dilbit treatment. *B. longirostris*, a small zooplankton that often dominates in contaminated environments (Fulton, 1988; Sterner and Schulz, 1988; Renberg et al, 1990; Vincent et al, 2017; Leppanen, 2018), was impacted the least by the presence of dilbit. They were the dominant zooplankton in the high-dilbit treatment at 54% (1.12 organisms per Litre); however, their absolute abundance did not change much – it was other species, particularly *H. glacialis,* that decreased in absolute abundance within the high-dilbit treatment. This species was dominant within the other two mesocosms and within Lake 240 proper but made up less than 6% of the

community in the high-dilbit treatment. *H. glacialis* has been observed to be impacted by contaminants, particularly by mining operations (Griffiths et al, 2018).

Cederwall et al (2019) reported a decrease in chlorophyll *a* and algal biomass in the control mesocosm relative to initial control concentrations as well and this may be indicative of enclosure-based effects, which will be discussed in detail in Section 5.2. The initial loss in algal biomass may have triggered the decrease in zooplankton biomass (loss of a diverse food source). Given the slight recovery in phytoplankton at the end of the study, it is certainly possible that zooplankton communities (particularly in the high-dilbit treatment) may have recovered to densities observed in Lake 240 and the Control mesocosm following the 11-day study. However, only one time point was evaluated for zooplankton and thus, recovery could not be evaluated.

Given the limitations on this study – no repetition, only one time point – it is difficult to define direct oil impacts on zooplankton. The zooplankton community was monitored for just 11 days and may not fully realize the long-term impacts of an oil spill or the potential for recovery. Juvenile copepods were not classified beyond “nauplii”, “CI to III copepodids”, “CIV to V copepodids” and there may have been subtle changes occurring at finer taxonomic levels.

The results suggest that *H. glacialis* may be more susceptible to the impacts of oil spills, whereas *B. longirostris*, may be more resistant. For a lake low in [Ca], this may present an issue. Due to the impacts of acid rain and a decrease in lake pH, species like *H. glacialis* are adapted for environments low in Ca^{2+} and are more abundant in soft water boreal lakes (like Lake 240 in NW Ontario; Jeziorski et al, 2012; Jeziorski et al, 2015).

Consequently, spills into these lakes may target *H. glacialis* and could cause impacts on lower and upper trophic levels in lakes that are relatively abundant in *H. glacialis*.

5.2 In-Lake Limnocorral Study

This thesis assessed impacts to three invertebrate groups as part of the large-scale BOREAL limnocorral study (**Objective 1**): (a) pelagic zooplankton, (b) benthic invertebrates present within the sediments and insect emergence, and (c) pleuston (water striders). Here, the trophic interactions associated with these communities are addressed alongside: (a) the impact of oil chemistry within the systems through polycyclic aromatic compounds (PACs), BTEX, and how the oil profile of these systems may or may not have influenced community-level and species-level changes; (b) the role of primary producers within the systems using chlorophyll *a* as a proxy for primary productivity, and; (c) the role of fish within the systems and how they may have confounded interpretation of the results observed. My overall conclusions are:

(a) The zooplankton response was largely determined by enclosure effects. Shifts to rotifer and juvenile-dominated communities and a decrease in biomass was seen in all limnocorrals, regardless of treatment. Variable predation by fish grazing also confounded these results;

(b) Benthic invertebrates showed no clear response following treatment, but sampling methods are likely implicated in the inability to fully capture the diversity of the benthic communities;

(c) Emergence impacts were clear, with sheen presence and oil volume strongly affecting insect emergence, and;

(d) Water striders were substantially impacted by even small amounts of oil.

A summary of the main findings of this thesis and the changes to the invertebrate communities following simulated spills of diluted bitumen are shown in **Figure 5.1**, and includes linkages and trophic interactions.

5.2.1 Zooplankton Response

Zooplankton biomass and abundance decreased across all limnocorrals, regardless of treatment. The decrease of zooplankton biomass regardless of treatment and in the controls simultaneously provides evidence that the enclosures were having a greater effect than the oil itself. Zooplankton densities within the limnocorrals were much lower than the lake itself when contrasting samples from the centre buoy in Lake 260 by the end of the study (**Table 4.3**; **Table 4.5**). Overall, it is difficult to disentangle the role of the enclosures from the oil as it relates to zooplankton dynamics.

A distinct change in copepod age groups was observed on Day 13; nauplii became dominant in the smaller dilbit volume treatments and the control whereas little change was observed in the greater dilbit treatments relative to the week before. This was brought about by a decrease in older life stages of copepods; nauplii did decrease in abundance but, relatively, were less impacted than the older copepods. Cross and Martin (1987) indicated minimal impact to marine copepod nauplii (particularly calanoid nauplii) following simulated spills of dispersed and non-dispersed oil. There was a clear

shift in copepod age structure favouring nauplii and a similar response was reported in the Baffin Island Oil Spill (BIOS) project (Cross & Martin, 1987). Following the Deepwater Horizon BP oil spill, a large decrease in zooplankton biomass was observed within the first few days of the spill (Abbriano et al, 2011). Sensitivity of certain taxa have been addressed in lab-based settings and following oil spills (Linden et al, 1987; Suchanek, 1993; Verrhiest et al, 2001; Seuront, 2010; Abbriano et al, 2011; Gerner et al, 2017), but many of these responses cannot be commented on given the similar changes to zooplankton reported within the FC and lower treatments when compared with the higher treatments.

Also observed was a shift in zooplankton community composition to a rotifer-dominated system – these organisms are much smaller than the cladoceran and copepod adults and juveniles and, although they are still relatively abundant within the systems, do not add much to biomass estimates. Rotifers have also been known to fare well in enclosed systems relative to their larger counterparts at the IISD-ELA (M. Paterson, pers. comm.). Enclosure effects and chaos in planktonic communities have been reported in Beninca et al (2008), identifying that predicting species abundance and community composition in enclosed system over the long-term can be difficult and noisy. Hanson et al (2007) indicate interactions between mesocosm zooplankton communities and dependence upon internal factors, such as predator interactions, can also drive observed trends. Shifts in zooplankton present in mesocosms have also been observed (Arnott et al, 2017) and may be a result of a loss of nutrients and external inputs into the system, periphyton growth, as well as shifts in fish grazing rates (Romare et al, 1999; Jack & Thorp, 2002).

Although zooplankton communities may be affected by the presence of limnocorrals, it was expected that zooplankton communities in the limnocorrals would deviate from the lake, but a relative collapse was not anticipated. The presence of walls induces periphyton development and may have encouraged a shift in zooplankton grazing behaviour (i.e. favouring the walls). This may have reduced zooplankton presence around the centre sampling port (a donut effect in zooplankton density throughout the limnocorrals), however, this cannot be proved as sampling only occurred from the centre. This introduced bias into the sampling technique (sampling via a pump system from the centre of the limnocorral) brought about by behavioural changes in zooplankton grazing habits. Regardless of differences attributed to limnocorral *effects*, any among limnocorral conclusions are still valid. All limnocorrals were constructed in the same manner and sampling occurred consistently among them. Zooplankton biomass crashes, like those observed here, have not been known to occur in limnocorrals at the IISD-ELA. *Daphnia* have fared well in 1-m diameter limnocorrals constructed using woven polyethylene (as with the BOREAL study) in Lake 227 at the IISD-ELA, with densities like that of the open lake epilimnion (Paterson et al, 2002). Zooplankton in sealed, deep limnocorrals (i.e. not open to the sediments) also showed no biomass crash, although abundances were smaller than the open lake in a study evaluating predator behaviour of *Chaoborus spp.* and *Mysis relicta* in Lake 239 at the IISD-ELA (MSc Thesis by Seckar, 2009). A zooplankton study looking at deep chlorophyll layers using deep limnocorrals also did not mention any zooplankton biomass crash within the closed systems (Pilati & Wurtsbaugh, 2003). In Lake 260, a limnocorral study with a similar design as the BOREAL project, employed the use of 2-

m diameter limnocorrals to evaluate impacts of ethinyl estradiol (EE2) prior to a whole-ecosystem study (Kidd et al, 2014). This study also observed no crash in zooplankton within the limnocorrals (M. Paterson, pers. comm.). Zooplankton faring well in limnocorrals/mesocosms are also reported elsewhere (Vanni & Findlay, 1990; Graham & Vinebrooke, 2009; Vincent et al, 2017). Ultimately, the major difference lies in fish abundance. As fish, particularly the fish observed within the limnocorrals, are planktivorous, there is a strong probability that their presence had a highly significant impact on zooplankton – possibly greater than the limnocorral effects. This is discussed below in Section 5.2.3.

5.2.2 Benthic Invertebrate Response

Much like the Kalamazoo River spill in 2010 (Fitzpatrick et al, 2015; Lee et al, 2015), surface dilbit within the BOREAL limnocorrals started to sink within the first month (Day 31 in the greatest dilbit treatment) of oil addition. This was also reported in the pilot study (Stoyanovich et al, 2019a; Cederwall et al, 2019) whereby oil began to sink within the first eight days of oil addition. Of initial concern under this scenario was long-term impacts to benthic communities and particularly to juvenile life stages that have the potential to be exposed to oil components present within the sediments for longer periods of time (Dupuis & Ucan-Marin, 2015; Abbriano et al, 2010). Unfortunately, benthic invertebrates were only evaluated within the sediments at the end of the study. At the time of completion of this thesis, oil chemistry data for sediments within the limnocorrals was not available, but there was a clear visible trend of more oil physically embedded in the sediments with increasing treatment. However, regardless of oil volume applied, the benthic community response seemed to follow no clear trend.

There were some differences in community composition noted among limnocorrals. It was observed that Ephemeroptera were only present within the reference sites, the FC, and the lowest treatment (1.5-L); however, this may have been a result of the sampling method employed and not necessarily related to the presence of oil. Ephemeroptera are more sensitive to contaminants than Chironomids and Dipterans (Klemm et al, 1990; Dos Santos et al, 2011). Components of oil, such as benzo(a)pyrene, have been reported to induce genotoxic and neurotoxic changes to Chironomid species in a lab-based setting (Vicentini et al, 2017). Ecdysis (moulting) has also been reported to be impacted by oil (Oberdorster et al, 1999; Song et al, 2017). However, no assessment of gene expression or individual toxicity to these organisms was assessed in this component of the BOREAL project.

When within-limnocorral variability is considered, this raises issues around drawing conclusions based on the benthic data presented here. Triplicate samples were taken in the 1.5-L limnocorral, providing a coefficient of variation of 50% for total benthic density. In demonstrating the impact of the sampling design, two chironomid species (*Tribelos sp.* and *Limnophyes sp.*) only appeared in the 18-L limnocorral. *Zavrelia sp.* was also only present within the 18-L limnocorral and was found in the third replicate for the 1.5-L limnocorral. Consequently, more extensive sampling (via triplicates) observed other species that would not have initially been found, suggesting that we did not capture the full extent of the benthic communities within the limnocorrals. Additionally, there was no organism found in the 2.9-L limnocorral Ponar samples, although organisms were known to be there given emergence trends and the presence of organisms within the emergence traps that same week. Although detectability was reduced given the

sampling method (i.e. rarer species may be missed), it can still be concluded that oil addition had no discernable impact on the benthic communities.

This study would have benefited from a more refined benthic sampling approach to address temporal variation (more than one sampling time point) and baseline monitoring prior to the spill (fall season before application). As previously discussed, it is likely that sampling did not capture all species present and could not give a strong idea of how some potentially key taxa (*Hexagenia*, Trichoptera, etc.) fared post-spill. Organisms that are present in low numbers may require more intense sampling due to spatial variability. Bartsch et al (1998) and Int Panis et al (1995) identified considerations for Ponar sampling. Specifically, changes in sediment type among sites may influence sampling efficiency and the quantification of samples, although in-field visual observations suggested no major differences in sediment types. Passive samplers, such as rock baskets or Hester-Dendy samplers, would also provide an assessment of temporal changes with limited disruption to the system (De Pauw et al, 1986; De Pauw et al, 1993; Merritt et al, 1996; Benoit et al, 1998; Kidd et al, 2014; Graves et al, 2019), though they would have risked oil fouling. Randomization when applying treatments also reduced uncertainty in concluding the lack of impact attributed to dilbit volume.

The decision to avoid oiled areas when conducting the benthic sampling (as outlined in the methods), may have also introduced a degree of bias in benthic assessments. The dilbit treatments with high volumes of oil added (180-L, 82-L) had visible patches of oil (tar mats) sitting on the sediments. In the 180-L limnocorral, tar mats covered upwards of 30% of the sediment surface based on visual assessment. As these areas were

being avoided, sample area would decrease with increasing oil volume. It is unlikely, although not clear, that benthic invertebrates were dwelling under these tar mats and is possible that they moved out to unimpacted areas (this has not been reported in the literature nor observed before). If this were the case, relative densities in these high dilbit treated limnocorrals may be overestimated as a result of the tar mats. This was not corrected here and limited interpretation of impacts to the benthic communities. This will be assessed in future work when more information is available on sediment-bound oil.

5.2.3 Role of Fish in Invertebrate Response

Among the most convincing impacts of fish within the limnocorrals was the presence of white sucker in one of them. The Near-Control (NC) contained four White Sucker at the start of the study, of which only one could successfully be removed prior to treatment. The potential impacts of their presence were observed when evaluating zooplankton total abundance in the Near-Control (**Appendix Figure B.1**). Rotifer communities were markedly elevated in the NC and peaked to well over 500 organisms/L near the end of the study. Fish are drivers of top-down trophic interactions in aquatic food webs – they are top predators and their presence can thereby dictate abundances of zooplankton and phytoplankton in boreal lakes (McQueen et al, 1986; Finlay et al, 2007).

Fish (finescale dace; *Phoxinus neogaeus*) were added to the limnocorrals on Day 21 and were removed at the end of the study (starting on Day 58); however, the number of fishes collected often exceeded numbers added (**Table 4.2**). Fish found within limnocorrals included fathead minnow and pearl dace in addition to the finescale dace added to the systems. It is likely that these species were present upon installation of the

limnocorrals. There are numerous implications of their continuous presence on the zooplankton and benthic communities. First, fish are important invertebrate predators and are well-known to strongly affect both zooplankton and benthic invertebrate abundance and community composition (Finlay et al, 2007). Uneven distribution of fishes among limnocorrals probably resulted in uneven predation pressures exceeding natural levels. Second, fish recycle considerable nutrients, which may have affected primary producers and bacteria in the enclosures, confounding bottom-up trophic interactions (McQueen et al, 1986).

Fishes were only (re)captured in 6 of the 8 limnocorrals (NC was excluded from the study and no fish were captured in the 82-L and 180-L limnocorrals). As an increase in oil volume was attributed to a decrease in fish presence (i.e. no fish captured in these two highest volume treatments), this likely had substantial impacts and confounded the ability to determine direct zooplankton impacts from dilbit addition. Fish density was highly variable across the test systems, ranging from a low of four fish captured in the FC (0 in 82-L and 180-L) to a high of 124 in the 5.5-L limnocorral. Additionally, there was no data collected on fish presence throughout the study so knowing the exact number of fish present at all time points was not possible (e.g. fish may have experience mortality over the duration of the study).

The size ranges among captured fish and the number collected from each limnocorral were large. Fish predation pressures have been observed to reduce copepod and cladoceran abundance, while increasing rotifer abundance as a result of reduced grazing by larger zooplankton. Romare et al (1999) demonstrated this using lake-based

mesocosms. Jack and Thorp (2002) also observed an increased density of *Polyarthra sp.* associated with a decline in *Diacyclops thomasi* in lotic systems as a result of intense grazing. They also indicated a corresponding increase in copepod nauplii with increased larval fish and young-of-the-year grazing pressure. Mesocyclops edax and other copepod zooplankton also graze heavily on rotifers (Brandl & Fernando, 1979). Their reduction, and the reduction in other predatory zooplankton in the limnocorral systems may also be implicated in the rise of rotifer dominance across the systems. However, these changes occurred in all limnocorrals, even where no fish (or very few) were recovered, indicating that the highest dilbit treatments (where no fish were collected) likely had fish grazing heavily at the start of the study where the crash in zooplankton biomass was observed. Not knowing when fish died, assuming they were present, further limits understanding of direct zooplankton impacts following the addition of oil.

5.2.4 Other Trophic Interactions and Environmental Parameters

Chlorophyll *a* and phytoplankton within the size range of 1 to 20 µm only showed a significant correlation with zooplankton abundance on Day 13 (regression outputs outlined in **Appendix Table B.1**). Day 13 (3rd July, 2018) showed the greatest correlations of chlorophyll, emergence (discussed below), and zooplankton total abundance (which was directly correlated with zooplankton biomass) when assessed based on volume of oil added. RDA outputs (**Figure 4.21**; **Figure 4.22**; **Figure 4.23**) provide evidence of a strong correlation between chlorophyll and the 180-L limnocorral, but no correlation was apparent on Day 69, further supporting the univariate data. It has been observed that aquatic systems can become eutrophic following oil spills

(Abbriano et al, 2011) and could be what was observed here, although to a lesser extent and not long-lasting.

Total dissolved phosphorous also showed no correlation with oil treatment throughout the study, indicating no long-term potential for eutrophic behaviour within the limnocorrals. Dissolved oxygen, however, was reduced in the 180-L limnocorral following dilbit addition. The decrease in DO appeared to have no great impact on the invertebrate communities and was likely confounded by impacts of fish presence and enclosure effects. This holds true for the other environmental and water quality parameters assessed. TDN was elevated directly following the spill and may have contributed to the spike in chlorophyll concentrations but did not remain at concentrations found immediately post-spill. Additionally, DOC was steadily increasing in the 180-L limnocorral at the end of the study, although the impacts of this on the biological communities and the cause of this increase are not clear at this time.

Temperature and light intensity within the limnocorrals were different at the start of the study. As the limnocorrals were established close to the northwest shoreline of Lake 260, light differences in the afternoon and evening were apparent. The daily light exposure was different among the limnocorrals attributed to (a) closeness to the shoreline, and (b) position along the shore. The 180-L limnocorral (located closest to the shoreline and farthest west) and the FC (located farthest east) had the greatest difference in temperature and light intensity both pre- and post-spill. The 1.5-L limnocorral was also located closer to the shoreline than the others and did see less sunlight. Light plays a role in both pelagic and benthic primary productivity and has

been observed to be positively correlated with fish production (Karlsson et al, 2009; Finstad et al, 2014). High DOC is also attributed to increased light attenuation and may have implications on benthic primary production (and thereby impacts on benthic invertebrate and zooplankton communities) as production is limited to the upper water column (Jones, 1992; Finstad et al, 2016). The relatively high DOC reported in the 180-L limnocorral did not seem to have any independent impact on light attenuation in this limnocorral. The impacts of light variability are also not clear relative to fish presence and limnocorral effects.

Changes in zooplankton biomass may have also been attributed to the large rainfall events that occurred between the 3rd sampling event post-spill (Day 6; 26th June, 2018) and the 4th sampling event (Day 13, 3rd July, 2018) with major rainfall events (more than 2 mm) on the 27th and 29th of June (**Figure 4.14**). During this time, we can see PACs spike and peak within the water column of all treated limnocorrals between Days 8 and 15, corresponding directly with the events observed here. It is likely that the rainfall – the first instance of rainfall post-spill – triggered disturbance of the oil sheen and mixing of the system and provided an influx of PACs into the systems, explaining the observed changes in zooplankton. No other environmental parameter assessed in this study seems to be implicated in the changes noted on Day 13. Given that no change occurs in the higher treatments based on species and life-stage response and that biomass drops in all limnocorrals, it is hard to definitively state that the changes in zooplankton are due to a PAC flux. No clear change is observed with an increase in BTEX within the water column over the first 96 hours following the oil addition.

5.2.5 Oil as a Physical Stressor: Implications for Pleuston and Emerging Insects

5.2.5.1 Threats to Emergence and Life Cycle Continuity

Emerging insects require a water-air interface free from obstruction to complete their life cycles, emerging as reproductive adults following a larval stage. As such, it was hypothesized that the presence of a surface sheen could limit emergence, and for organisms that could pass through the air-water interface, affect oviposition. This would ultimately limit fecundity and reproductive success of benthic communities. In this study, it was observed that the presence of oil beyond 5.5-L (or a nominal 0.07 L/m^2 based on the surface area of the limnocorrals, assuming the oil spread out evenly across the water's surface) had significant impacts on insect emergence, reducing total emergence by near half by the end of the 90-day period. Over the study duration, pre-spill emergence exceeded 70% in the two highest treatments based on a one-week sampling effort. This contrasts to the control, where this value was less than 6%. Results from the multivariate (PRC) and regression analyses were consistent with the univariate data. The regression-based design provided a strong linear model that fit the emergence data. The reference sites deviated from limnocorral sites, although this was likely an artifact of the limnocorrals which prevented movement of benthos along the sediments, and fish presence. This was observed among benthic and zooplankton data as well.

The presence of oil had a substantial impact on organisms that passed through the surface of the water. Emergence impacts contrasted with the benthic data reported herein, which suggested that the larval organisms dwelling within the sediments were

not substantially affected based on the amount of oil applied. Species-level changes also showed no trend associated with amount of oil applied on Days 76 and 77. It is possible that insect communities that have a terrestrial life stage may be able to sustain themselves even if emergence (and therefore, reproduction) is limited, noted by the continued presence of larval organisms. This would need to be evaluated in a fully closed system over a long period of time to understand at what point benthic communities could no longer sustain themselves due to a decrease in fecundity, a consequence of emergence impairment. In a real-world scenario, it is likely that this would not occur based on surface sheening alone – natural systems are not closed and reintroduction of organisms from other regions is possible.

Formation of tar balls and subsequent tar mats may have confounded these data. As mentioned previously, it is possible that the tar mats induced a migration of the benthic communities to oil free areas. The emergence traps were positioned along the north west edge of the limnocorrals, an area which saw the greatest accumulation of tar mats and surface oil. Corresponding with the need to further understand surface sheen impacts on oviposition and emergence comes the need to understand any migratory potential of benthic organisms following submergence of dilbit. This is a confounding factor that cannot be addressed here as no biotic data was collected on sediments with heavy oiling.

Surface sheens are the first component to be cleaned following an oil spill (via use of absorbent materials and skimming devices) so for an oil spill to sit contained within one area of the water's surface, like in the BOREAL project, is unlikely. Additionally, surface

sheen thickness can still not be accurately determined, so the impacts of sheen thickness were not assessed here (but quantified based on oil volume and an assumed consistent behaviour across the limnocorrals). The hypotheses made here (i.e. surface sheens limit reproduction and may be the driver of benthic impacts following oil spills) still need to be evaluated further. It Is apparent that oil volume is directly implicated in reduced emergence but the mechanism by which this is occurring cannot be stated definitively.

5.2.5.2 Pleustonic Organism Impacts

Water striders live on the water's surface using fine hairs to maintain surface tension and allow them to stride across the water; however, they require intact hairs on their legs to maintain their movement and elevation (Kaitala, 1987). Disruption of this mechanism may lead to immobility and death, as was observed following the addition of oil to systems where water striders were present. Water striders were not observed within all treated limnocorrals for several weeks but were present in high densities outside of the limnocorrals and within the control limnocorrals, an observation that was the impetus for the additional work conducted here.

Much like the Kalamazoo River Spill (Kalamazoo, Michigan, 2010) boreal lake environments may be more susceptible to sustained impacts following an oil spill than marine environments given their extensive riparian and wetland environments (Lee et al, 2015). Riparian environments and shoreline development (D_L; the shoreline to surface area ratio) are far greater in inland lakes and rivers relative to larger bodies such as the Laurentian Great Lakes and oceans. Productivity is greater in these riparian

and littoral environments – a factor associated with increased light penetration, nutrient availability and interaction with the land – therefore, diversity and species densities are higher. Water striders and other pleustonic organisms such as whirligig beetles tend to favour these environments given that they are more sheltered (less fetch) and have greater productivity (more food availability) (Nummelin et al, 1984; White, 2009). Spills into these environments, therefore, may have important impacts for these insect communities based on the data presented herein suggesting effects from even small amounts of oil.

Water striders lay eggs on exposed rocks and macrophytes and overwinter in riparian environments (i.e. under rocks or debris on and around the shoreline). Several species of water striders cannot fly – water striders are wing polymorphic and thereby have wings, have the potential to develop wings, or do not develop wings at all; these traits can be dependent on habitat, food availability, and season (Kaitala, 1987). Striders present in more permanent environments with consistent food sources (such as a pristine freshwater boreal lake) tend to not be long-winged, or winged at all (Jardine et al, 2005; Fairbairn & King, 2009).

Water striders communities may not be able to find refuge from oil spills. The least amount of oil added in this study that saw a substantial effect on striders occurred at nominal 17 uL in 5.73 m^2 (or 2.97 uL/m^2). This is 25,000-fold lower than the 5.5-L limnocorral based on surface area to oil ratios. If an oil spill were to cover the surface of a small lake (these conditions would be possible on a calm evening on a lake with little fetch), it is likely that a resident water strider population could be highly impacted on that

lake. Riparian environments and shorelines are also impacted following an oil spill and may inhibit spill evasion, resulting in strider mortality.

More work is needed to ascertain the long-term impacts of an oil spill on water strider communities as acute assessment indicates great impacts at small volumes of oil. Work should focus on understanding recovery and recolonization of a water body by striders under natural conditions based on the evidence presented here.

5.2.6 Chitobiase as a Proxy for Secondary Production Estimation

The chitobiase data generated from the limnocorrals was difficult to interpret due to low detection and associated noise. Although coefficient of variations based on subsampling are adequate, limits of quantitation had large ranges across limnocorrals and treatment days. Observed chitobiase values often fell below these limits and the method detection limits, reducing the number of useful samples for developing secondary production estimates. An interference assay was conducted and showed no difference in a L260-proper water sample following production of standards using FC and 180-L water; this ruled out any factors associated with PACs and other oil components interfering with the chitobiase assay.

Much of the data at the start of the study were above the methods limits, albeit low, which contrasts with Day 42 and 70 where almost all points were below the detection limit. Zooplankton biomass was also low beyond Day 13 post-spill and may be the reason why chitobiase measurements were too low to be quantified via the chitobiase assay. Emergence also dropped following Day 13 which may have been attributed to a

decrease in benthic biomass (see above). It appears that with low invertebrate biomass, the utility of chitobiase as a useful proxy for secondary production estimates in boreal environments is limited. The assay does not have enough resolution to accurately and consistently estimate secondary production in oligotrophic systems like Lake 260 and the limnocorral systems within it. Sastri et al. (2013) published results using the chitobiase method *in situ* in a mesotrophic lake. Relative zooplankton abundance was greater in Lac Croche than in Lake 260, which may explain their success.

The chitobiase assay cannot discern species-specific changes in the arthropod community. This functions in lab-based studies as the species present are controlled so impacts of a stressor on individual species can be evaluated (as in Richards et al (2008)). Although chitobiase offers ease of understanding total production among these communities, understanding energy shifts following contaminant exposure would be invaluable. Oil spills affect aquatic systems in a variety of ways (surface oil, oil presence on sediments, TPAH and other oil components within the water accommodated fraction, etc.) and reliance on this assay independent of traditionally taxonomic approaches would not realize these changes.

5.2.7 Summary

As evaluated in the Introduction (Chapter 1), little information exists on invertebrate changes following oil exposure in freshwater using whole-ecosystem approaches. More research is needed that addresses dilbit spill impacts using a whole ecosystem approach due to the interesting behaviour of dilbit in freshwater. Most current research has looked at oil spill impacts using lab-based studies. Focus should be on

understanding how food web components interact with one another following oil spills and if this has long-term impacts on the ecosystem. This would allow researchers to understand the intricacies involved in oil spills. This study is one of the first to evaluate how invertebrate communities are impacted under field conditions that consider all food web components and the natural weathering of dilbit in freshwater systems. This study looked at three main objectives and several accompanying hypotheses and are revisited below.

*Evaluation of **Objective 1** outcomes (Characterize the response of invertebrate communities (arthropods) to experimental dilbit spills under field conditions at the IISD-Experimental Lakes Area through analysis of productivity, community diversity, biomass, and function by abiotic and biotic measures. Additionally, a broader lens will be applied to this research by contrasting effects on invertebrates to changes in fish populations and phytoplankton, corresponding with changes in the fate and post-spill concentration of dilbit throughout the water column.):*

Hypothesis 1-A proposed that community diversity among benthic invertebrates, emerging insects, and zooplankton would all decrease with increasing dilbit volumes and concentrations of various oil components (e.g. TPAHs, BTEX components). **Hypothesis 1-B** proposed that invertebrate production would be impacted by changes in primary production resulting from impacts on light penetration and both bottom-up (loss of nutrients and a food source) and top-down (decreased zooplankton grazing on primary producers) interactions. **Hypothesis 1-C** suggested that the health of the added fish within the limnocorrals would impact the invertebrate communities via top-down

grazing pressures and subsequent loss of a food source, resulting in bottom-up impacts on fish populations. Each hypothesis as it relates to **Objective 1** is discussed below.

No clear, sustained, impact – based on volume of oil added, TPAC concentration, and BTEX concentration within the first 96 hours of the study – was observed for zooplankton communities within the Lake 260 limnocorrals (**H1-A**). Day 13 changes in limnocorral biota suggest that rainfall may have triggered a PAC influx into the water column and resulted in a temporary proliferation of phytoplankton; a drop in zooplankton biomass followed. However, these changes did not last and an overall decrease in biomass within the limnocorrals was noted, regardless of treatment.

Emergence reductions of > 50% were noted at volumes as low as 5.5 litres of dilbit. Contrasting with the benthic data, it is likely that the surface oil has a great impact on the benthic communities relative to the oil components present within the water column (**H1-A**). Although the impacts of oil spills on oviposition and other reproductive endpoints still need to be assessed, **H1-A** (specific to emergence) is supported by this work, suggesting that surface sheen impacts probably pose a significant impact. The long-term impacts associated with this still need to be evaluated. As well, future studies should attempt to understand how the presence of tar mats affects colonization by benthic invertebrates and the implications on emergence rates should be assessed.

Day 13 spikes in chlorophyll and, thus, primary production suggested that decreases in zooplankton may have been inducing changes in phytoplankton biomass and vice versa (**H1-B**). However, these changes were not long lasting and may be attributed to fish presence and uneven grazing rates among limnocorrals.

Limnocorrals were observed to deviate from Lake 260 proper, based on benthic abundance and collected emerging insects. Relative to Lake 260 samples collected at centre buoy and in reference sites for emergence and benthic invertebrates, abundance was much lower in the limnocorrals, however this is expected in limnocorral studies. Uneven grazing pressures of fish, with fish numbers ranging from 0 to 124 collected at the end of the study, had large consequences for the invertebrate communities, limiting our ability to understand how oil directly impacted the biota. **H1-C** suggested fish would have an impact, although the impacts observed here were not attributed to oil presence directly but were a result of study design flaws and an uneven presence of fish within each limnocorral. As such, **Objective 1** could only partially be addressed. These hypotheses could not be definitively addressed due to the changes in all limnocorrals attributed to uneven fish grazing.

Further analysis needs to be conducted that combine all environmental and biotic parameters to fully understand the changes that occurred within the limnocorral systems. This includes compiling all fish data (cellular changes, gene expression, etc.), further analysis into phytoplankton species composition and associated impacts to the rest of the food web. Nutrient data (such as DOC and TDN changes observed) need to be evaluated alongside microbial and phytoplankton data to understand how the lower food web was changing. However, there are a number of limitations in study design and study outcome that will limit further analyses and include : (a) loss of zooplankton biomass and increased rotifer dominance within all limnocorrals, regardless of treatment; (b) the imbalance in fish communities within the limnocorrals, and; (c) the

lack of temporal understanding of benthic community change, limiting understanding of acute benthic community impacts.

*Evaluation of **Objective 2** outcomes (Characterize the impacts of surface oil on water strider [Family: Gerridae] movement and survivorship using small and large tanks.):*

Hypothesis 2 proposed that small amounts of oil, creating a surface sheen, would have substantial impacts on water strider populations due to physical smothering causing immobility and death. Physical impacts of oil present on the water's surface were clear – even small amounts of oil had substantial, long-term impacts on water striders (**H2**). This was supported by two studies evaluating water strider exposure to surface oil, indicating a loss of 50-100% of water striders at volumes of oil as low as 11 uL in the small-scale study and 17 uL in the large-scale study. As such, **H2** was supported, however, the long-term impacts associated with this still need to be evaluated.

*Evaluation of **Objective 3** outcomes (Assess a new method of quantifying secondary productivity in a lake environment under field conditions at the IISD-Experimental Lakes Area.):*

Hypothesis 3 proposed that chitobiase production would be positively correlated with total arthropod (zooplankton and benthic invertebrate) biomass and could therefore be used to evaluate impacts of an oil spill on total community production. Given the low zooplankton and benthic invertebrate biomass present within oligotrophic, lentic systems, and particularly when contrasting the limnocorrals with Lake 260 proper, chitobiase measures cannot accurately and consistently provide estimates of

invertebrate biomass. The rate of chitobiase production could not be determined in several sampling events due to high sample variability and low detection (often below method detection limits). Chitobiase as a tool for assessing changes in secondary production following anthropogonic inputs may be useful in more eutrophic systems where biomass is greater and chitobiase detection is improved. Improving method detection limits may also support its use. **H3** was therefore not supported due to issues with sample detection and low arthropod biomass in the limnocorrals.

Figure 5.1: Summary of the invertebrate response following simulated spills of diluted bitumen to freshwater limnocorrals

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Appendix A
Biotic Data Sets

A.1 Zooplankton Abundance Data

The accompanying data file includes abundance data for zooplankton assemblages collected from the nine limnocorrals in Lake 260. Data includes abundance from Day -14 to Day 76 of the study and is separated by taxa and limnocorral.

Zooplankton_BOREAL2018_TABlack.csv

A.2 Emergence Data

The accompanying data file includes abundance data for emerging insects collected weekly from emergence traps within nine limnocorrals and three references sites in Lake 260 proper.

Emergence_BOREAL2018_TABlack.xlsx

A.3 Benthic Invertebrate Data

The accompanying data file includes count data for benthic invertebrates collected on Day 76 and Day 77 of the BOREAL study from nine limnocorrals and three references sties in Lake 260 proper.

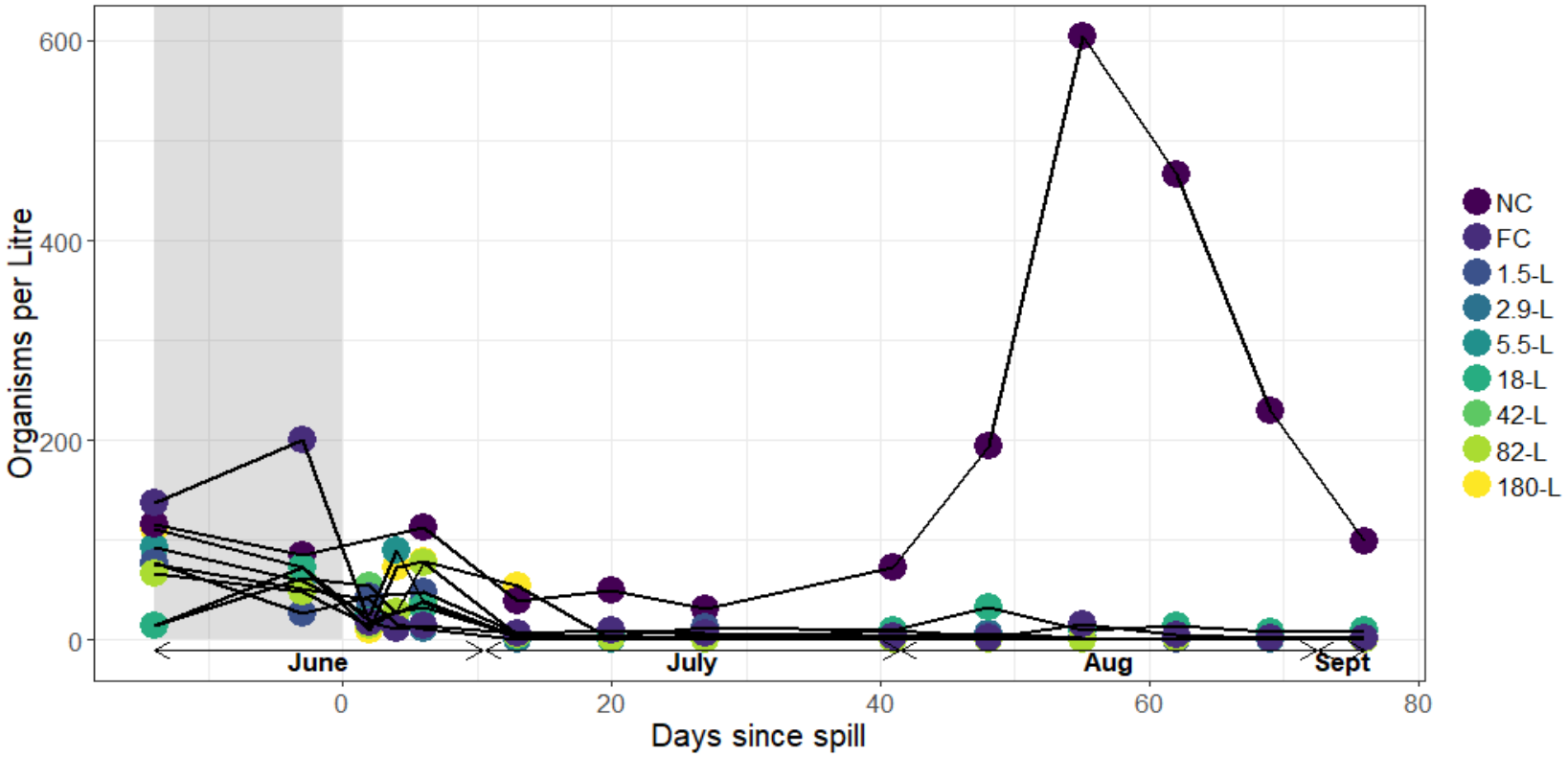
Benthics_BOREAL2018_MWhite%TABlack.xlsx

A.4 Chitobiase Data

The accompanying data files include chitobiase data generated from water samples taken from nine limnocorrals in Lake 260 between Day -14 and Day 76 of the BOREAL 2018 study. Data includes fluorescence outputs and standard curve values used in determining chitobiase degradation rate and chitobiase standing activity.

Chitobiase Data Files_TABlack

Appendix B
Supplemental Tables and Figures

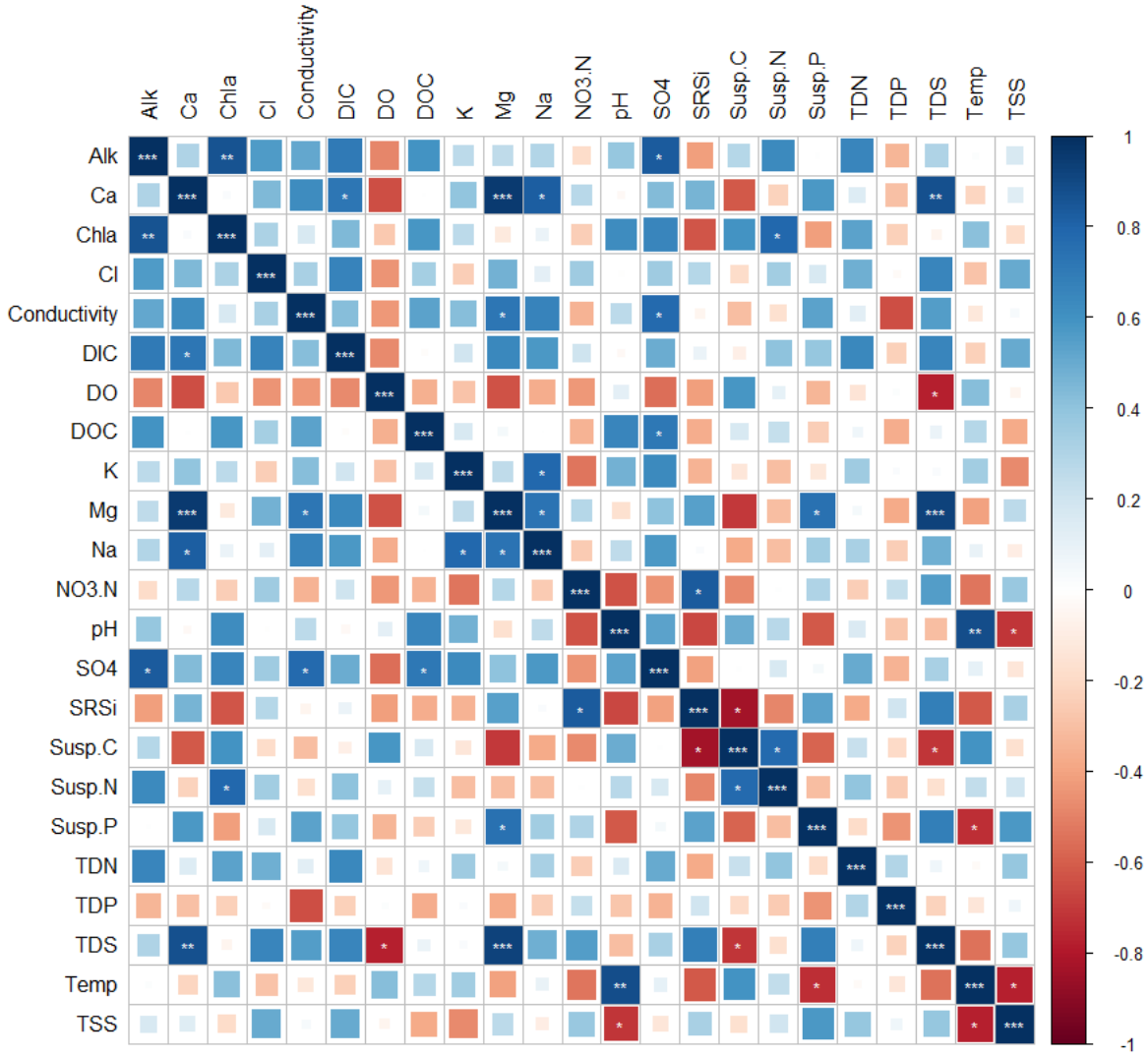


Appendix Figure B.1: Total zooplankton abundance over time within nine limnocorrals, including the near-control (NC). Shaded region represents the time before dilbit application. Zooplankton were monitored for 90 days, from Day -14 (6th June, 2018) to Day 76 (4th September, 2018).

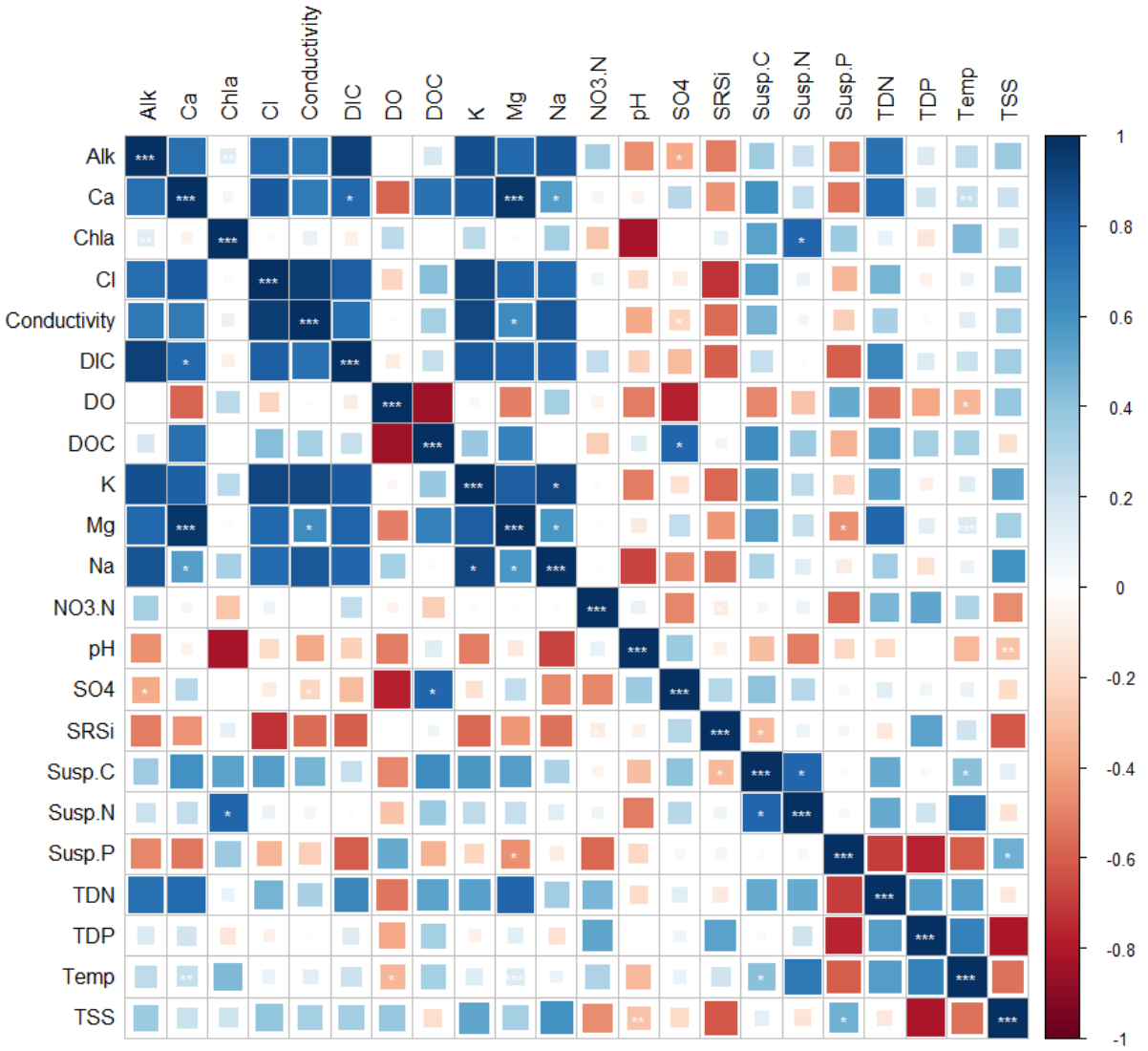
Appendix Table B.1: Regression outputs following multiple linear regression (linear model = zooplankton total abundance ~ mean chlorophyll *a* + fraction of edible phytoplankton (1-20 µm) biomass).

Date	Coefficients	Estimate	Standard Error	*T*	*p*
Day -3	(Intercept)	124.24	240.91	0.516	0.628
	Chlorophyll a (µg/L)	30.19	66.42	0.454	0.669
	Phytoplankton, 1-20 µm	-0.35	0.43	-0.816	0.452
Day 13	(Intercept)	-34.38	17/38	-1.978	0.105
	Chlorophyll a (µg/L)	17.54	5.3	3.312	*** 0.021**
	Phytoplankton, 1-20 µm	-0.07	0.10	-0.665	0.536
Day 69	(Intercept)	4.48	4.48	1.000	0.363
	Chlorophyll a (µg/L)	-1.46	3.24	-0.451	0.671
	Phytoplankton, 1-20 µm	$4.7x10^{-4}$	0.01	0.034	0.974

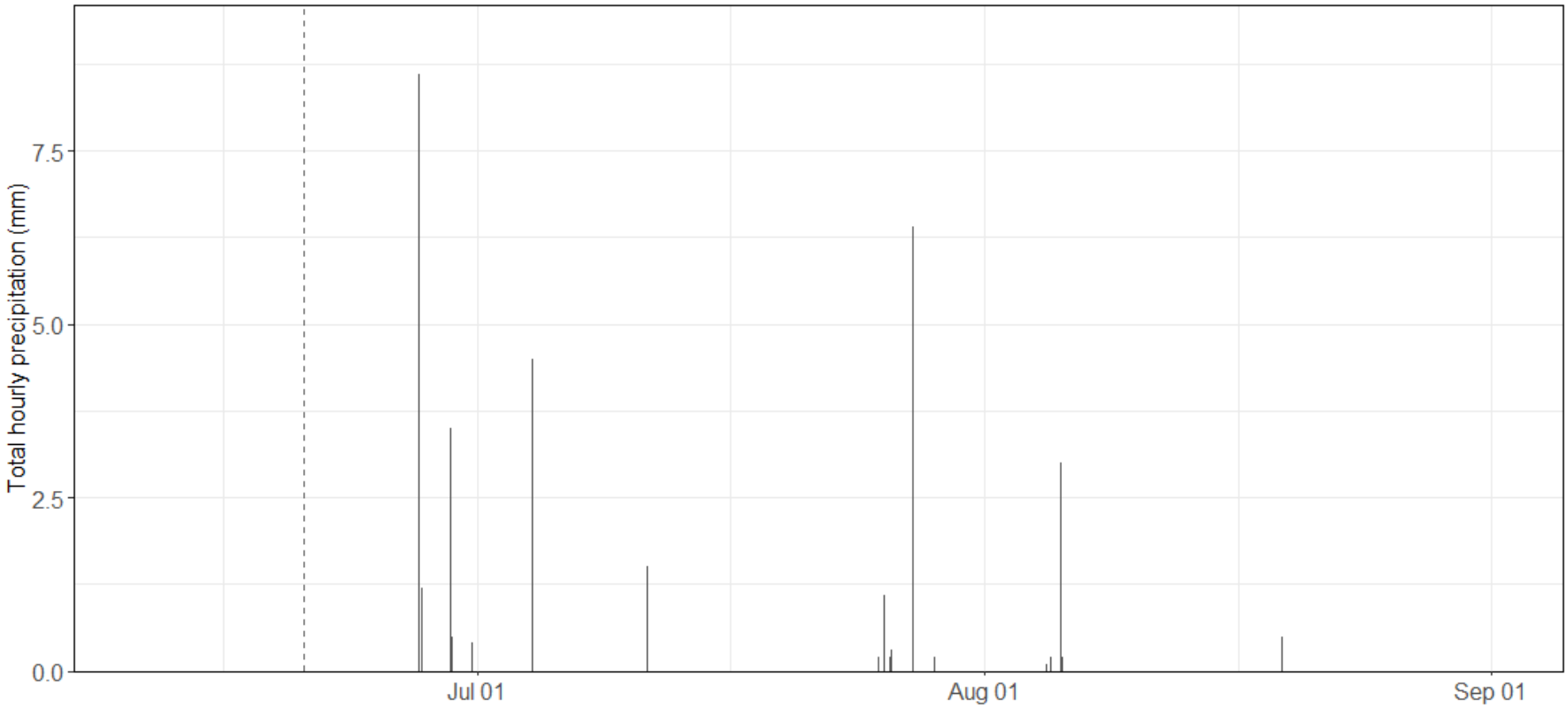
* Phytoplankton data were collected and analyzed by J. Cederwall.



Appendix Figure B.2: Correlation matrix for the measured water chemistry, water nutrient, and physical parameters within the Lake 260 limnocorrals on Day -3 (17th June, 2018). Red colour indicates a negative correlation and blue colour indicates a positive correlation. Shade of colour indicates the magnitude of the correlation. Variables assessed include: alkalinity (Alk), cations (Ca 2+, K +, Mg 2+, Na +), chlorophyll a (Chla), anions (Cl -, SO4 2-), conductivity (Cond), dissolved inorganic carbon (DIC), dissolved oxygen (DO), dissolved organic carbon (DOC), nitrate (NO3 -), pH, soluble reactive silica (SRSi), suspended carbon (Susp C), suspended nitrogen (Susp N), suspended phosphorus (Susp P), total dissolved nitrogen (TDN), total dissolved phosphorus (TDP), total dissolved solids (TDS), temperature (Temp), and total suspended solids (TSS).



Appendix Figure B.3: Correlation matrix for the measured water chemistry, water nutrient, and physical parameters within the Lake 260 limnocorrals on Day 69 (28th August, 2018). Red colour indicates a negative correlation and blue colour indicates a positive correlation. Shade of colour indicates the magnitude of the correlation. Variables assessed include: alkalinity (Alk), cations (Ca 2+, K +, Mg 2+, Na +), chlorophyll a (Chla), anions (Cl -, SO4 2-), conductivity (Cond), dissolved inorganic carbon (DIC), dissolved oxygen (DO), dissolved organic carbon (DOC), nitrate (NO3 -), pH, soluble reactive silica (SRSi), suspended carbon (Susp C), suspended nitrogen (Susp N), suspended phosphorus (Susp P), total dissolved nitrogen (TDN), total dissolved phosphorus (TDP), total dissolved solids (TDS), temperature (Temp), and total suspended solids (TSS).



Appendix Figure B.4: Total rainfall reported on Lake 260 via a weather station set up near the limnocorral site. Rainfall reported here is from 6th June, 2018 to 4th September, 2018.

Appendix C
Chitobiase Assay Protocol

Methodology for Chitobiase Assay. Initially created by Mark Hanson, modified by Scott MacKenzie on August 17, 2011 and Matthew Randell on January 26, 2015.

Preamble

This assay was originally developed by Hanson and Lagadic (2005) as a modification of the method used by Sastri and Roff (2000) to measure chitobiase released by zooplankton and benthic invertebrates, both in terms of ambient levels and rates of production. Hanson and Lagadic (2005) felt that chitobiase activity could be used as an indicator of ecosystem health. The modifications made here will be combined with arthropod biomass data to attempt to investigate a link between chitobiase activity and overall arthropod biomass. Chitobiase is one of two moulting enzymes used to cleave chitin polymers that comprise the exoskeleton of arthropods. This assay allows for the measurement of standing chitobiase activity, as well as the rate of chitobiase production, from field systems. In order to compare effectively between lotic sites and systems, discharge rates should be known in order to correct for volume. To compare between lentic systems the overall volume should be known and used as a correction factor. In this way, the total amount of chitobiase being produced can be calculated. The assay works best in systems with higher microbial activity and thus higher degradation rates, but by adjusting the filtering times (see Section 7.) it can also be used in systems with lower microbial activity. The time points required should be determined prior to formally implementing a study. Prior to starting any experiment, the user should practice and confirm their ability to perform the assay proficiently. The described methodology can be modified for laboratory sampling.

1. Reagents

1. MUF-NAG (4-methylumbelliferyl N-acetyl-β-D-glucosaminide)
   Product Number: M2133-250MG (Sigma)

2. Chromasolv (2-methoxyethanol)
   Product Number: 270482-1L (Sigma-Aldrich)
   Grade: ≥99.9%, for HPLC

3. Citric Acid, anhydrous
   Product Number: 42356-5000 (Acros)
   Grade: 99.6%, reagent ACS

4. Sodium Phosphate Dibasic, anhydrous
   Product Number: S374-500 (Fisher)
   Grade: Certified ACS

5. Sodium Hydroxide
   Product Number: S318-500 (Fisher)
   Grade: Certified ACS

6. MUF (4-methylumbelliferyl acetate)
   Product Number: M0883-5G (Sigma)

7. Millipore 18μΩ water (Milli-Q®-Synthesis)

2. Reagent Equipment

1. 50mL Pyrex Jars No. 5139550 (2 - for 0.3 mM MUF-NAG substrate in citrate phosphate buffer and 5 mM stock of MUF in 2-methoxyethanol) (Via Fisher, Catalogue # 13-646-21)

2. 100 mL Pyrex Jar No. 51395100 (for 5 mM Stock of MUF-NAG in 2-methoxyethanol) (Via Fisher, Catalogue # 06-423-2A)

3. Magnetic stir plate and 2 magnetic stir bars

4. 1L Clear glass bottles (4) (one each for citric acid solution, phosphate dibasic solution, sodium hydroxide solution and citrate phosphate buffer)

5. 100mL graduated cylinder

6. 25mL amber Pyrex Jars No. 5139525 (11 for standards) (Fisher, Catalogue # 13-646-20)

7. Scale, accurate to 0.0001g

8. 100-1000 µL micropipetter (Eppendorf, via Fisher, Catalogue # 21-371-13) with tips (Fisherbrand, Catalogue # 02-707-402)

3. Assay Equipment

1. Spectrometer (Molecular Devices – Spectramax M2)

2. SoftMax Pro v5 (Spectrometer software)

3. 100mL beakers (2) (one for Milli-Q water and one for sodium hydroxide solution)

4. 20-200µL micropipetter (Eppendorf, via Fisher, Catalogue # 21-371-10) with tips (Fisherbrand, Item # 02-707-450)

5. Black, polystyrene, 96 well assay plate (Costar #3915, via Fisher, Catalogue # 07-200-590)

4. Field Equipment

1. 20mL syringes (1 per water sample collected) (Fisher, Catalogue # 03-377-24)

2. 0.2µm syringe filters (4 per water sample collected) (Filtropur – Sarstedt, Catalogue # 83.1826.001)

3. 20mL scintillation vials (7 per water sample collected) (Fisher, Catalogue # 03-377-23)

4. 4 – 1L Mason jars (three for collecting water samples, one for straining sample into)

5. 80µm mesh sieve (to fit over mouth of Mason jar)

6. Coolers (2 – 1 labelled “Ambient” and 1 labelled “Cold”)

7. Ice pack (enough to keep “Cold” cooler cold)
8. Maximum/Minimum Thermometers (2) (one for each cooler)

5. Solutions Required

1. 5 mM Stock of MUF-NAG in 2-methoxyethanol (Chromasolv) stored at -20°C (Clear liquid). Use magnetic stir bar to dissolve MUF-NAG in Chromasolv.
   MUF-NAG MW = 379.36 g
   Mass of MUF-NAG = 250 mg
   Volume of Chromasolv = 132 mL

2. 0.25 N NaOH Solution (pH 14.1)
   5 g of NaOH
   500 mL Millipore 18 μΩ water

3. 0.1 M Citric Acid Solution
   9.6 g Citric Acid, anhydrous
   500 mL Millipore 18 μΩ water

4. 0.2 M Dibasic Sodium Phosphate Solution
   14.1 g Sodium Phosphate Dibasic Anhydrous
   500 mL Millipore 18 μΩ water

5. 0.15M Citrate Phosphate Buffer pH 5.5 (See Appendix I)
   65 mL of 0.1 M Citric Acid Solution
   85 mL of 0.2 M Dibasic Sodium Phosphate Solution
   150 mL Millipore 18 μΩ water

6. 0.3 mM MUF-NAG substrate in citrate phosphate buffer
   1 mL of 5 mM MUF-NAG stock in 16 mL of citrate phosphate buffer

7. 4.12 mM Stock of MUF in 2-methoxyethanol (Chromasolv) (Clear liquid) for standard curve (Table 1.). Use magnetic stir bar to dissolve MUF in Chromasolv.
   MUF MW= 218.21 g
   Mass of MUF = 45.0 mg
   Volume of Chromasolv = 50 mL

6. Creating Standards for Quantification

Ideally these standards would be made using field water (as opposed to Milli-Q water in the citrate phosphate buffer), but if the field water shows no evidence of interference (see Section 16) then Milli-Q water in the buffer is fine.

To do the serial dilutions pre-label all the jars (25mL amber Pyrex), add the listed (Table 1) volume of pH 5.5 citrate phosphate buffer to its corresponding jar. Starting with the highest concentration add the MUF solution via pipette (follow the order of Table 1 from top to bottom). After the addition of MUF solution close the jar, gently turn it over and back to mix the solution then, using a new pipette tip transfer the listed volume to the corresponding jar (next highest concentration).

Each standard is stored in the dark in the fridge (4°C).

Table 1. Serial dilutions for MUF standards - Store refrigerated (4°C) in the dark.

Starting Concentration of MUF solution (nM)	Volume (mL) of MUF solution (first column)	Volume pH 5.5 citrate phosphate buffer added (mL)	Final Concentration (nM)
4 120 000 (4.12 mM stock)	0.02	19.98	4120
4120	9.94	10.06	2028
2028	5	15	512
512	5	15	128
128	10	10	64
64	10	10	32
32	10	10	16
16	10	10	8
8	10	10	4
4	10	10	2

To get the 40 nM QC standard take 0.19mL of 4120 nM MUF stock and add 19.81mL pH 5.5 citrate phosphate buffer.

7. Field Sample Collection

1. Rinse a 1L Mason jar three times in the water from which the sample will be taken.
2. Fill the jar 30 cm below the surface of the water.
3. Transfer enough water through the mesh sieve to another Mason jar to rinse it. Repeat three times. Make sure to leave enough in original Mason jar that it is at least half full.
4. Pour remaining water through the mesh sieve into the rinsed Mason jar.
5. Using a 20mL syringe, draw up sample water and discard three times (to rinse syringe).
6. Fill four pre-labelled glass scintillation vials (for time 1, 3 and 6 hours, as well as the interference control sample) with the syringe. Make sure there is minimal headspace.
7. Attach a syringe filter, press the plunger of the syringe until a few drops come out, then fill the fifth labelled glass scintillation vial (time 0).
8. Be sure not to exceed the maximum volume or pressure capacity of the syringe filter, e.g. get blow through of microbes or prevention of enzyme passing through.
9. Put the unfiltered samples, except the interference control sample, into the “Ambient” cooler containing a thermometer, the temperature should be as close to the temperature of the water from which the sample came as possible.
10. Put the filtered sample and the interference control sample into the “Cold” cooler containing ice packs and a thermometer, the temperature should be as close to 4°C as possible.
11. After one hour filter the time 1-hour sample as in 5) and put into “Cold” cooler. Repeat for times 3 and 6 hours.
12. Upon arrival to the laboratory put all filtered samples in the fridge (4°C) and all unfiltered samples into the environmental growth chamber (same temperature as the water from which the samples were taken, dark).
13. At the same sites as water samples are collected, temperature, dissolved oxygen, conductivity, pH and discharge should also be measured to help explain any differences between sites.

Note: At one randomly selected site the above steps should be conducted three times, i.e. there are three independent replicates from a single river location. This will provide an estimate of the variability in the methodology and of the chitobiase activity in one site. There should also be a minimum of one field blank of Milli-Q water that is a 20 mL scintillation vial, filled in the laboratory prior to going out in the field that is processed as if it is a field sample (i.e. filtered) at a random site to give an estimate of the cross contamination occurring during transport.

8. Laboratory Sample Analysis

All the samples from one site are run on one microplate, along with a standard curve and a QC concentration (See Section 7). The sample blanks are used to differentiate between background fluorescence for the samples themselves and fluorescence due to chitobiase activity releasing MUF during incubation with the MUF-NAG substrate.

1. Allow samples and all reagents to reach 25°C (in the dark) in the spectrometer, an environmental growth chamber or equivalent.
2. Make sure to layout the assigning of wells (blanks, standards, incubated samples, etc...) prior to beginning the assay (see Appendix V for an example).
3. Take a 100 µL subsample of the filtered sample water and transfer to eight wells of a 96 well microplate.
4. Transfer 100 µL of the 0.3 mM MUF NAG in citrate phosphate buffer to four of the wells per sample.
5. Agitate the plate using the spectrometer.
6. Allow reaction to incubate at 25°C for 60 minutes.
7. Add 50 µL of 0.25 N NaOH to all the wells with sample, standard solution or QC solution to stop the reaction.
8. Add 100 µL of the 0.3 mM MUF NAG in citrate phosphate buffer to the four wells per sample that were not incubated with it. (These are the sample blanks. See Section 11)
9. Measure with a spectrofluorometer at 360 nm excitation and 450 nm emission within 10 minutes of NaOH addition.
10. Activity will be expressed as nM MUF liberated per hour, or nmol/L MUF liberated per hour, after determination of the MUF concentration using the standard curve. See Section 12 for sample calculations.

If more than one microplate needs to be incubated at the same time this can be done in the environmental chamber in the dark. Cover with a microplate slip to minimize evaporation and possible contamination.

9. Standard Curve

The standard curve is created using MUF in a dilution series as described in Section 7. A standard curve is required for each microplate run. It is recommended that the standards are added to the microplate prior to the addition of samples so that the sample incubation time is more accurate.

This assay uses citrate phosphate buffer (as the blank or zero) and 2, 4, 8, 16, 32, 64, 128 nM concentrations (the 40 nM standard is used for QC). A new study should confirm that samples will fall within the range of the standard curve

1. Add 100 µL of DI water to 27 of the microplate wells.
2. Add 100 µL of each standard solution to 3 wells (see Appendix V for suggested layout of the microplate).
3. Add 50 µL of 0.25 N NaOH after the 60-minute incubation at 25°C (same time as when it is added to the sample wells).
4. From this data a standard curve is derived (See Section 12 for sample calculations).

10. Sample Blanks

This is required to calculate the chitobiase activity per hour for every sample run (See Section 11). It separates the background fluorescence of the sample from the fluorescence due to the enzyme activity that releases MUF.

1. 0.1 mL sample (same as is being used in the assay)
2. 0.1 mL 0.3mM MUF-NAG in pH 5.5 citrate phosphate buffer
3. 0.05 mL 0.25 N NaOH

11. Calculations

All calculations are done in MS Excel and plotting can be done in SigmaPlot (preferred) or MS Excel.

Standard Curve (See Appendix II for an example):

1. Calculate the mean fluorescence for each concentration.
2. Subtract the mean fluorescence of the blank (citrate phosphate buffer) from the mean of each standard.
3. Plot the fluorescence versus concentration.
4. Determine the slope and r^2 of the trend line.

Chitobiase activity per hour (See Appendix III for an example):

1. Calculate the mean fluorescence of the four wells per sample.
2. Subtract the mean of the unincubated sample from the mean of the corresponding incubated sample.
3. Use the equation of the trend line from the standard curve to determine the equivalent concentration of MUF released in the sample (equal to the chitobiase activity per hour).

Rate constant of chitobiase production (See Appendices III and IV for examples):

1. Graph $\ln(C/C_o)$ vs. time where C is the chitobiase activity at time i and C_o is the chitobiase activity at time 0.
2. Add trend line to the graph.
3. Slope of trend line is the rate constant for the degradation of the chitobiase.
4. It is assumed that the system from which the sample was taken was at equilibrium, therefore the chitobiase rate constant for production is equal to the inverse of the rate of degradation, by taking the positive value of the slope of the degradation curve, the rate of chitobiase production is obtained (in terms of the amount of chitobiase activity in the form of MUF produced per hour per hour).
5. Remember, the slope is a rate constant.

12. Chitobiase in Relation to Discharge or Volume

The discharge, or volume, at each site must be considered because it is in effect a dilution of the chitobiase. Since this assay is used to determine the difference in arthropod biomass between sites, standardization by discharge must occur. This is simply done by multiplying the discharge of the stream at a site, or volume of the lake/pond, by the chitobiase activity found at the same site.

13. Method Detection Limits and Limits of Quantitation

To determine the method detection limits (MDL) blank (citrate phosphate buffer) samples are run as if they were field samples (see Section 9), except that instead of using four wells each of incubated and non-incubated samples, seven wells of incubated buffer are used instead as recommended for environmental chemistry analyses (Wisconsin Department of Natural Resources, 1996).

The MDL is calculated using the standard deviation of the blanks multiplied by the Student's t value for a 99% confidence level (with 7 wells the Student's t value is 3.143).

The 95% confidence interval for the MDL is obtained by multiplying the MDL above by percentiles of chi square over degrees of freedom.

Therefore:

Lower Confidence Limit = 0.64 * MDL
Upper Confidence Limit = 2.20 * MDL

The limit of quantitation (LOQ) is determined by multiplying the standard deviation of the 7 aliquots of the blanks by 10.

14. QA/QC

The guidelines below are to provide the user with as much confidence in the results that are obtained as possible.

1. The coefficient of variation must be less than 5% for all the wells for each treatment of each sample run (i.e. 4 wells of incubated sample from Site X); otherwise that sample must be rerun.
2. All responses in the microplate should be screened for possible outliers amongst the wells for a sample and removed if justified (e.g. statistically or based on methodological errors, etc...)
3. There will be a minimum of three (preferably four) reps per sample in each assay run, as well as for each standard in the standard curve.
4. The r^2 for the standard curve must always be greater than 0.98, otherwise the standard curve must be rerun.
5. There should be no significant chitobiase activity found in the blanks.
6. The 40 nM QC for the standard curve should be within 4 nM of its expected concentration.

15. Measuring Field Water for Interference with Fluorescence and Chitobiase Activity

It is imperative that the field waters be assessed for their potential to interfere with fluorescence of the MUF tag released by the enzyme, as well as the potential of the water to denature or impair chitobiase activity. Both could result in inaccurate interpretation of the data generated.

To do this, follow the steps in Section 8. but instead of using DI water use the interference control sample water. The slope of the standard curve should be equal to the slope of the original standard curve, but the y-intercept will vary depending on the microbial activity and standing chitobiase activity.

A spike and recovery should also be done. This is done by following the steps in Section 8, but instead of using 100µL of MUF-NAG solution add 100µL of the 40nM QC MUF Standard. The MUF equivalent of these wells should be 40 nM higher than those that weren't spiked. If it is less than what would be expected by the 40 nM QC Standard alone, it is an indication of interference occurring.

16. General Advice

Avoid using plastic containers (either jars or vials) because the enzyme may adhere to them, resulting in a reduction in measured activity.

Preliminary data show that it is possible to store filtered samples at 4°C for approximately 5 days without compromising measured chitobiase degradation rates, but storage of samples should be minimized whenever possible.

If reusing containers to store samples, be sure to acid was them between uses.

17. Literature Cited

Hanson, M. L. and Lagadic, L. 2005. Chitobiase as an indicator of aquatic ecosystem health. *Aquatic Ecosystem Health & Management* 8(4):1-10.

Sastri, A. R. and Roff, J. C. 2000. Rate of chitobiase degradation as a measure of development rate in planktonic Crustacea. *Canadian Journal of Fisheries and Aquatic Sciences* 57: 1965-1968.

Wisconsin Department of Natural Resources Laboratory Certification Program, 1996. Analytical detection limit guidance & laboratory guide for determining method detection limits. PUBL-TS-056-96 33pp.

Appendix I: Citrate Phosphate Buffer Recipe

Citrate Phosphate Buffer

A: 0.1M solution of Citric Acid
B: 0.2M solution of Dibasic Sodium Phosphate

** x mL of A + y mL of B diluted to a total of 100 mL

x	y	pH
44.6	5.4	2.6
42.2	7.8	2.8
39.8	10.2	3.0
37.7	12.3	3.2
35.9	14.1	3.4
33.9	16.1	3.6
32.3	17.7	3.8
30.7	19.3	4.0
29.4	20.6	4.2
27.8	22.2	4.4
26.7	23.3	4.6
25.2	24.8	4.8
24.3	25.7	5.0
23.3	26.7	5.2
22.2	27.8	5.4
21.0	29.0	5.6
19.7	30.3	5.8
17.9	32.1	6.0
16.9	33.1	6.2
15.4	34.6	6.4
13.6	36.4	6.6
9.1	40.9	6.8
6.5	43.5	7.0

Adjust pH with NaOH or HCl accordingly

Appendix II: Typical Standard Curve

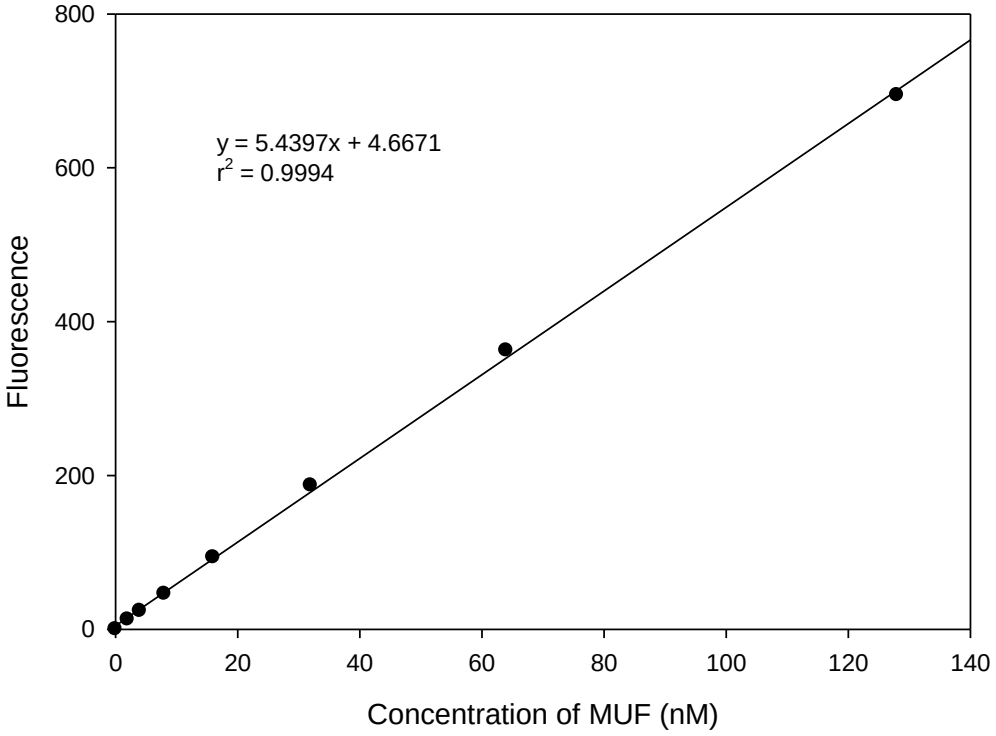


Figure 1. An example of a typical standard curve obtained following the steps in Section 9.

Table 1. Data used to create the standard curve in Figure 1. The blank is the 0nM MUF standard (pH 5.5 citrate phosphate buffer).

Standard (nM MUF)	Fluorescence	Fluorescence above blank
0	25.3554	0
2	37.88546667	12.53007
4	49.07046667	23.71507
8	71.36673333	46.01133
16	118.8208	93.4654
32	212.1062667	186.7509
64	387.6582	362.3028
128	719.6007333	694.2453

Appendix III: Typical Results

Table 2. Example of the data provided from the florescence assay.

	Time (Hours)						Mean	St. Dev.	CV
Incubated	0	721.8862	747.163	747.5586	760.4242		744.258	16.13667	2.168156
	1	681.7366	688.4418	702.8962	699.7018		693.1941	9.837675	1.41918
	3	614.8022	631.5522	631.9342	635.9046		628.5483	9.372978	1.49121
	6	512.1202	522.4752	524.6642	522.4		520.4149	5.628619	1.081564
Unincubated	0	342.179	346.5928	347.4754	340.4946		344.1855	3.37971	0.981944
	1	347.7334	344.8232	351.3682	347.5214		347.8616	2.687024	0.772441
	3	346.8492	346.9186	346.3576	347.4536		346.8948	0.448511	0.129293
	6	343.221	343.5698	345.5446	342.6634		343.7497	1.253475	0.364648

Table 3. Example of fluorescence values (from above) converted into an equivalent concentration of MUF by using the standard curve in Appendix II. Difference is the difference between the incubated and unincubated samples. $\ln(C/C_o)$ is used to create the degradation curve in Appendix IV.

Time (Hours)	Incubated Mean	Unincubated Mean	Difference	MUF Equivalent (nM)	$\ln(C/C_o)$
0	744.258	344.1855	400.0726	72.7	0
1	693.1941	347.8616	345.3326	62.6	-0.14901
3	628.5483	346.8948	281.6536	50.9	-0.35594
6	520.4149	343.7497	176.6652	31.6	-0.83243

Appendix IV: Typical Degradation Curve

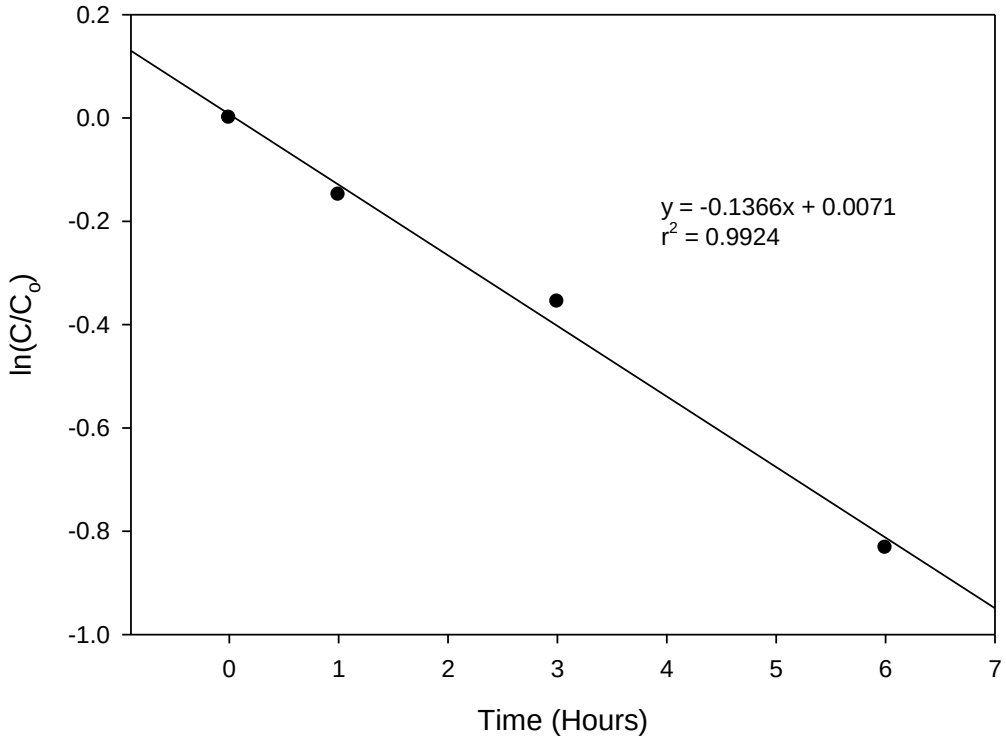


Figure 2. An example of a degradation curve obtained by following the steps in Section 11. From this curve the degradation rate of chitobiase is estimated at -0.1366 nM MUF/hour^2 and therefore the estimated rate of chitobiase production is 0.1366 nM MUF/hour^2 (slope of the curve multiplied by -1).

Appendix V: Layout of Microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample A Time 0, Incubated				Sample A Time 0, Non-incubated				40 nM QC Standard	Citrate Phosphate Buffer		
B	Sample A Time 1 Hour, Incubated				Sample A Time 1 Hour, Non-incubated					2nM MUF Standard		
C	Sample A Time 3 Hours, Incubated				Sample A Time 3 Hours, Non-incubated					4nM MUF Standard		
D	Sample A Time 6 Hours, Incubated				Sample A Time 6 Hours, Non-incubated				Spike and Recovery	8nM MUF Standard		
E	Sample B Time 0, Incubated				Sample B Time 0, Non-incubated					16nM MUF Standard		
F	Sample B Time 1 Hour, Incubated				Sample B Time 1 Hour, Non-incubated					32nM MUF Standard		
G	Sample B Time 3 Hours, Incubated				Sample B Time 3 Hours, Non-incubated				Empty Wells	64nM MUF Standard		
H	Sample B Time 6 Hours, Incubated				Sample B Time 6 Hours, Non-incubated					128nM MUF Standard		

Appendix D
Water Strider Bioassays: Oil Mass Determination

Appendix Table D.1: Droplet masses used to determine nominal oil volumes added to each water strider tank. Oil used was a Cold Lake Winter Blend (CLB-W) with a density of 0.9215 g/m^3.

Droplet #	Droplet Mass (g)
1	0.0049
2	0.0051
3	0.0052
4	0.0058
5	0.0053
6	0.0056
7	0.0054
8	0.0055
9	0.0051
10	0.0052
11	0.0053
12	0.0052
13	0.0051
14	0.0056
15	0.0050
Average Droplet Mass	**0.0053**
Droplet Standard Deviation	**0.0002**
Coefficient of Variation	**0.0458 (4.58%)**