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Evaluating the Potential Impacts of Municipal Wastewater Effluent on Benthic Macroinvertebrates in the Upper Bow River

Sutherland, Aphra

UNIVERSITY OF CALGARY

Evaluating the Potential Impacts of Municipal Wastewater Effluent on Benthic Macroinvertebrates in the Upper Bow River

by

Aphra Sutherland

A THESIS

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Abstract

Municipal wastewater effluent (MWWE) is a common, high-volume, point source effluent that is commonly released to urban aquatic systems. MWWE is a complex mixture containing nutrients and many emerging chemicals of concern (ESOCs), which may vary due to treatment type, receiving environments, and the population served. Outputs of MWWE have been associated with nutrient enrichment (eutrophication) but as populations grow and knowledge of ESOCs improves, it is crucial to understand how nutrient enrichment, and potential interactions of nutrients and ESOCs affect aquatic ecosystems. This study provides updated data on the basal aquatic food web of the Bow River in urban areas of its higher reaches (Canmore and Calgary) through characterizations of the benthic macroinvertebrate assemblages in the mainstem and experimental stream systems associated with Calgary's Pine Creek wastewater treatment plant. Changes to benthic macroinvertebrate assemblages on a longitudinal gradient and cumulative exposure to MWWE in the Bow River were statistically significant and were generally associated with nutrient enrichment (particularly phosphorus). Through the use of different sampling methodologies, MWWE exposures in the experimental streams suggest that the high-quality treated effluent produced by the Pine Creek facility causes consistent, but divergent, cumulative effects in relation to background Bow River source water. In both projects, community metrics that identify types of taxa found in assemblages also described changes to communities more effectively than traditional metrics of urban disturbance such as diversity and richness.

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To all my grandparents, for encouraging and supporting all my academic pursuits

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List of Symbols, Abbreviations, & Nomenclature

ACWA – Advancing Canadian Water Assets

AIC – Akaike's Information Criterion

BOD – Biological Oxygen Demand

BREHA – Bow River Ecosystem Health Assessment

BWP – Bowness Park (site)

CDS – Canmore Downstream (site)

CoC – City of Calgary

COCH – Cochrane (site)

CPOM – Coarse Particulate Organic Matter

CUB – Cushing Bridge (site)

CUS – Canmore Upstream (site)

D – Diptera

dbRDA – Distance based Redundancy Analysis

EEM - Environmental Effects Monitoring

EPT – Ephemeroptera Plecoptera Trichoptera

ESOC – Emerging Substance of Concern

FPOM – Fine Particulate Organic Matter

GRB – Graves Bridge (site)

HBI – Hilsenhoff Biotic Index

MWWE – Municipal Wastewater Effluent

NARS – National Rivers and Stream Assessment

NCA – Nose Creek Adjacent (site)

NMDS – Non-Metric Multidimensional Scaling

PERMANOVA – Permutational Multivariate Analysis of Variance

PMF – Policeman's Flats (site)

RCC – River Continuum Concept

SGI – St. Georges' Island (site)

SIMPER – Similarity Percentage

TKN – Total Kjeldahl Nitrogen

TN – Total Nitrogen

TOC - Total Organic Carbon

TP – Total Phosphorus

WWTP – Wastewater Treatment Plant

Chapter 1: Introduction

1.1 General Background

1.1.1 Biomonitoring Organisms in the Basal Aquatic Food Web

The practice of monitoring the riverine ecosystem health has been increasingly informed by recognizing the complexity of the surrounding landscape, which is shaped by both natural processes and anthropogenic influences (Wen et al. 2023). Lotic freshwater habitats vary dramatically over significant spatial gradients, due to shifts in the surrounding ecoregions and smaller geomorphic features (Allan 2004, Lemm et al. 2021). However, rivers also feature unique connectivity over long distances, which can have significant impacts on the fate of point and non-point source aquatic anthropogenic contaminants (Vörösmarty et al. 2010). While impacts from increasing urbanization, industrialization, and human-driven climate shifts are important to monitor, identifying how natural changes in surrounding landscapes additionally alter river ecosystems is necessary to accurate interpretation of biomonitoring program data. A common measure of health in aquatic systems is observation of aquatic biota that can represent cumulative conditions in aquatic ecosystems (Fig. 1.1) (Effert-Fanta et al. 2022, Linares et al. 2023).

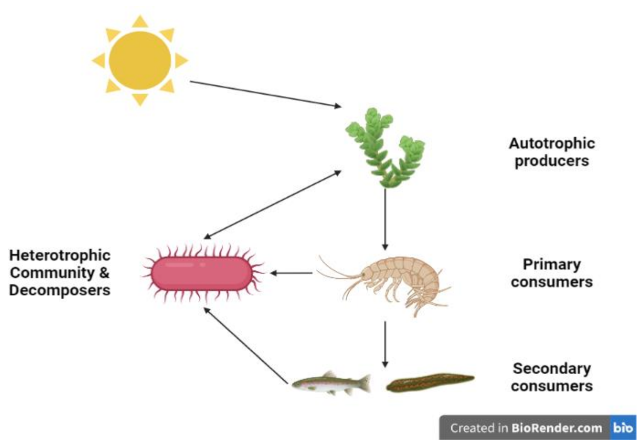


Fig. 1.1 A simplified freshwater food web following energy transfer through trophic levels

The major energy input pathways to aquatic trophic systems may be primarily allochthonous: relying on producer input from the riparian area, or autochthonous: relying on producers growing within the stream. Riparian vegetation entering aquatic systems primarily supports the food web through decomposition. Aquatic autotrophic producers are typically macrophytes (aquatic plants) and periphyton (algal assemblages containing fungal and bacterial symbiotic partners). Primary consumers in streams are generally fish or macroinvertebrates, which includes larval forms of terrestrial insects, other arthropods, and annelids. Secondary consumers in aquatic systems may be predatory macroinvertebrates, fish, or other larger animals. When assessing the health of aquatic ecosystems, examining the health of communities can provide important information regarding the effects of both regional conditions and potential contaminants (Buss et al. 2014). Information can be gathered from larger community-level indices, involving measures such as richness, diversity, and dominance of taxa or functional groups (Juvigny-Khenafou et al. 2021). Alternatively, monitoring can also take place using more physiological measurements of health, such as growth or survival (Juvigny-Khenafou et al. 2021). When well defined, changes in the overall trophic dynamics of an aquatic system can often indicate the condition of other components of the system (Johnson 1993, Herman and Nejadhashemi 2015).

In trophic dynamics, defining the relative importance of top-down predator controls versus bottom-up controls via availability of primary producers is difficult to establish. However, artificial stream experiments manipulating nutrient availability have demonstrated shifts in biomass at all trophic levels, rather than just basal or top levels, which underlines the importance of prioritizing bottom-up approaches in lotic systems where nutrients are a main pollutant (Forrester et al. 1999, Dodds 2007). The fate of nutrients in lotic systems has been theorized as a spiral, where nutrients are displaced downstream until they are taken up into the trophic system

(Newbold et al. 1981). Correspondingly, the basal food web has been shown to be extremely sensitive to changes in nutrient contamination, as nutrient uptake occurs initially in primarily sessile producers, although at varying spatial distances (Lutscher and McCauley 2013). Additionally, as contaminants may be imbibed through trophic interactions, as well as through environmental exposure, the condition of all the basal food web is critical to determining early signs of potential bioaccumulation in higher-level organisms, both aquatic and terrestrial (Burket et al. 2020). These additions can exert pressure for change on lower-trophic status organisms and primary producers have the potential to directly impact the viability of organisms higher up in aquatic food webs. Endpoints stemming from basal organisms such as periphyton and macroinvertebrates, therefore, may be used as bioindicators for the overall aquatic system. Shifts to these groups can indicate imminent issues for larger consumers, inclusive of both humans and organisms directly important to societal practices (Kroll et al. 2021).

1.1.2 The River Continuum Concept

Lotic systems, such as rivers, pose unique challenges in assessing impacts, due to their dynamic nature and length. When predicting effects of cumulative pressures on rivers, hypotheses are often anticipated through the context of initial effects studies that characterize the system (Arciszewski et al. 2017). However, it is also helpful to situate these experimental results in the context of a null model. As rivers flow downstream, there are shifts in their ecological systems unrelated to possible anthropogenic influences. This can complicate attribution of observed changes in rivers to specific disturbances or contaminants. The River Continuum Concept (RCC) provides a basic, unimpacted river system model, which can be used as a key starting point for predicting ecological status of basal food web constituents at various river sites (Vannote et al. 1980). The RCC posits that river systems are shaped by a tendency to maximally reduce energy inefficiencies. A river can be considered "a continuous gradient of physical conditions," and the RCC provides a model for the prediction of how biological parameters will assert themselves so as to use energy most efficiently in those particular physical conditions. The RCC makes broad predictions, based on river size (via Strahler Stream Order), on the dominance of autotrophic or heterotrophic production, the size of particles of organic matter, and the assemblage of functional feeding groups of consumers present in rivers as they flow down from their headwaters (Fig. 1.2) (Vannote et al. 1980).

There have been innovative additions regarding the RCC in recent years that are applicable to the interpretation of biological parameters while biomonitoring riverine systems. A consideration of distance from headwaters, in addition to stream order, has been suggested to address variation in tributary patterns and surrounding environmental (Sánchez-Hernández 2023). The River Wave Concept, built using the RCC and other models, establishes river characteristics as existing in "waves" of material and energy moving downstream differently in various hydrological areas (Humphries et al. 2014). Also, the concept of abrupt "discontinuities" as a natural part of the continuous gradient described in the RCC can be applied to specific natural phenomenon within rivers, such as lakes or tributaries, which show significant localized change in assemblage of macroinvertebrates (Doretto et al. 2020). Discontinuities can also be applied to anthropogenic breaks, such as dams or point source pollution inputs, which similarly, can cause abrupt shifts in the communities present in the local ecosystem (Doretto et al. 2020). Similarly, other adaptations of the RCC model describe rivers as "patches" separated by major discontinuities (Doretto et al. 2020). Particularly among these concepts, identifying the presence of discontinuities related to nutrient point source inputs will be important in clarifying the importance of these pollutants to their receiving systems.

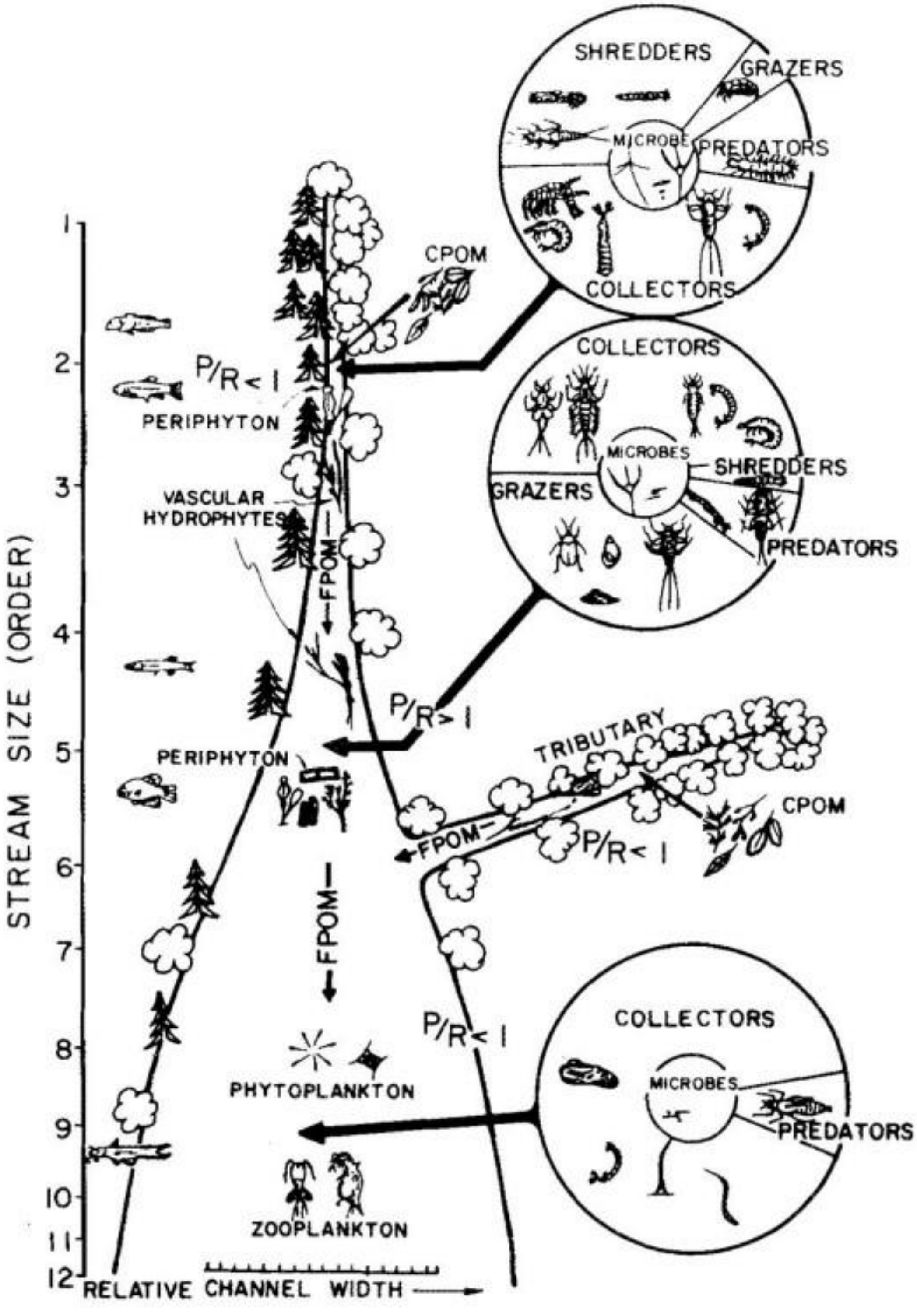


Fig. 1.2. A visual representation of the gradient related changes observed as the basis of the River Continuum Concept (Vannote et al. 1980).MWWE and Aquatic Basal Food Webs

1.1.3 Components of Municipal Wastewater Effluent

The handling of waste products generated in densely populated urban areas has seen dramatic evolution over recent decades. From a societal standpoint, proper handling of sewage is critically linked to a reduction of burden from disease and disease related mortality (Naik and Stenstrom 2012). From an ecological perspective, sewage treatment can also mitigate serious changes in habitat quality and organism health clearly observed with the release of raw effluent in Canada (Holeton et al. 2011). The treatment process in large, continuously discharging wastewater treatment plants (WWTPs) in Canada generally includes a primary treatment to separate out liquid and solid components with a clarifier (Fig. 1.3). Municipal wastewater effluent (MWWE) released into rivers is derived from the liquid treatment, which in addition to the clarifier, is exposed to a bioreactor where micro-organisms break down excess nutrients. Canadian WWTPs typically include a secondary treatment, which involves a second clarifier to further separate solids and filtration. When available, additional tertiary treatment utilizes UV disinfection.

Current regulations for municipal wastewater in Canada address the condition of the effluent prior to release into the aquatic systems but do not require testing of biotic variables in the river where effluent is released. Effluent volume, acute lethality, and carbonaceous biochemical oxygen demand are measured, as well as levels of suspended solids, total residual chlorine, nitrogen, total ammonia, and pH (Environment and Climate Change Canada 2013). Even when treated, the volume of MWWE produced is considerable, with an effluent volume comparable to the volume of wastewater derived from other major industrial processes (Chambers et al. 1997). MWWE is generally released directly into rivers, lakes, and oceans and contains relatively high levels of nutrients (primarily nitrogen and phosphorus-based compounds) compared to the receiving environment (Chambers et al. 1997). This disparity has identified MWWE as a significant pollutant in these aquatic systems, primarily due to the introduction of excess nutrients, but also as a reaction to introduced pathogens, potential antimicrobial resistance, and other numerous contaminants (Holeton et al. 2011, Eftim et al. 2017, Fouz et al. 2020).

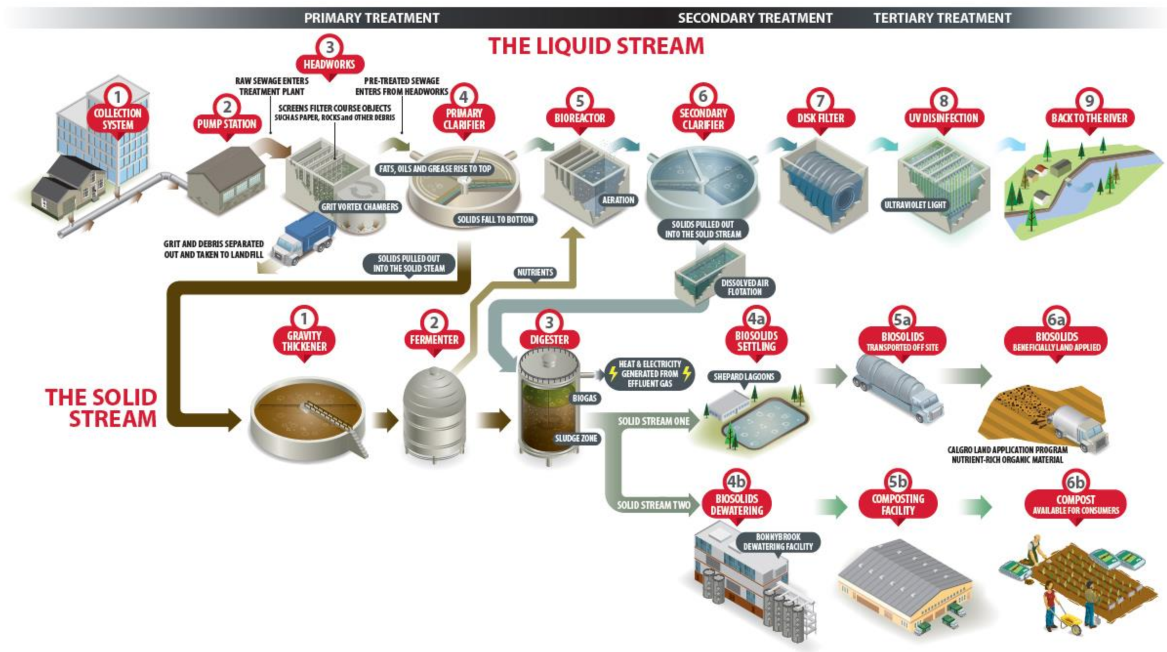


Fig. 1.3. The tertiary wastewater treatment process in the City of Calgary (City of Calgary 2023)

Nitrogen and phosphorus are major nutrients that are essential to the growth of photosynthesizing organisms, but their relative availability has the potential to cause massive shifts in growth and development patterns at all levels of the aquatic food chain when natural levels and limitations are manipulated by the addition of MWWE (Gücker et al. 2006). In extreme cases, nutrient inputs from MWWE are associated with depressions to the oxygen diel cycle and the process of eutrophication, where oxygen availability in water is severely limited due to excessive growth of primary producers including macrophytes and algae (Holeton et al.

2011). This can drastically affect the viability of aquatic ecosystems, particularly affecting biodiversity and productivity, although less severe effects are often associated with increases in size and abundance of some organisms, due to increased food availability (Aristone et al. 2022). While improved nutrient removal treatments have reduced severity of some of these outcomes, recent research shows the presence of many Emerging Substances of Concern (ESOCs) that are strongly associated with MWWE and are an additional, growing concern (Chambers et al. 1997, Deblonde et al. 2011, Holeton et al. 2011).

Most ESOCs (roughly quantified at ~40 000 unique compounds) are not clearly governed under nationalized Canadian guidelines due to a lack of knowledge of their individual and interactive impacts (Diamond et al. 2011). While many of these chemical mixtures are derived from industrial processes and their associated runoffs, a significant portion of ESOCs enter aquatic systems through MWWE from WWTPs (Deblonde et al. 2011). A significant number of the substances emerging from MWWE fall under the umbrella of pharmaceuticals and personal care products, including examples such as painkillers, antibiotics, and diabetes medication. Many of these ESOCs not only survive WWTP treatment but remain active after release into aquatic systems. Many pharmaceuticals are specifically designed to promote effects on organisms with the correct receptors, meaning that their potential for interaction with the ecosystem is high, especially animals and specifically vertebrates (Deblonde et al. 2011, Geiger et al. 2016).

Crucially, when stemming from MWWE, compounds are released not individually but rather as complex cocktails of different ESOCs (Geiger et al. 2016). Complex mixtures can act in a number of ways: they may be additive, where combined effects are equal to their independent effects; they may also be antagonistic and their combined effects mask effects of one or the other; or they may be synergistic and produce unexpected effects beyond what was predicted for either substance alone (Howard and Webster 2009). For example, the antagonistic tension between the increase of productivity associated with nutrients and toxicological effects on organisms associated with many contaminants can mask the true effect of either (Chambers et al. 1997). Many ecotoxicological studies examine compound toxicity (through endpoints such as survival or reproductive output) individually at a variety of doses and pair this information with production volume of these substances to assign impact potential (Diamond et al. 2011). While this provides important in-depth data on the relative toxicity of studied ESOCs, this type of study struggles to quantify theoretical cumulative assessment of environmental impacts (Berger et al. 2016). The magnitude of ESOCs underlines the need to ascertain current cumulative impacts through in situ research, in addition to inferring potential impacts through laboratory identified modes of action.

1.1.4 Monitoring MWWE in Aquatic Basal Food Webs

MWWE can also indirectly affect the basal food web through changes in the overall environmental condition of the aquatic system. Eutrophication has been established to significantly shift the availability of dissolved oxygen and diel cycles in aquatic systems (Holeton et al. 2011). Increases in production can change light regimes, which can affect the success of all organisms throughout the food chain (Vannote et al. 1980, Hill et al. 2010). Temperature has also been demonstrably affected by wastewater inputs, typically raising the overall temperature of a system (Holeton et al. 2011). Variation in the hydrograph is also a necessary consideration for assessing MWWE contaminants, including ESOCs, as concentrations vary with river volume (Bai et al. 2018).

As low-level consumers, benthic macroinvertebrates are more ubiquitous than higher trophic level organisms, with quicker generational turnover, and yet are still relatively long-lived, allowing them to provide important comparative insights into multi-year conditions between sites (Johnson 1993, Johnson and Hering 2009). Nutrient inputs are also a significant driver of benthic macroinvertebrate health, where direct nutrient impacts typically increase the presence of herbivorous macroinvertebrates (Forrester et al. 1999, Aristone et al. 2022). However, certain macroinvertebrates that are sensitive to changes in oxygenation, temperature, or sedimentation can be negatively impacted by the presence of MWWE. This can be seen in changes to benthic macroinvertebrate abundance or changes to community composition and particularly in changes in overall diversity (Berger et al. 2016, Peralta et al. 2020). Effects of ESOCs on benthic macroinvertebrates are not as well investigated as those in higher level consumers, such as fish. However, demonstrable exposure impacts typically show negative outcomes to benthic macroinvertebrates, through direct toxicological effects, indirect reproductive impacts, or shifts to available food (Kidd et al. 2014, Bundschuh et al. 2020, Konschak et al. 2020, Lencioni et al. 2020). Because of the wide suite of ESOCs present in MWWE, it may be necessary to address impacts to the basal food web through multiple endpoints. Identifying differences in the effects of nutrients, more associated with changes in community structure, and effects of ESOCs, which typically are associated with negative impacts to fitness traits such as survival and fecundity, may allow for more effective detangling of the effects of complex MMWE mixtures.

1.2 The Bow River Watershed

1.2.1 MWWE in the Bow River

The 25 000 km^2 Bow River watershed located in Southern Alberta is home to over 1.5 million people (Stewart and Bennett 2017). It provides 33% of the province's population with freshwater, making it a crucial resource for agriculture, industry, power, and drinking water (Stewart and Bennett 2017). The Bow River is a nival system associated with high flow events during spring melt and lower flows during times of low precipitation and high temperatures (Whitfield and Pomeroy 2016). High flow events, such as the relatively recent flood of 2013, cause morphological, biological, and chemical change in the river, such as re-setting biological communities by scouring away aquatic plants and depositing sediments (Milner et al. 2018). A dynamic system like the Bow River presents challenges in monitoring anthropogenic impacts but its high-quality condition also provides a unique opportunity for examining point source impacts.

High-level categorization of stream features in the RCC is based on Strahler Stream Order. The Bow River around Calgary falls into the upper portion of medium sized streams. Medium sized streams are characterized in the RCC as having a lessened reliance on terrestrial inputs, higher autotrophic production, and a shift in size of particulate organic matter from coarse (CPOM) towards fine (FPOM) (Vannote et al. 1980). Relatedly, there will be an increased presence of grazing macroinvertebrates and a reduction in shredders (Vannote 1980). An examination of the Oldman and South Saskatchewan River (of which the Bow River is a tributary) demonstrated that macroinvertebrate communities along the river historically aligned reasonably well with predictions from the RCC in that macroinvertebrate functional feeding groups generally reflected a shift from reliance on CPOM towards FPOM along a downstream gradient (Culp and Davies 1982). However, the narrow and shaded headwater streams described by the RCC does not fit all streams well, which could affect the patterns observed in a river like the Bow, that starts out relatively wide and shallow (Wiley et al. 1990).

Most agricultural pressure on the Bow River occurs downstream of the City of Calgary (CoC), emphasizing the importance of exposure to MWWE in the upper reaches, which occurs at a number of point sources between its headwaters and below the CoC. The largest WWTPs in this area are found in Banff, Canmore, and the part south of the CoC. Within the CoC, a number of tributaries (notably the Elbow River, Nose Creek, and Fish Creek) flow into the Bow bringing MWWE, agricultural, and industrial footprints from other areas. Notably, MWWE is also released from a WWTP on the Kananaskis River and from a number of smaller treatment plants (including sewage lagoons) both on the Bow mainstem (at Exshaw) and within tributaries, such as Nose Creek.

The CoC has been proactive with municipal wastewater management, adding targeted removal of phosphorus and nitrogen compounds in MWWE to their WWTPs between 1987 and 1990. This addition corresponded with a large reduction in nuisance macrophytes below WWTP effluent discharges after treatment improved (Sosiak 2002). Currently, all major WWTPs up to and within the CoC employ tertiary treatment regimes, and while nutrient contamination still occurs, nutrients levels in the resulting effluent are below acceptable nationally defined limits (Environment and Climate Change Canada 2013). However, testing in the Bow River above and below WWTPs suggests that only select ESOCs are removed by the CoC's treatment system with higher occurrences and concentrations of ESOCs occurring downstream (Chen et al. 2015).

1.2.2 Relevant Background in the Bow River

There are several parameters that can dictate how long it will take a river to show recovery from point source nutrient inputs. Previous Bow River projects have demonstrated that recovery distances for WWTP effluent inputs are significant. Biological, and chemical tracers in the Bow River in 2002 and 2003 projects demonstrated that recovery to pre-input nutrient concentrations and biomass ranged from 30-50km downstream from WWTPs (Hogberg 2004). Additionally, anthropogenically traced nitrogen stable isotope levels in the Bow increased dramatically downstream of the Bonnybook WWTP and did not recover until 80-100km downstream, although agricultural inputs may also prolong this pattern (Hogberg 2004). This is relevant in understanding the role that WWTPs have on downstream sites, from both Canmore to the CoC and sites within the CoC itself.

Additional background investigating the condition of the Bow River around WWTPs includes 2013 research on dissolved oxygen regimes, which demonstrated the presence of hypoxic pockets in the river during overnight periods of low oxygen, often associated with the increased macrophyte growth downstream of WWTPs (Chung 2013). This is relevant to both autotrophic and consumer constituents of the basal food web. Other studies have investigated how fish may be affected by wastewater, demonstrating that MWWE, overall, seemed to have little effect on stress (cortisol) responses in fish in the Bow River, but any endocrine disruptors likely present in MWWE did appear to have significant effect on hormone imbalances and gonad development (Henderson 2014, Patel 2018, Lazaro-Côté et al. 2018). Notably, one study demonstrated reproductive impairment at a site a small distance upstream from the CoC's most upstream WWTP, underlining the importance of performing monitoring on a broader source of stressors in a finer spatial resolution in the Bow (Patel 2018).

The most recent surveys of macroinvertebrate communities in the Bow River occurred in 1989, 1992, and 2006 but most work occurred above and below CoC limits, with little focus on changes occurring within the city limits, especially around point source inputs (Powell 2008, Saffran et al. 2009). The largest survey of benthic macroinvertebrates available focused on the role of ecozones on macroinvertebrate communities, tracking shifts in community assemblages between the mountains, foothills, and prairies in three Albertan rivers, including the Bow. This project demonstrated that environmental variables driving communities in this area, such as conductivity and depth, may be different in different ecozones (Powell 2008). Collectively, these previous studies provide some information on the changes in benthic macroinvertebrate community composition and diversity along the river; however, no predictive relationships were derived in relation to the role of MWWE.

The Bow River, fed from relatively pristine mountain headwaters in Banff National Park flows through three distinct ecoregions and has large spatial gaps between MWWE inputs that allow for potential recovery as well as MWWE inputs close together that allow for investigation of cumulative impacts. This Bow River, therefore, provides an interesting study paradigm for assessing the complexity of MWWE in lotic environments. Nutrient inputs have been established to show shifts in the basal food web, particularly in autotrophs, but generally treatment quality is high and nutrient inputs are less notable than other places in Canada and the rest of the world. However, ESOC presence has only begun to be examined and initial results indicate that current treatment processes are unreliable in removing them from MWWE and they do have some effect on organisms (fish) in the Bow River. An examination of the basal food web in the Bow River will provide additional information in determining the role that the increasing presence of ESOCs will play in aquatic ecosystems.

1.2.3 Advancing Canadian Water Assets Facility

The City of Calgary is unique in the complementary existence of experimental stream mesocosms at the CoC Advancing Canadian Water Assets (ACWA) facility. The role of artificial stream experiments has been increasingly highlighted in national Canadian biomonitoring programs as a way to unify the results of highly controlled laboratory experiments and results from large and complex natural systems (Liber et al. 2009, Alexander et al. 2020). These experiments represent an important first step in addressing which contaminants, and at what ecotoxicologically relevant concentration, have a negative direct or indirect impact on the biota of the receiving, natural environment (Dubé et al. 2002). Crucially, they also provide the ability to control many physicochemical factors such as flow or light availability (Alexander et al. 2020). This complements the abilities to replicate experiments and to control dose or mixtures when exposing the system to a stressor, which can be a key part of detangling the effects of components in complex mixtures (Harris et al. 2007, Juvigny-Khenafou et al. 2021). Additionally, artificial stream experiments using macroinvertebrate endpoints, as opposed to larger organisms (such as fish or amphibians), are less expensive to set up, easier to source materials for, and overall, more conducive to future replication and extension (Alexander et al. 2020).

The naturalized streams at the ACWA facility provide triplicate MWWE exposure regimes for three different MMWE treatments (ultraviolet disinfection, ozonation, and reverse osmosis) as well as control streams using Bow River source water only. The streams were constructed in 2015, using a smaller tributary of the Bow River, Jumpingpound Creek, as a basic model and the streams were specifically designed to eliminate differences in stream morphology and water flow between replicates (Jackson 2020). While MWWE is piped into streams in the form of the final effluent, the facility provides the ability to control dose. This offers a unique opportunity for examining potential dose-specific ESOC-nutrient effects on complex basal food webs that are difficult to replicate in a lab scenario and expensive to test on whole watersheds (Jackson 2020).

1.3 Study Objectives and Significance

1.3.1 Study Objectives

The fundamental design and objectives of this study are guided by the multi-tiered Environmental Effects Monitoring (EEM) framework for aquatic receiving environments (Hewitt et al. 2003). The EEM approach is based on cyclical, field-based evaluations of the receiving environment to first determine whether measurable biological or ecological effects exist when the facilities in question are in compliance with discharge regulations. This study contributes to the first comprehensive EEM-based cycle examining the responses of benthic macroinvertebrate assemblages to a gradient of MWWE exposure in the Bow River and in a complimentary controlled exposure experiment conducted at the ACWA experimental stream facility. The study focuses on identifying the extent and magnitude of observed effects on a range of community-based metrics and ecological endpoints with the intent to quantify and recommend the most effective and efficient sampling and analytical methods for assessing MWWE exposure as a driver of changes in the basal aquatic food web. The objectives of the two inter-related projects in this thesis are as follows:

- Describe and quantify the current status, as well as the presence and magnitude of difference, in key ecological endpoints associated with benthic macroinvertebrate assemblages exposed to a gradient of MWWE in the Bow River.
- Develop predictive relationships (initiate investigation of cause) that link MWWE related nutrients and/or ESOCs exposure measured benthic macroinvertebrate assemblage responses.
- Discuss the advantages and disadvantages of various experimental designs and benthic macroinvertebrate assemblage sampling methods in assessing the responses of benthic macroinvertebrate assemblages to MWWE exposure.

These objectives are explored over the following two chapters and a final synthesis chapter:

- Chapter 2 examines the responses of benthic macroinvertebrate community assemblages in the Bow River along a longitudinal gradient of MWWE exposure.
- Chapter 3 examines the responses of macroinvertebrates community assemblages of macroinvertebrate families in the ACWA experimental streams at the Pine Creek WWTP

in Bow River (control) and Pine Creek MWWE exposed streams using two different macroinvertebrate sampling methods.

1.3.2 Significance

In 2020, the Bow River Ecosystem Health Assessment (BREHA) was established as a multi-disciplinary research initiative, intended to evaluate the health of the Bow and overall South Saskatchewan River watershed. As a part of this initiative, this study aims to form direct connections to other related studies, including examination of fish health responses and fate and transport of ESOCs, with data taken from similar areas at a similar time. Early stages of these projects can be observed in a report on ESOCs in the Bow River (Arlos et al. 2023). The information provided by these projects is intended to inform the development of a new, integrated monitoring toolkit for the Bow River watershed. To best address the complex relationship between landscape variation and anthropogenic influences that can affect lotic systems, the methods and study design of biomonitoring programs should be judiciously chosen for unique settings. This is reflected in the multiple statistical and analytical approaches used in this thesis to assess the measured benthic macroinvertebrate community responses to MWWE exposures. Benthic community metrics are assessed through the use of a combination of summary statistics, visualizations, and qualitative descriptions. Additionally, these metrics can contribute to early cycles of the development of Environmental Effects Monitoring (EEM) programs, which rely on baseline measurements of systems to determine natural variability when setting triggers for changes in monitoring and management (Kilgour et al. 2007). Significance testing is utilized to represent the experimental aspects of the project and multivariate techniques are used to guide exploration of potential environmental drivers. Overall, this varied approach to analysis provides multiple potential paradigms to inform future community-based monitoring programs.

The large number of ESOCs present in complex MWWE mixtures are a significant analytical barrier to rapid determination of the potential to affect the biological and ecological structure and function of aquatic ecosystems. Assessments of biological endpoints, in conjunction with water quality parameters, will provide the necessary context for understanding observable outcomes on *in situ* organisms and the overall ecosystem health.

Additionally, to date, few studies have specifically examined the potential impacts of complex mixtures of MWWE inputs in the Bow River system, particularly following infrastructural updates to WWTP infrastructure, and following the major flooding event of 2013. There have also been no recorded studies examining effects of MWWE on the basal food web that are able to compare river results to those done in a more controlled experimental stream setting with the ability to manipulate effluent exposure.

Chapter 2: Assessing Ecosystem Health for Potential Impacts of Municipal Wastewater Effluent in the Bow River through Benthic Macroinvertebrate Community Composition

2.1 General Background

2.1.1 Assessing MWWE in Aquatic Ecosystems

Rivers will often change hydrological and physicochemical properties in predictable patterns as they flow from their headwaters, generating the expectation that biological communities and associated ecological processes will not also remain identical at all points in the drainage basin, regardless of anthropogenic impact (Allan 2004). This pattern has been theorized in many models but with differing characteristics, ranging from the gradual change described in the River Continuum Concept (RCC) to a patchwork system described in updates to the RCC and related models (Vannote et al. 1980, Doretto et al. 2020). However, physicochemical conditions in flowing ecosystems are increasingly understood as a cumulation of natural surroundings and anthropogenic pressures, such as the relation of urbanization to shifts in channel morphology and water flow in urban environments (Townsend et al. 2003, Downs and Piégay 2019). This can lead to unpredictability in the effects of stressors, depending on the landscape surrounding them, and initiates further potential for varying results due to interactions with other combinations of stressors (Townsend et al. 2008, Hroch 2022). Biological assemblages are integral in assisting to identify how natural and anthropogenic stressors combine and interact to affect the overall aquatic ecosystem characteristics.

Shifts in a diverse suite of physiochemical parameters related to both small changes in the surrounding habitat and larger changes in the surrounding geomorphology, ecozone, and land-use have been established to translate into significant variation in biological communities, such as changes to abundance patterns of benthic macroinvertebrate assemblages (Allan 2004, Herman and Nejadhashemi 2015). Additionally, considerations are not only spatial but also temporal, as some organisms will show legacy effects of land use, such as deforestation, decades after the initial impact (Linares et al. 2023). Many studies have identified common impacts of specific core water quality parameters but, by building on this knowledge and identifying patterns of change in benthic macroinvertebrate assemblages over a large spatial area, it is possible to identify key stressors for a particular system (Hroch 2022). Notably, benthic macroinvertebrates experimental work has identified assessments of benthic macroinvertebrate assemblages to be a good predictor of changes related to nutrient concentrations (Johnson 1993, Juvigny-Khenafou et al. 2021, Hroch 2022). This has also been demonstrated with particular emphasis in oligotrophic mountain streams (Johnson and Hering 2009). A common source of nutrient enrichment in streams and rivers, especially oligotrophic mountain waterways not associated with agriculture, is treated sewage from urban centres.

Physiochemically, the presence of treated sewage, typically called municipal wastewater effluent (MWWE), is mostly clearly associated with increases in total suspended sediments, as well as nutrient concentrations (typically nitrogen and phosphorus), which can both strongly influence in-stream productivity and oxygenation (Holeton et al. 2011). Specifically for benthic macroinvertebrates, changes to suspended sediments and sedimentation rate in lotic environments can alter light and nutrient availability, adjusting the growth of in-stream production and affecting the decomposition rates (Jones et al. 2012, Piggott et al. 2012). High sedimentation rates in some environments can even eliminate habitat for benthic macroinvertebrates by filling in interstitial spaces in the substrate and changing flow patterns (Wood and Armitage 1997, Jones et al. 2012). Changes to oxygenation have also demonstrated strong impacts on benthic macroinvertebrate assemblages, with low oxygen systems showing decreased presence of benthic macroinvertebrates sensitive to pollution (Dos Reis Oliveira et al. 2019).

In addition to understanding how changes in nutrients result in changes to sediments and oxygen regimes, which affect basal food web responses, there is a growing need to understand how aquatic ecosystem health is affected by increasing discharges of emerging substances of concern (ESOCs) in MWWE (including pharmaceuticals, personal care products and other chemical pollutants) (Holeton et al. 2011). Depending on the municipal wastewater source, ESOC concentrations are highly varied following treatment and part of complex chemical mixtures (Cleuvers 2004). Combined, these qualities make ecosystem level assessments difficult to perform and understand on the basis of individual chemical modes of action (Diamond et al. 2011). Specifically in relation to understanding the both the acute and chronic effects on benthic macroinvertebrate assemblages, there remains a poor understanding of how ESOCs contribute to adverse impacts since they have less-studied physiology (Kidd et al. 2014).

Given this complexity, one approach that could be used to better ascertain ESOC related impacts is focusing on observed effects of higher concentration ESOC groups with taxon-specific outcomes, which may allow potential effects to be discerned while in a nutrient-ESOC mixture. For example, one chemical group with some evidence of direct effects on benthic macroinvertebrate assemblages are analgesics (such as ibuprofen), which demonstrate notable toxicity in invertebrates compared with other commonly occurring ESOCs (Lencioni et al. 2020). Additionally, analgesics are associated with greater toxicity to grazers and detritivores than predatory species, which disagrees with broad assumptions stemming from traditional classifications of pollution tolerant taxa (Lencioni et al. 2020). Similarly, antibiotics (such as ciprofloxacin), have been shown to affect decomposition of leaf litter – a common benthic invertebrate food source (Konschak et al. 2020). While benthic macroinvertebrates showed relatively high toxicity tolerance, microorganisms associated with leaf breakdown (largely fungi) showed significantly decreased abundance, and this was found to directly affect nutrient intake and size of a representative shredder macroinvertebrate (Konschak et al. 2020). Both these groups of ESOCs show effects at concentrations that exceed those normally found in ecosystems, but laboratory studies are limited in examining the effects of chronic exposure to mixtures, such as potential bioaccumulation (Richmond et al. 2018).

2.1.2 Assessing Impacts on Benthic Macroinvertebrates

A common ecological endpoint used to assess aquatic ecosystem health is examining the changes in benthic community assemblages and function in relation to exposure of the environmental stressor(s) of concern. Alterations in benthic macroinvertebrate assemblages are often assessed for abrupt changes through spatial and temporal changes in taxonomic composition or functional feeding group classification (Buss et al. 2014, Herman and Nejadhashemi 2015). Benthic macroinvertebrate assemblages may be described through a number of endpoints. Density and abundance measure the relative sizes of communities on a spatial or temporal measurement and can also be utilized for discrete groups within overall communities (Brown 2001, Herman and Nejadhashemi 2015). Some specific taxa may also be used to identify environmental change based on pre-determined tolerance for various conditions and pollutants. This is often applied to a set of common insect orders whose larvae are primarily aquatic: Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies) (often referred to together as EPT). EPT taxa are generally considered pollution-intolerant, while the aquatic larvae of the true flies, order Diptera, are typically extremely pollution tolerant (Herman and Nejadhashemi 2015). However, variation of tolerance within these orders has led to the development of additional indices such as the Hilsenhoff Biotic Index, which assigns scores to taxa (usually at the Family level) based on their tolerance to pollution and provides an overall score for the community (Hilsenhoff 1988). Alternatively, assemblages can be assessed by the number of distinct groups at any consistent taxonomic resolution (richness), how evenly distributed taxa are among groups (evenness), how dominant any dominant or specific taxa are, and how diverse the overall assemblage is (often measured through an index such as the Shannon-Weiner diversity index) (Buss et al. 2014).

Choosing relevant ecological endpoints for biomonitoring is important to interpreting changes in the basal food web, especially with a complex effluent such as MWWE that contains not only nutrients but also a shifting, diverse complement of additional ESOCs. Environmental Effects Monitoring (EEM), used in Canada, aims to define targeted biological endpoints over a range of long-term experiments. The mandate of EEM programs is to develop protocols for biomonitoring specific pollutants on the understanding that unique environments and discharges may require specific strategies for biomonitoring (Walker et al. 2003). For benthic macroinvertebrates in lotic systems exposed metal mining, EEM has defined relevant endpoints to include density and taxa richness, evenness (Simpson’s), diversity (Simpson’s) and site similarity (Bray-Curtis) (Environment and Climate Change Canada 2012). Similarly, pulp and paper EEM guidelines suggest the use of benthic macroinvertebrate endpoints such as total and taxon specific densities, taxa richness, evenness (Simpson’s), similarity (Bray-Curtis), and taxa presence/absence (Environment and Climate Change Canada 2010). There is not yet an EEM framework developed for MWWE in Canada, but previous EEM frameworks, especially for related discharges such as pulp and paper that are also primarily nutrient-based, can provide important insights for choosing new endpoints.

2.1.3 Benthic Macroinvertebrates in the Bow River

The upper Bow River is a relatively pristine river, flowing from Bow Glacier in Banff National Park in Alberta, Canada. In its headwaters, the Bow River is cold, fast, and relatively oligotrophic (Bowman et al. 2007). Between the river's headwaters and the Bassano Dam, the river flows through two diverse ecozones: montane cordillera and prairies. The surrounding landscape is likely to influence the benthic macroinvertebrate community in the area, alongside shifts observed as part of a natural longitudinal gradient, as would be generally predicted by conceptual river models such as the River Continuum Concept and its derivatives (Vannote et al. 1980, Allan 2004, Powell 2008). Due to additional protections regarding urbanization and industrial development in National Parks, the upper reaches of the Bow River are not highly exposed to anthropogenic effluents, which above the city of Calgary (CoC), largely consist of MWWE, which, as established, is primarily monitored due to potential impacts from the nutrients released from the associated organic matter.

Previous work in the Bow River has established that only mountainous sites were significantly distinct from foothill and prairie sites in terms of benthic macroinvertebrate assemblages, and only some indices describing the assemblages were able to differentiate defined ecoregions, such as EPT abundance (Powell 2008). Typically, overall changes in assemblage track relatively well onto RCC predictions, where sites in the lower stream order, mountain ecoregions showed higher abundances of shredder taxa and gatherer/filterer representatives increased downstream as the stream order increased (Culp and Davies 1982, Powell 2008). However, changes in benthic macroinvertebrate assemblages were not always linear, as in site similarity was not consistently organized following the longitudinal gradient of the river, and the environmental variables that drove changes in benthic invertebrate abundance varied between ecoregions (Powell 2008). In subsequent studies, benthic macroinvertebrate assemblages on the Bow River have shown evidence of nutrient enrichment downstream of Calgary, as well as more general perturbation around Cochrane (Saffran et al. 2009, Robinson et al. 2009). However, most previous research focusing on benthic macroinvertebrates was not experimentally designed to identify differences surrounding MWWE outflows.

There has been a higher volume of research regarding producer (periphyton and macrophyte) growth in the Bow River, particularly temporally bracketing a significant nutrient removal upgrade for Calgary's WWTP treatments between 1982 and 1990. This upgrade reduced nuisance levels of macrophytes in the Bow River in southern Calgary but had little effect on periphyton biomass as observed concentrations of phosphorus compounds remained high enough to prevent limitation below WWTPs (Sosiak 2002). Macrophytes were also significantly scoured away in a large flood in 2005 (Saffran et al. 2009). Evidence of overall nutrient enrichment in the whole ecosystem was also reduced following the previously mentioned upgrades in the late 20th century, but regular sampling of the Bow River around MWWE outflow sites has not been recently prioritized in the area, particularly for benthic macroinvertebrate assemblages (Saffran et al. 2009). Comparatively to other components of MWWE, ESOCs have only been regularly monitored (monthly) in the Bow River at a limited number of sites since 2018 by the City of Calgary; however, their potential impacts on benthic macroinvertebrate assemblages or functional traits have not been explored.

From a water quality perspective, results typically show that ESOC levels in the Bow River are consistently below levels of acute toxicity but removal of many ESOCs through WWTP processes is reliably incomplete, particularly for certain types of ESOCs (Chen et al. 2015). Recent exploration of ESOCs in the Bow River emphasized the role of seasonal patterns in assessing ESOC presence and importance, as several examples of ESOCs showed high annual variation in concentration such as DEET from insect repellant, an antibiotic (sulfamethoxazole), and an antidepressant (O-desmethylvenlafaxine) (Arlos et al. 2023). This reinforces the paradigm observed in other systems that suggest that cultural patterns and river water volume are key to interpreting potential impacts of ESOCs in freshwater ecosystems (Bai et al. 2018). Overall, ESOCs generally occurring in the highest concentrations in the Bow River were the artificial sweetener sucralose, antidiabetic metformin, and caffeine, as well as previously mentioned insect repellants, antidepressants, and antibiotics (Arlos et al. 2023).

These results emphasize the need to continue monitoring changes to the Bow River over time. Notably, populations in Calgary have increased by approximately 25% by 2021 since the last available widespread sampling on Bow River benthic macroinvertebrate communities in 2006. Flows in the Bow River are variable. The system has seen one major flood since this time (2013) and several years of historic hydrographic highs and lows. In recent years, including 2021, overall river flows have reached historical lows in the late summer and early fall. This emphasizes the importance of identifying shifts and patterns in the river following increases in MWWE volume that come with increased population, which is projected to continue to increase (Saffran et al. 2009).

2.1.4 Objective, Hypothesis, and Guiding Questions

The objective of this chapter is to identify and describe observed changes in benthic macroinvertebrate community assemblages in relation to a longitudinal river gradient with multiple point source municipal wastewater effluent (MWWE) exposures in the Bow River, Alberta. This work was done across the open water season (May, September, November) to compare and confirm appropriate sampling periods for the endpoints under investigation. I hypothesize that the most significant changes in benthic macroinvertebrate assemblages over the longitudinal gradient will be observed at sites immediately below point source WWTPs. These changes in benthic macroinvertebrate assemblages will be associated most strongly with changes in nutrient-related water quality parameters, spatially related to MWWE exposure. This broad hypothesis will be addressed through several key guiding questions:

- Are there distinct regions along the longitudinal gradient related to the presence of point source MWWE exposure, ecozone, or other anthropogenic stressors?
- What is the current status of key ecological endpoints associated with benthic macroinvertebrate assemblages at sites along the longitudinal river gradient in the Bow River?
- Are there significant differences in benthic macroinvertebrate assemblages (i.e. community composition metrics) among reference sites, sites at different points on the longitudinal river gradient, and potentially impacted sites below point source MWWE exposures?
- Can predictive relationships be developed that link system drivers (such as MWWE related nutrients or ESOCs) to benthic assemblage responses?

2.2 Methods

2.2.1 Laboratory and Field Methods

Water quality parameters in the Bow River were monitored correspondingly to sampling events in May, September, and November. Grab water samples for nutrients (TOC, TN/TKN, and TP) from May 5-7, 2021 (specific sampling dates in Table A.1 and specific site locations in Fig. 2.1) were taken using a triple rinse protocol grab sample. Samples were analyzed at the Pine Creek Analytical Laboratory at ACWA. A VWR handheld conductivity meter was used to take in situ measurements of conductivity and temperature. Grab samples for nutrients (TOC, TKN, and TP) from September 22-24, 2021 (specific sampling dates in Table A.2 and specific site locations in Fig. 2.2) were taken using a triple rinse protocol grab sample. Samples were analyzed at the Bureau Veritas Environmental Services Laboratory, Calgary. Specific conductivity, turbidity, and temperature were taken using a YSI sonde (6600 V2). Water grab samples for nutrients (TOC, TKN, and TP) for November 3-7, 2021 (specific sampling dates in Table A.3 and specific site locations in Fig. 2.3) were taken using a triple rinse protocol grab sample. Samples were analyzed at the Bureau Veritas Environmental Services Laboratory, Calgary. Specific conductivity, turbidity, and temperature were taken using a YSI sonde (6600 V2). The City of Calgary's Watershed Monitoring Program, which performs monthly water quality sampling at several long-term sites, was used to augment the data collected in all three months, as well as data provided from EPCOR for their long-term monitoring sites in Canmore (inclusion of external data detailed in Tables A.1, A.2, and A.3) (Fig. 2.1, Fig. 2.2, Fig. 3.3). ESOC data was sampled by the City of Calgary as part of a pilot ESOC monitoring program taking monthly grab samples (Arlos et al. 2023) (Specific sampling dates in Table A.5).

Field work was conducted at three general locations (Canmore, Cochrane, Calgary) on the Bow River, Alberta within a natural gradient of MWWE exposure. Sites were established at key points surrounding natural features (e.g. tributaries), anthropogenic features (e.g. dams, urban areas), and point source MWWE exposure (e.g. WWTPs) (summarized in Table A.5.).

The substrate was characterized using a time-saving adaptation of the 100-pebble count from the Substrate Characteristics section of the CABiN field manual for wadable streams (Environment and Climate Change Canada 2017). Fifty rocks were systematically and randomly chosen by taking the boot-end rock every 2 steps. Rocks were classified using Wentworth substrate classes of the length of the longest axis, rather than measured precisely. This was performed at all sites in May and September, meaning sites sampled in both months have an overall n of 100 and sites sampled in only one month have an overall n of 50.

Benthic invertebrate samples were taken using an adapted CABiN protocol (Canadian Aquatic Biomonitoring Network). Three replicates were taken at each site using a 3-minute travelling 400 µm kicknet and frozen until processing (Environment and Climate Change Canada 2017). Replicates were sampled from longitudinal sections of either an appropriately sized overall distance (6x river width) or the entire selected reach and performed moving from upstream to downstream. Kicknet samples were subsampled using a 355 µm sieve to evenly distribute biomass before removing excess water and dividing into even parts. Subsamples ranged from 3.125% to 100%, depending on the overall abundance of taxa. A subsample was required to contain a minimum of 100 organisms for identification and was resampled until the sample either met that threshold or was 100% identified to establish distribution of (Hickley 1975, Wrona et al. 1982). Samples were multiplied by the coefficient of subsampling fraction to standardize sorting effort before analysis. Taxa were identified to the lowest practical unit, usually family, primarily following *Aquatic Invertebrates of Alberta* (Clifford 1991). Sites and dates of benthic macroinvertebrate samples are identified in Table A.4.

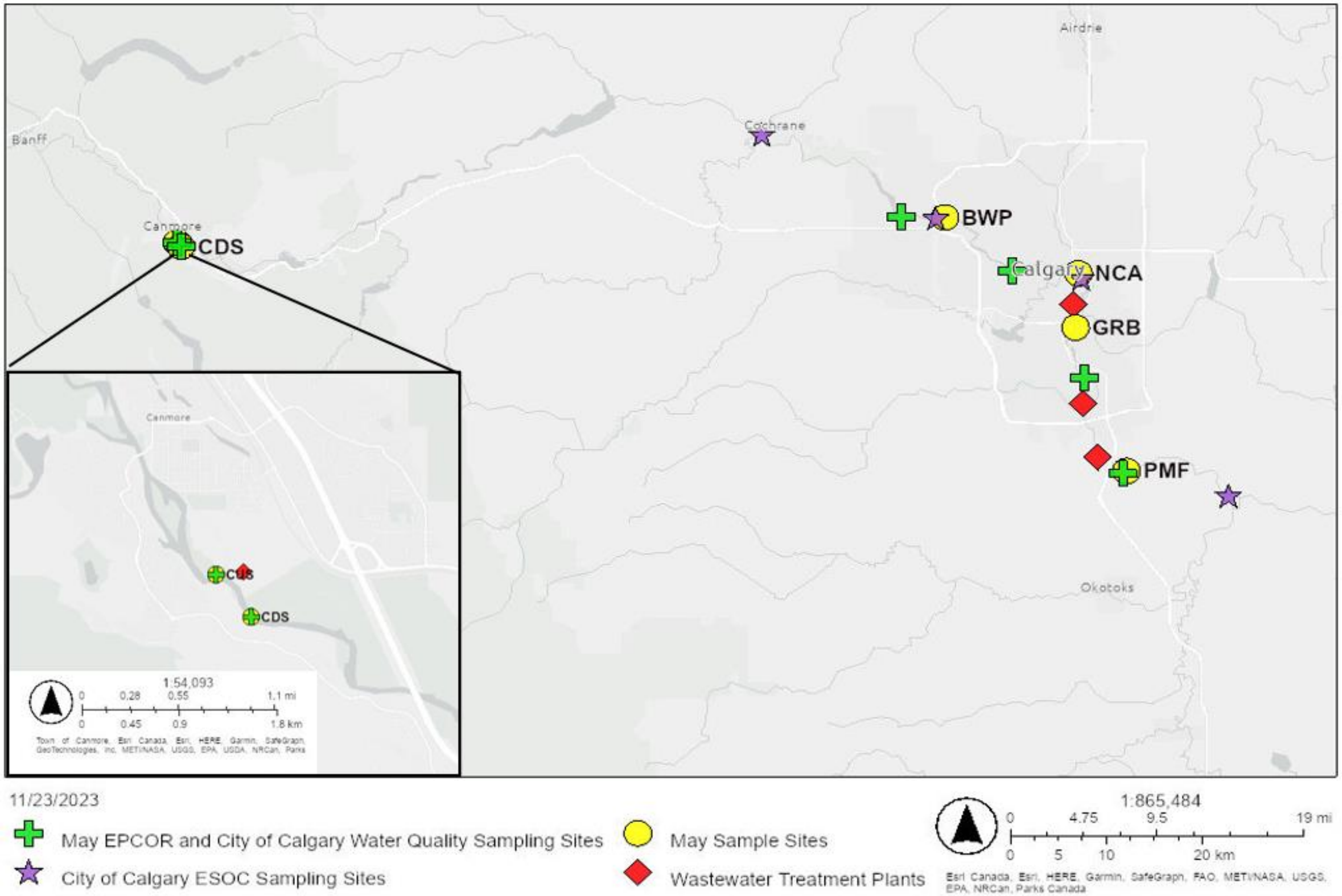


Fig. 2.1. May 2021 sample sites in Canmore and Calgary for benthic macroinvertebrates and associated parameters, nearby large WWTPs, sites where the City of Calgary or EPCOR took water quality samples that were used in analysis, and where ESOCs were measured (CUS – Canmore Upstream, Canmore Downstream – CDS, BWP – Bowness Park, NCA – Nose Creek Adjacent, GRB – Graves Bridge, PMF – Policeman's Flats).

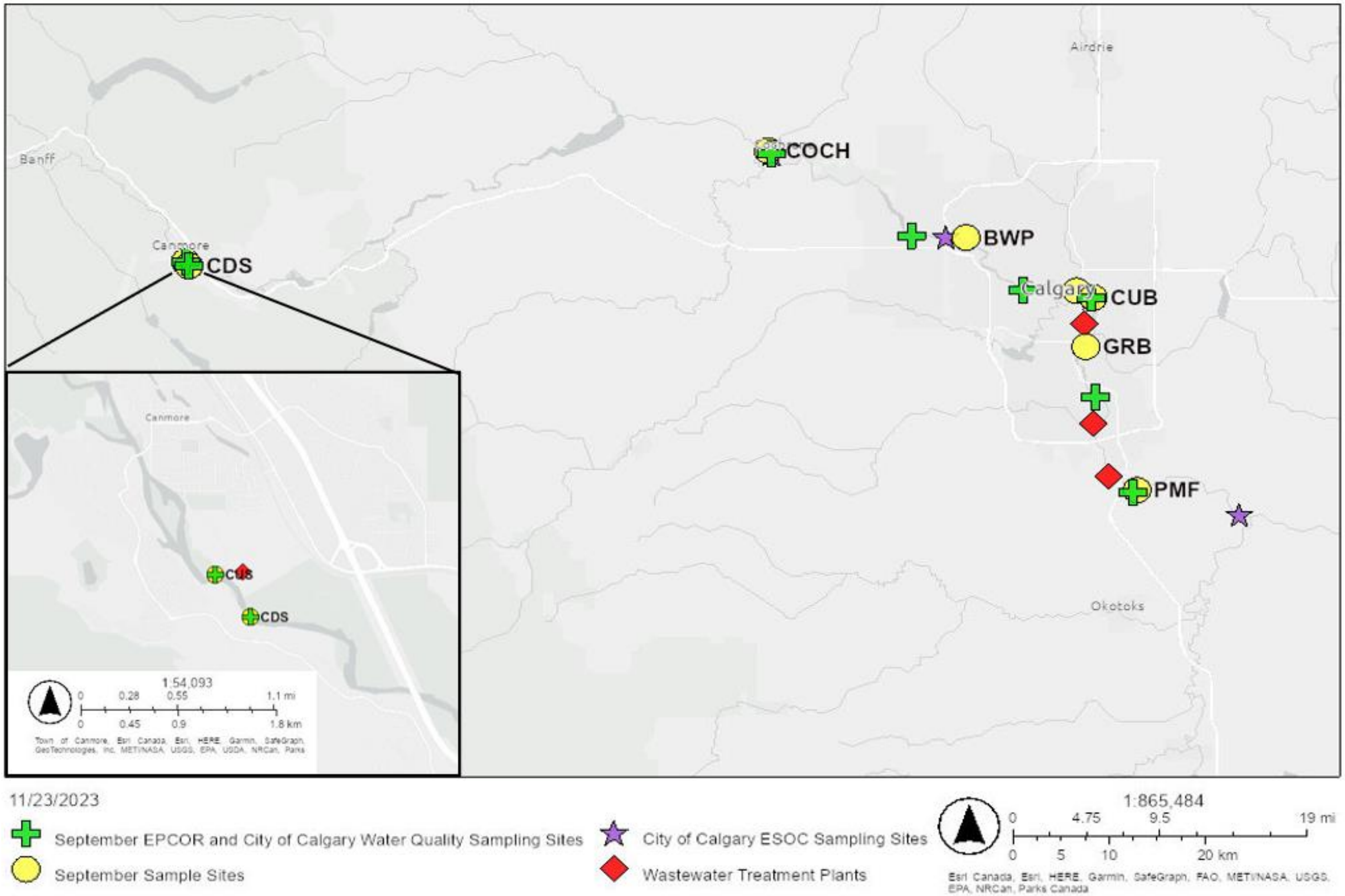


Fig. 2.2. September 2021 sample sites in Canmore and Calgary for benthic macroinvertebrates and associated parameters, nearby large WWTPs, sites where the City of Calgary or EPCOR took water quality samples that were used in analysis, and where ESOCs were measured (CUS – Canmore Upstream, Canmore Downstream – CDS, COCH – Cochrane, BWP – Bowness Park, SGI – St. Georges' Island, CUB – Cushing Bridge, GRB – Graves Bridge, PMF – Policeman's Flats).

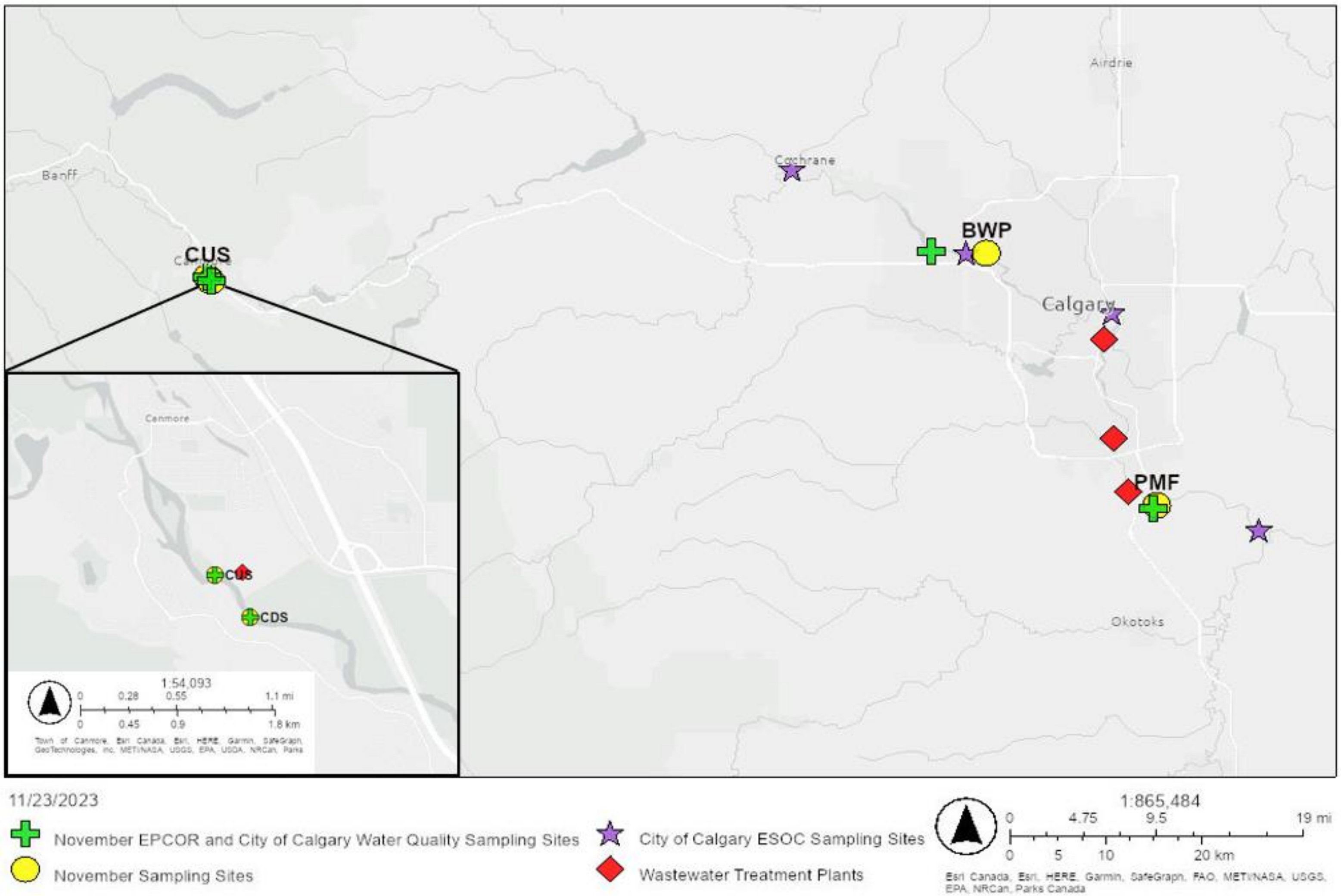


Fig. 2.3. November 2021 sample sites in Canmore and Calgary for benthic macroinvertebrates and associated parameters, nearby large WWTPs, sites where the City of Calgary or EPCOR took water quality samples that were used in analysis, and where ESOCs were measured. (CUS – Canmore Upstream, Canmore Downstream – CDS, BWP – Bowness Park, PMF – Policeman's Flats).

2.2.2 Data Analyses

Objective 1: The first step in the analysis was identifying key differences in benthic macroinvertebrate assemblages among reference sites, sites at different points on the longitudinal river gradient, and potentially impacted sites below point source MWWE exposures. All analyses were performed using RStudio (R version 4.3.2). A cluster analysis, using the `vegdist` function from the vegan package and the `hclust` function from the stats package, assessed similarity among replicates in Beta-diversity based on the Bray-Curtis difference matrix (Oksanen et al. 2022, Hroch 2022). Once similar groups of sites were defined in relation to location on the longitudinal gradient and proximity to point source MWWE exposures, sites were combined to form longitudinal river regions. The remainder of the analyses were performed using these regions for comparative purposes. Bray-Curtis distances for sites within regions were used to calculate non-metric multidimensional scaling (nMDS), using the `metaMDS` function from the vegan package, which spatially represented replicate similarity at the regional level for each month (Oksanen et al. 2022).

Objective 2: To identify the current status of key endpoints associated with benthic macroinvertebrate assemblages at sites along the longitudinal river gradient in the Bow River, Alpha-diversity for macroinvertebrate assemblages was calculated within each defined region. Each region was assessed using a range of ecological metrics. This included metrics summarizing total abundance (using the `rowSums` function), taxonomic richness (using the `specnumber` function from the vegan package), Shannon-Weiner diversity (using the `diversity` function from the vegan package), evenness, and % dominance of the dominant taxa. The percent composition of dominant taxonomic groups was also calculated, including the total percentage of Orders known for low pollution tolerance, typically referred to as EPT

(Ephemeroptera, Plecoptera, Trichoptera) (Buss et al. 2014). The proportion of Diptera, an Order known for high pollution tolerance, was also calculated. Lastly, the Hilsenhoff Biotic Index for each defined region was calculated and a categorical rating of stream quality was assigned (Haney 2013).

Objective 3: Only at this stage, prior to significance testing of differences between the river-reach regions, was benthic macroinvertebrate data was $\log_{10}$ transformed to reduce influence of zeroes in the dataset (Chin et al. 2016). Transformed data was used for the remainder of the analysis. After assumptions were tested, the testing was then performed using matrices of invertebrate communities within regions using the `adonis2` function from the vegan package in R to perform one-way PERMANOVA (999 permutations) and, where appropriate, pairwise comparisons with a Bonferroni correction, using the `pairwise.adonis` function ($p < 0.05$) (Anderson 2017, Arbizu 2020, Oksanen et al. 2022). A follow-up SIMPER analysis determined which taxa were most responsible for differences between sites using the `simper` function from the vegan package (Oksanen et al. 2022, Hroch 2022).

Objective 4: Lastly, using physicochemical data at sites sampled for benthic macroinvertebrate communities, multivariate analyses were performed to develop predictive relationships that link system water quality drivers (MWWE related nutrients or ESOCs) to benthic assemblage responses. ESOC data was selected to represent the regions indicated by the cluster analysis through sites with available monthly grab sample data from the City of Calgary. ESOCs were combined into groups for each monthly average, based on previous classification in the Bow River (Table A.5) (Arlos et al. 2023). However, due to the smaller number of sites with ESOC data available, ESOC data were not included in subsequent multivariate analyses in order to prevent restriction of sample size. Other physicochemical parameters representing environmental conditions at sites were summarized using a combination of data gathered in the field at the time of sampling and data from long-term sampling programs at the CoC and EPCOR (Table A.1, A.2, and A.3). To avoid overparameterization, parameters chosen were first selected for their biological relevance to benthic macroinvertebrate community composition and relation to MWWE exposure. Following an explorative assessment of data visualizations, for the open-water season physicochemical data set (all months), parameters were transformed using a $\log_{10}$ transformation to meet the necessary test assumptions and then were investigated for significant differences using permutation tests ($p < 0.05$). Following this, to identify what parameters were primarily responsible for variation in the benthic macroinvertebrate community, a dbRDA was performed using the transformed physicochemical data and a SIMPER reduced benthic macroinvertebrate matrix of Bray-Curtis differences. The dbRDA was performed using the `dbrda` function from the vegan package, which automatically normalized the physicochemical matrix. Constrained inertia represents variation in the benthic macroinvertebrate assemblages explained by the physicochemical environmental data. Type III ANOVA tests were used to identify if the overall model was significant and/or what physicochemical parameters were involved($p < 0.05$) (Oksanen et al. 2022). A stepwise model function involving both forward and backward selection and AIC was also used to identify the most parsimonious model given physicochemical matrix using the `ordistep` function from the vegan packaged (Oksanen et al. 2022).

2.3 Results

2.3.1 Cluster Analysis & NMDS

For May 2021 benthic macroinvertebrate samples (analyzed at the replicate level at each site to find regions), Canmore upstream and downstream (CUS and CDS) replicates were partially separated into discrete groups but were both most similar to each other. Canmore sites were most similar to sites in the northern part of Calgary (above the first WWTP in Calgary) (BWP and NCA). The sites least similar to other sites were sites downstream of one or more WWTP in the south part of Calgary (GRB and PMF), which were distinct from one another but were least similar to all other site replicates (Fig. 2.4).

September 2021 benthic macroinvertebrate samples had similar groupings, with Canmore site replicates being distinct from each other upstream and downstream of Canmore's WWTP (CUS and CDS) but overall, most similar to each other. Cochrane (COCH), a site added in September sampling, is distinct from other sites further south in northern Calgary but was part of the overall cluster of Calgary sites above Calgary's WWTPs. Within this cluster, Bowness Park (BWP) was distinct from the other two sites, which did not show clear distinctions (SGI and CUB). These two sites were spatially very close together, but SGI was situated after the Elbow River confluence and before the Nose Creek confluence and CUB was situated after both confluences. All these sites were more similar to one another than to the sites below one or more Calgary WWTP in southern Calgary (Fig. 2.5).

November 2021 sampling, which only involved four sites, followed the same pattern. Canmore upstream and Canmore downstream were distinct but most closely related. All Canmore sites were more similar to the northern Calgary site (BWP) than to the southern Calgary site (PMF) below all three of Calgary's WWTPs (Fig. 2.6).

Cluster analysis of Bow River sites based on a Bray-Curtis differences for every site replicate consistently sorted sites into similar groups (Fig. 2.4, Fig. 2.5, Fig. 2.6). Overall, both sites in Canmore were generally similar, although upstream replicates clustered together more, as did downstream replicates. Sites upstream of Calgary’s most upstream WWTP (Bonnybrook) were more similar to both Canmore sites than to the sites downstream of one or more WWTP in Calgary (GRB and PMF), despite being much closer spatially to the other Calgary sites. Generally, sites were most similar to their replicate sites, except in the northern part of Calgary in September.

The site groupings emerging from this analysis define larger segments of river regions that have similar benthic invertebrate assemblages and are used to as the basis for further comparisons in this chapter. These river-reach regions are: 1) Canmore Upstream (all months); 2) Canmore Downstream (all months); 3) Cochrane (September only); 4) Northern Calgary (all months, some varying sites); 5) Southern Calgary (all months, some varying sites).

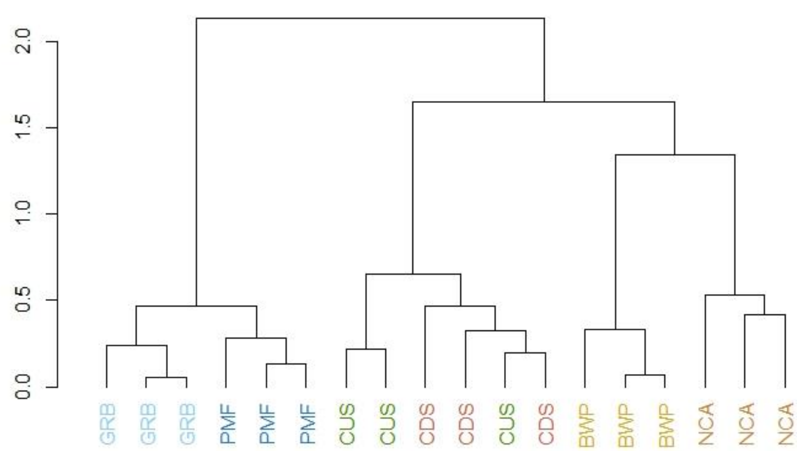


Fig. 2.4. Dendrogram representation of similarity between all replicates at all sites sampled in the Bow River in May of 2021. Similarity was calculated using Bray Curtis differences. Sites with lower differences are grouped more closely together. Branches distance on the y-axis indicates difference.

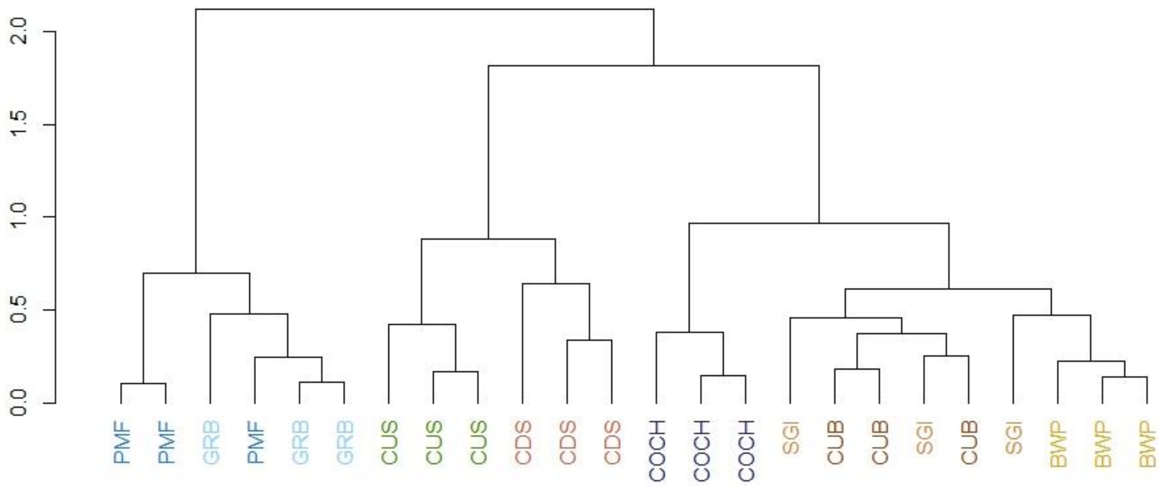


Fig. 2.5. Dendrogram representation of similarity between all replicates at all sites sampled in the Bow River in September of 2021. Similarity was calculated using Bray Curtis differences. Sites with lower differences are grouped more closely together. Branches distance on the y-axis indicates difference.

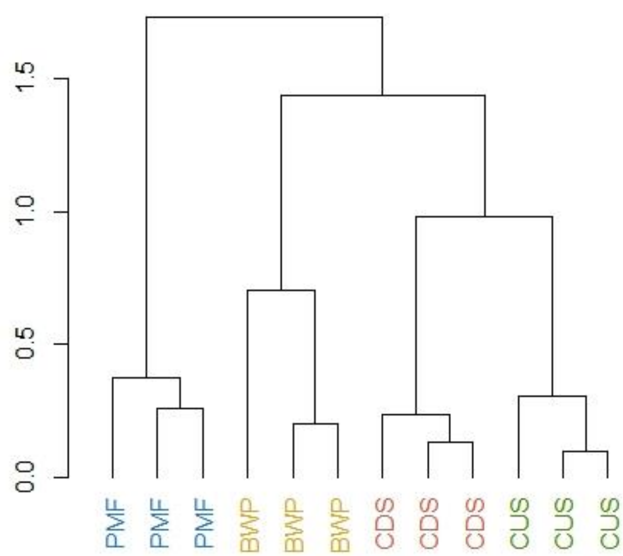


Fig. 2.6. Dendrogram representation of similarity between all replicates at all sites sampled in the Bow River in November of 2021. Similarity was calculated using Bray Curtis differences. Sites with lower differences are grouped more closely together. Branches distance on the y-axis indicates difference.

NMDS analysis was subsequently used to spatially represent the similarity of the regions defined by the cluster analysis. An NMDS performed for May 2021 benthic macroinvertebrate samples identified distinct clusters of sites within regions with an acceptable stress value <0.2 (stress = 0.14). Canmore upstream (red ellipsis) and downstream (green ellipsis) sites were grouped independently but were closely clustered overall, while North Calgary (blue ellipsis) sites clustered a similar distance from Canmore and South Calgary (purple ellipsis) sites (Fig. 2.7). An NMDS performed on September 2021 samples had generally similar clusters of sites within regions (stress = 0.14). Canmore sites were closely clustered; however, upstream (red ellipsis) and downstream (green ellipsis) sites were not distinct clusters showing overlap (Fig. 2.8). North Calgary and Cochrane were not differentiated, but south Calgary was distinctly separate from all other sites. This was similarly noted in November 2021, where Canmore upstream and downstream were not distinct and South Calgary was most distinctly separated from all other sites (Fig. 2.9) (stress = 0.11).

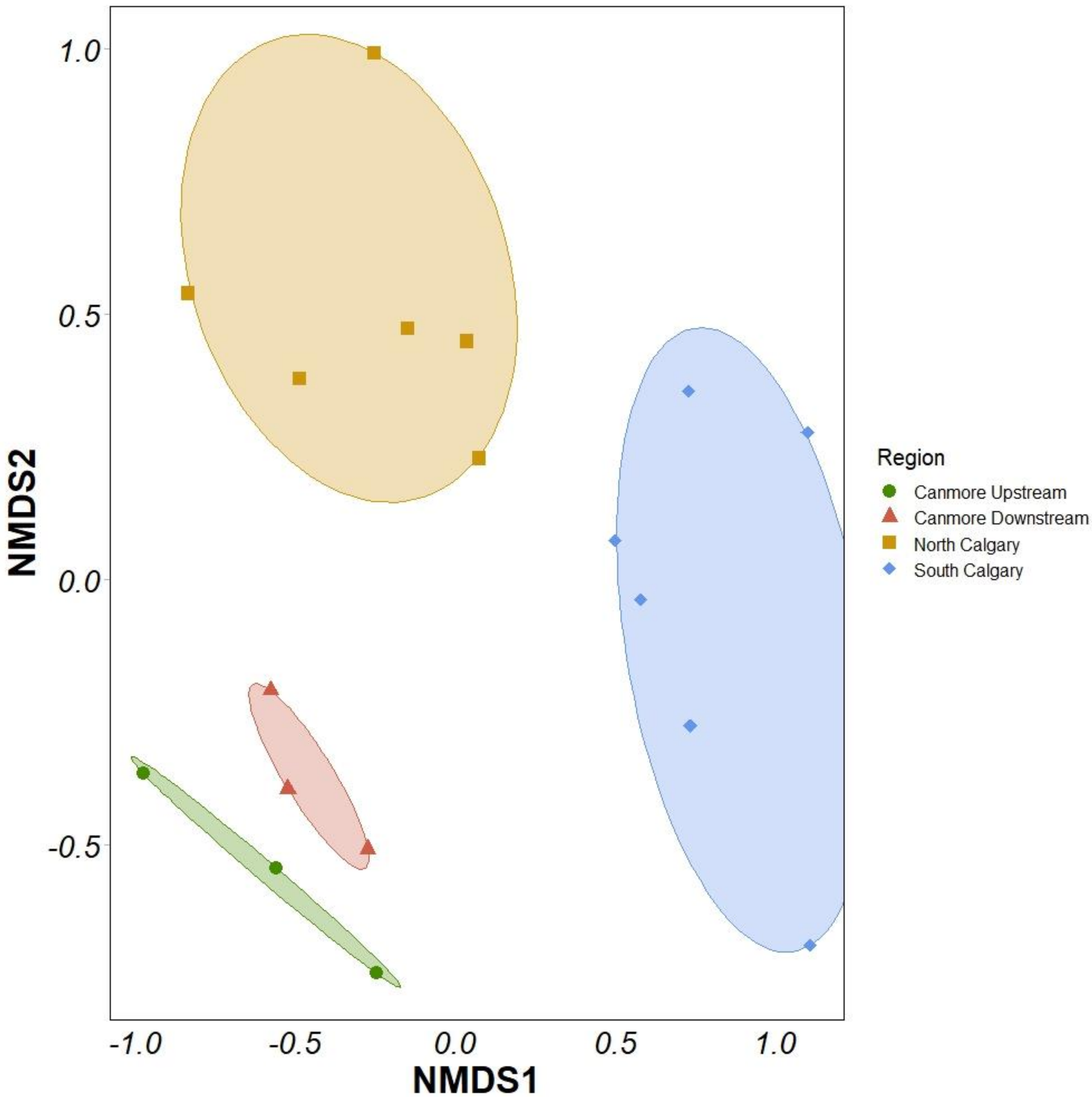


Fig. 2.7. Spatial representation of similarity between all replicates of all samples of benthic macroinvertebrate assemblages from May 2021. Sites are colour coded to the region indicated by the cluster analysis rather than individual sites. The nonmetric multidimensional scaling (NMDS) is based on Bray-Curtis dissimilarities where sites closer together are more similar and ellipses indicate spatial spread of the overall region.

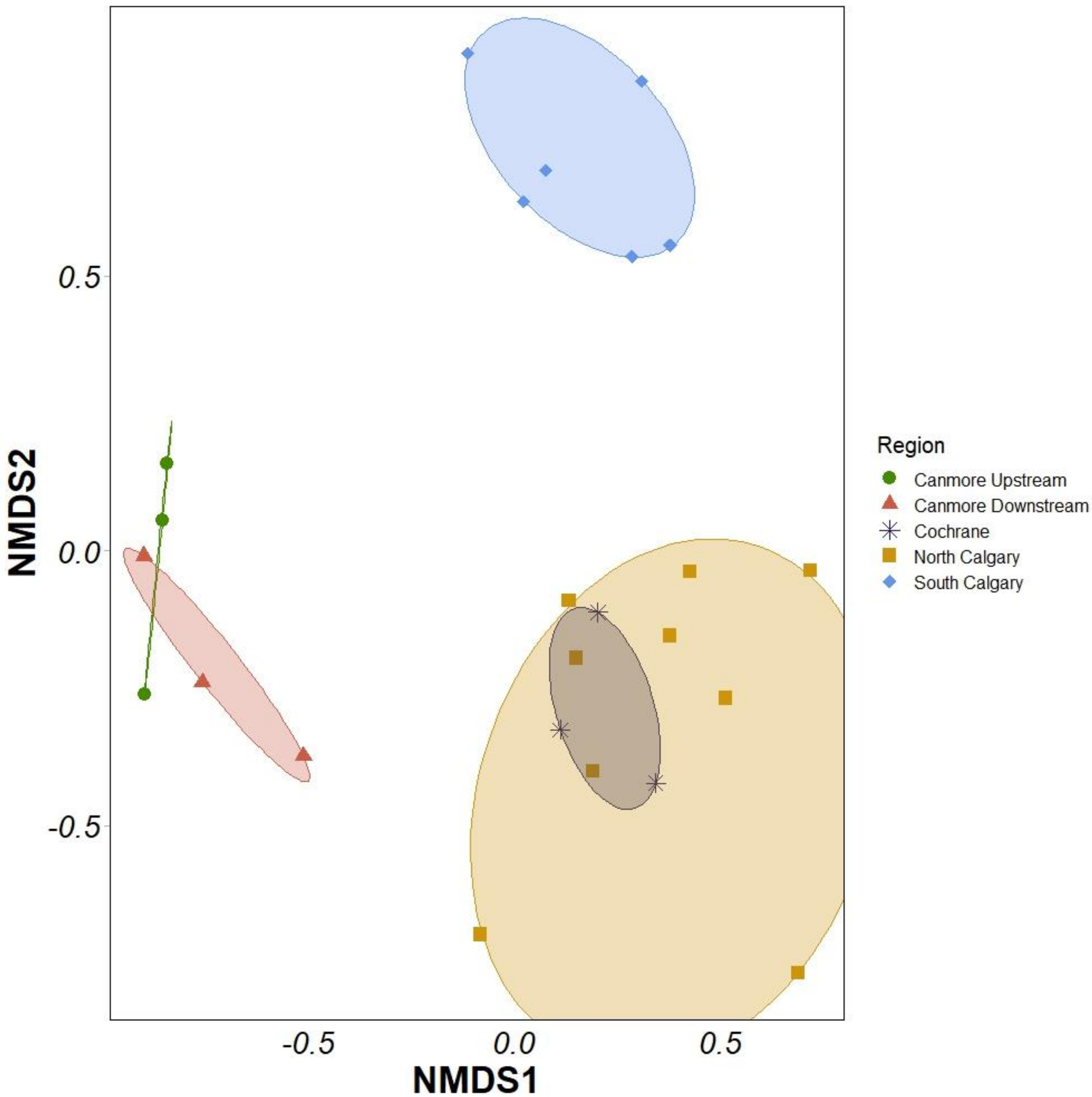


Fig. 2.8. Spatial representation of similarity between all replicates of all samples of benthic macroinvertebrate assemblages from September 2021. Sites are colour coded to the region indicated by the cluster analysis rather than individual sites. The nonmetric multidimensional scaling (NMDS) is based on Bray-Curtis dissimilarities where sites closer together are more similar and ellipses indicate spatial spread of the overall region.

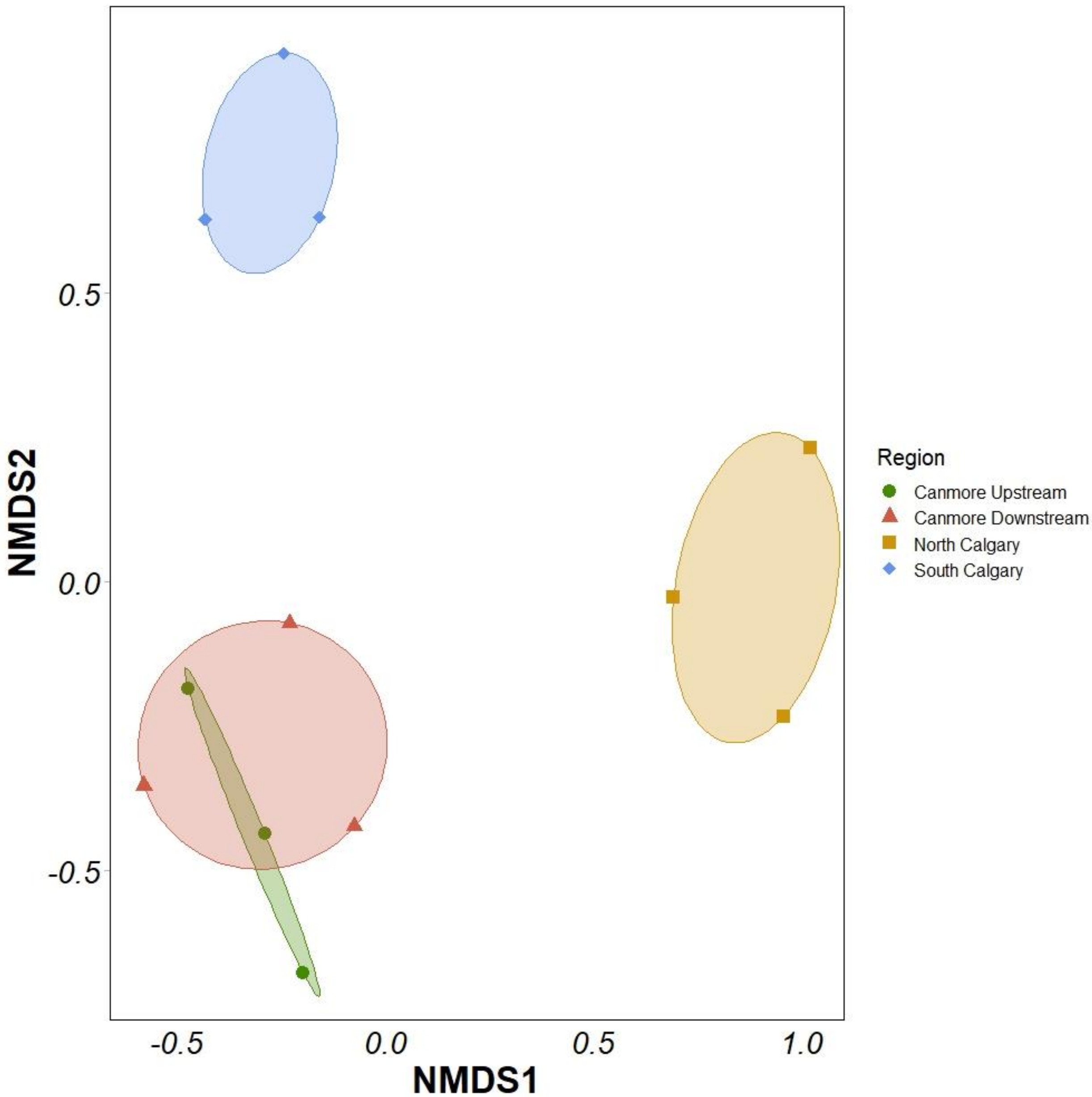


Fig. 2.9. Spatial representation of similarity between all replicates of all samples of benthic macroinvertebrate assemblages from November 2021. Sites are colour coded to the region indicated by the cluster analysis rather than individual sites. The nonmetric multidimensional scaling (NMDS) is based on Bray-Curtis dissimilarities where sites closer together are more similar and ellipses indicate spatial spread.

2.3.2 Biotic Indices

Most of the benthic invertebrate diversity in Bow River sites in May 2021 is represented by EPT and Diptera taxa, with cumulative other taxa making up 1% or less of the total community assemblage. Generally, EPT remained dominant over Diptera until the South Calgary region, downstream from the first, Bonnybrook WWTP. More specifically, Canmore upstream and downstream sites do not show notable variation in relative EPT:D composition. However, there is a shift in relative abundance of EPT between Canmore and Calgary in the slight increase in Ephemeroptera (~55-58% to 63%) compared to Plecoptera, which consistently decreases in each river-reach region moving downstream (from ~31% to 22% to just ~4%). Trichoptera makes up a relatively small proportion of the overall community at all sites but does show slight increases downstream of both treatment plants compared to their upstream counterpart (1% to 3% in Canmore and 5% to 8 % in Calgary). Diptera generally increase in relative abundance moving upstream to downstream, although there is a consistent increase after the downstream Canmore site. Most notably, South Calgary shows large increases in the proportion of Diptera, shifting from ~27% in North Calgary to ~83% in South Calgary sites. (Fig. 2.10).

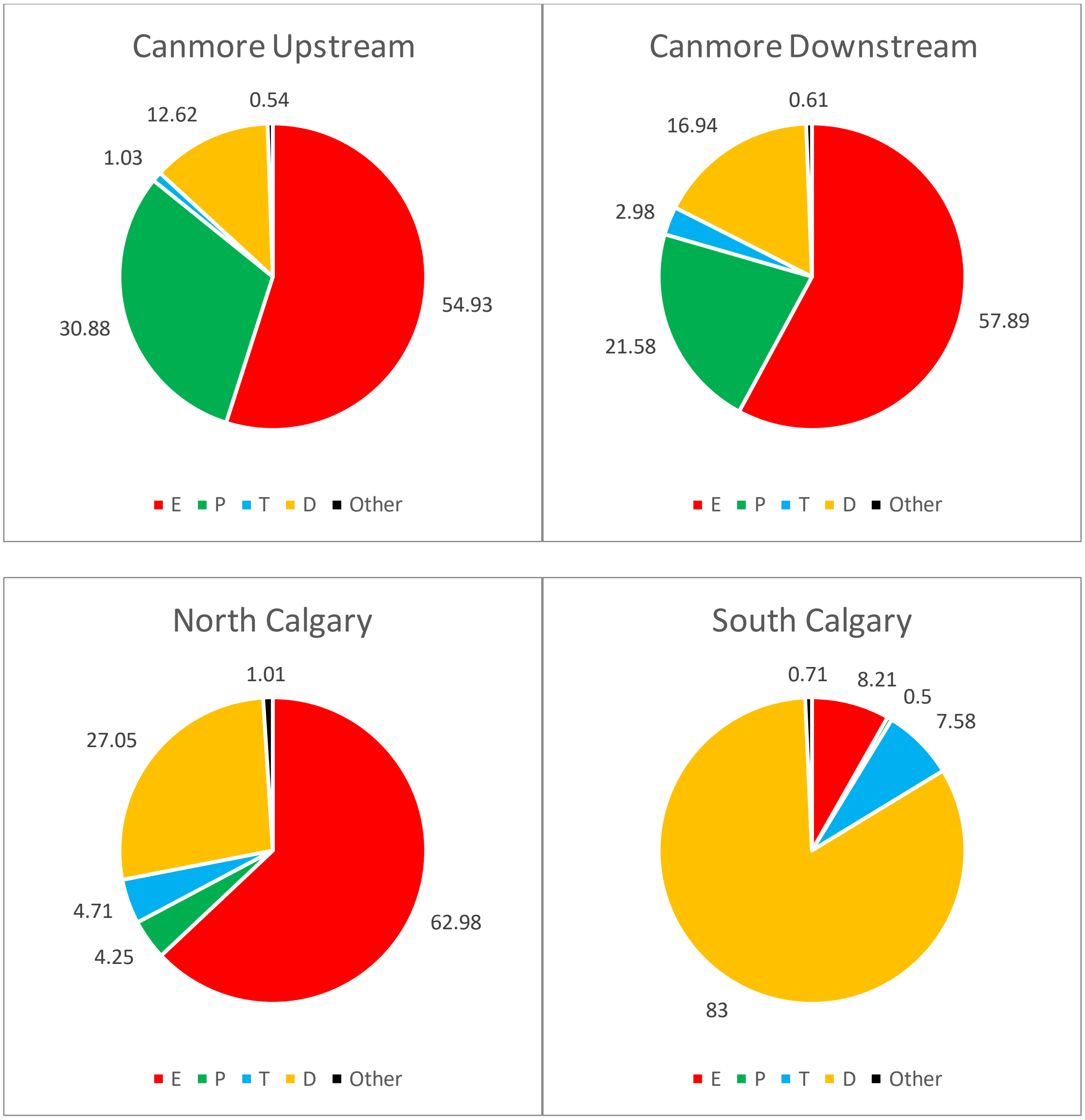


Fig. 2.10. In May of 2021, the relative contribution (%) in the overall benthic macroinvertebrate community assemblage of key, coarse (mainly Order) taxonomic groups including EPT (Ephemeroptera (E), Plecoptera (P), Trichoptera (T)) taxa, Diptera (D) taxa, and cumulative other organisms (O).

Similar patterns were observed in samples taken in September of 2021. Other taxa not part of EPT or Diptera were more represented, making up almost 8% of organisms in Cochrane and 7% in North Calgary. However, their contribution was below 3% in all other sampling regions. EPT were more represented in the benthic macroinvertebrate community than Diptera in all regions; however, the margin of dominance decreases dramatically in the South Calgary region. The EPT:D ratio between upstream and downstream Canmore also changed more notably in September than in May, with Diptera changing from making up 2% of the population upstream to >30% downstream. This was accompanied by reductions in Trichoptera and Ephemeroptera families in these sites, with the most dramatic decrease observed in Ephemeroptera (from 70% to 35%). The relative abundance of Plecoptera doubled downstream of the Canmore WWTP (13% to 26%) but were relatively rare at all sampling locations in within the City of Calgary. The same increase in Ephemeroptera between Canmore and Calgary was also observed but was not reflected in the Cochrane site that falls between these sites, which had the highest proportion of Trichoptera of any region (46%). Diptera generally increased in regions below WWTPs, showing up in high proportions at both the Canmore downstream site (30%) and at the sites in South Calgary (42%). There is a slight reduction in Diptera dominance between Canmore downstream (~30%) and Cochrane/North Calgary (13-17%). EPT are still dominant in the South Calgary region, unlike in May, primarily due to the increased presence of Trichoptera, while the relative proportions of Ephemeroptera and Plecoptera are greatly reduced (Fig. 2.11)

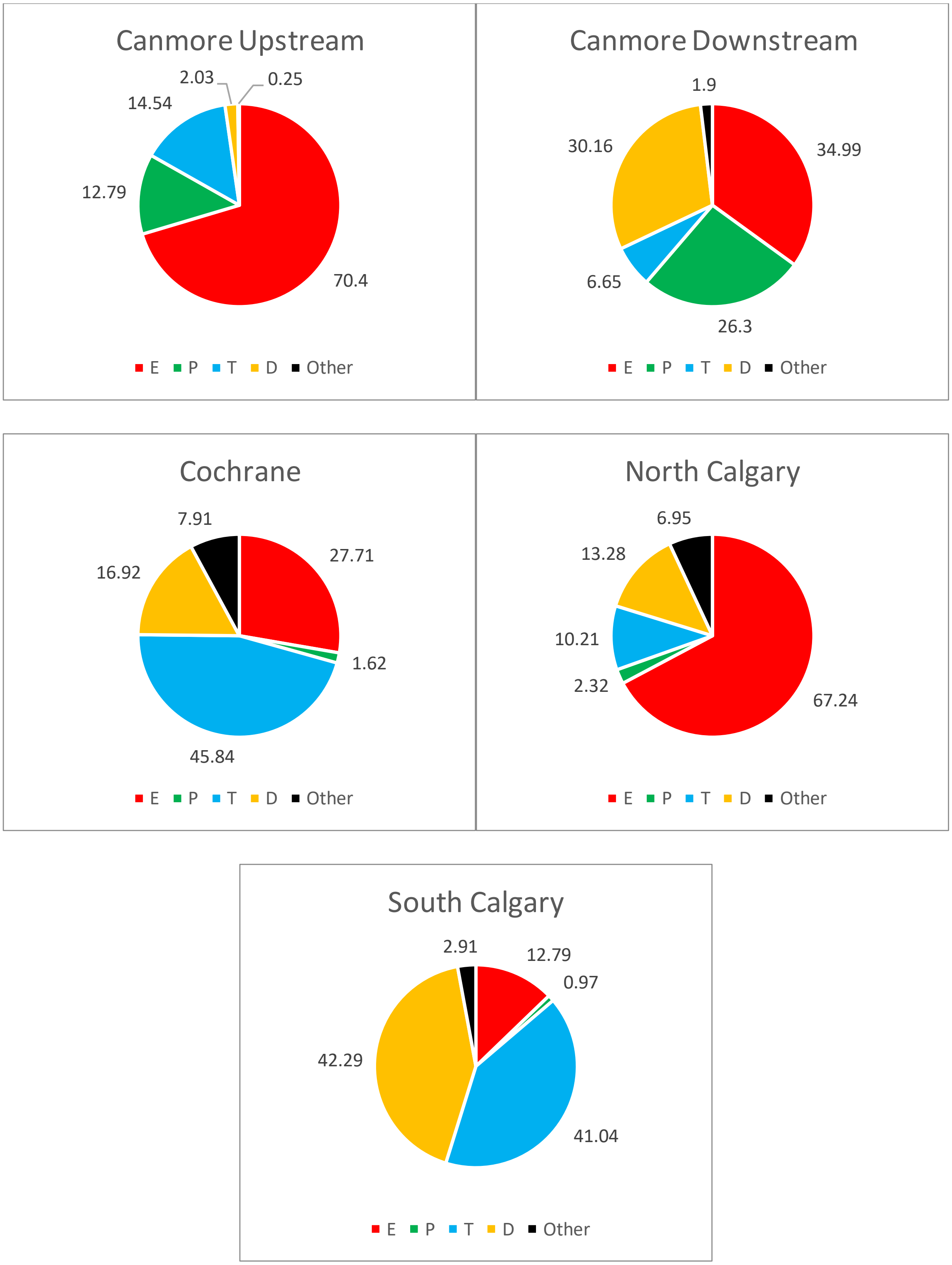


Fig. 2.11. In September of 2021, the relative contribution (%) in the overall benthic macroinvertebrate community assemblage of key, coarse (mainly Order) taxonomic groups including EPT (Ephemeroptera (E), Plecoptera (P), Trichoptera (T)) taxa, Diptera (D) taxa, and cumulative other organisms (O).

The benthic assemblage patterns observed in November 2021 were similar to the previous two months. EPT were dominant compared to Diptera or any other organisms (always less than 3% of total community) in all regions except South Calgary (here just PMF), where Diptera made up just over 50% of the total community. Ephemeroptera showed a similar pattern to May samples and September samples excepting Cochrane, with the highest relative abundances (67-81%) in the Canmore Upstream site and the North Calgary site BWP) and showing reductions in the corresponding nearby downstream sites. Like in previous months, Plecoptera were most represented downstream of the Canmore WWTP but are generally quite reduced in Calgary (~1% of total community). Trichoptera were similarly represented at all sites until South Calgary (PMF), where they increase by approximately a factor of four to almost 20% of the total population. Diptera was the highest proportion of the population downstream of WWTPs in Canmore and in Calgary, which is similar to the September samples (Fig. 2.12).

Overall, the relative abundance patterns in EPT across the sampling time periods and regions showed a major shift only in the South Calgary region, with all months and regions showing that EPT represents around 70% of the community in all regions in May, September, and November. This drops to around 50% in South Calgary for September and November and is even lower in the spring May sampling, hovering at a mean proportion of just less than 25% (Fig. 2.13). This pattern is correspondingly seen in the % Diptera changes in the same regions. Diptera contributions to the community are around 25% until the South Calgary region, where they consistently make up >50% of the total population in all sampling months (Fig. 2.14). However, within the EPT species, some differences between sites and time periods were observed. Particularly, Trichoptera presence in September was much higher than May and November in region, but particularly South Calgary.

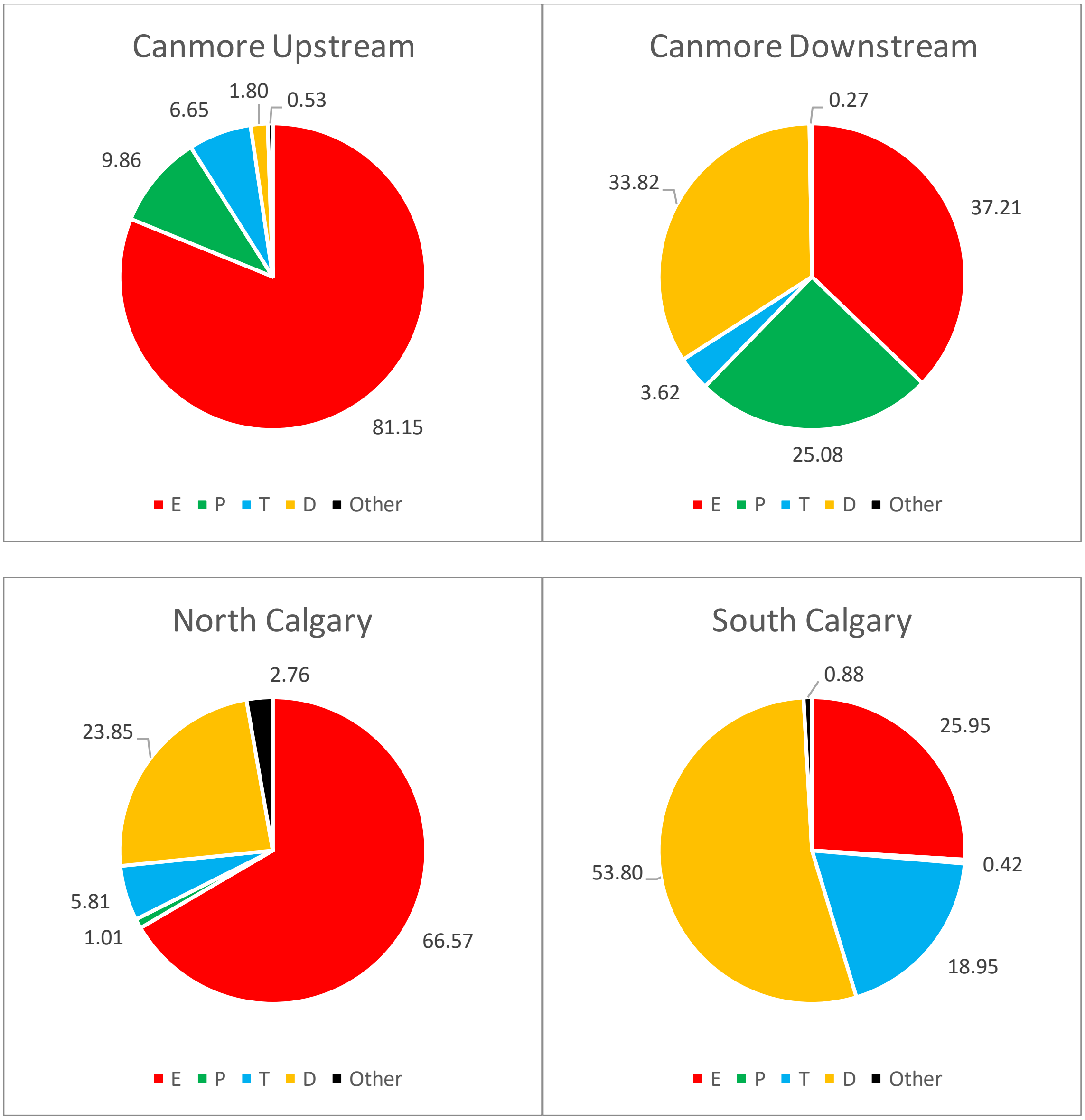


Fig. 2.12. In November of 2021, the relative contribution (%) in the overall benthic macroinvertebrate community assemblage of key, coarse (mainly Order) taxonomic groups including EPT (Ephemeroptera (E), Plecoptera (P), Trichoptera (T)) taxa, Diptera (D) taxa, and cumulative other organisms (O).

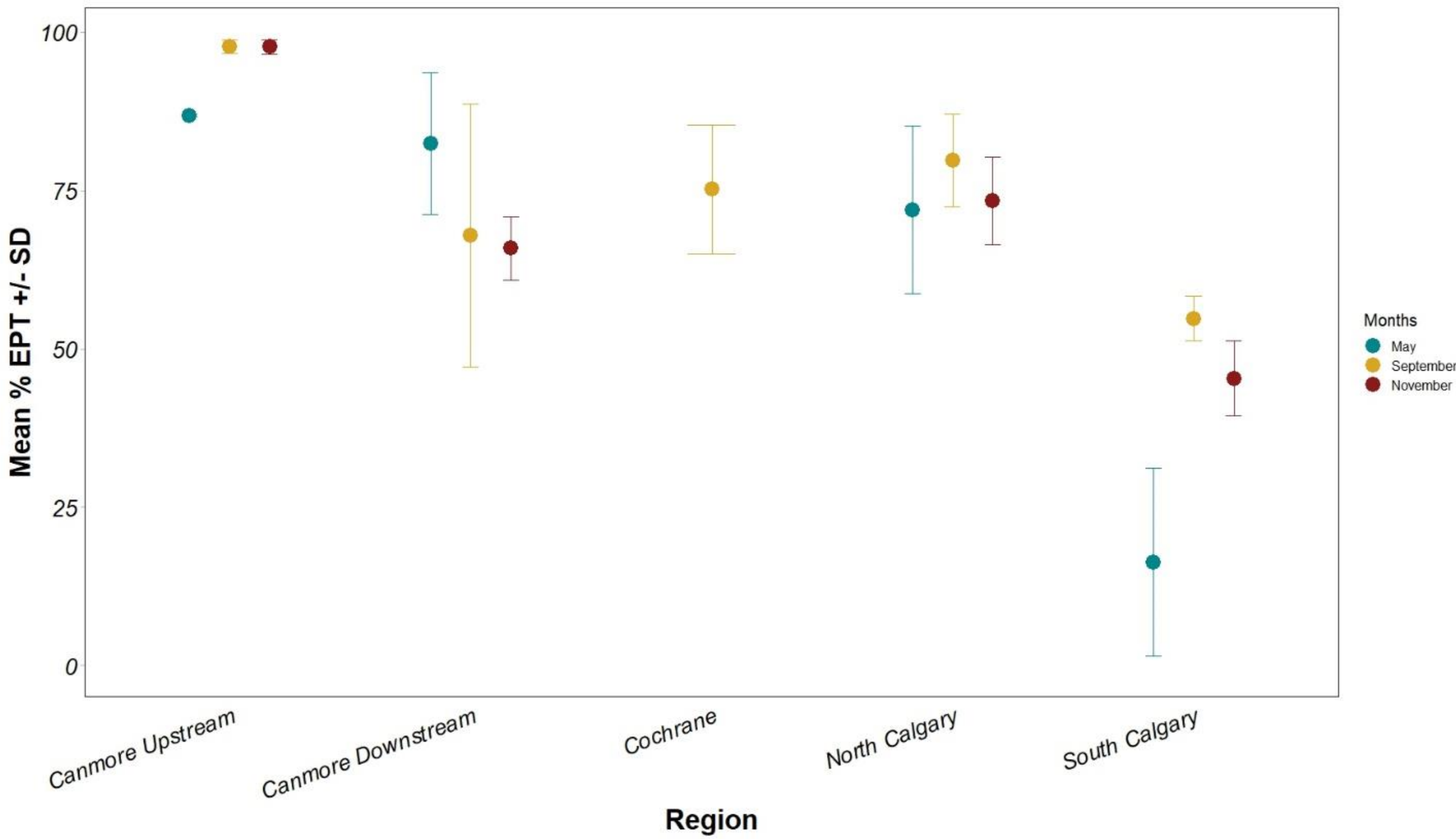


Fig. 2.13. The monthly mean percentage of EPT taxa in the macroinvertebrate assemblage (% of total community +/- 1 standard deviation). %EPT was calculated from the abundance of taxa from the Orders Ephemeroptera, Plecoptera, and Trichoptera divided by the total number of taxa identified in each replicate. All replicates for each site in the defined regions were averaged. Note that sites sampled for each region differ slightly in different months and the Cochrane region/site was only sampled in September). Regions are arranged by most upstream (Canmore upstream) on the far left and most downstream (South Calgary) on the far right.

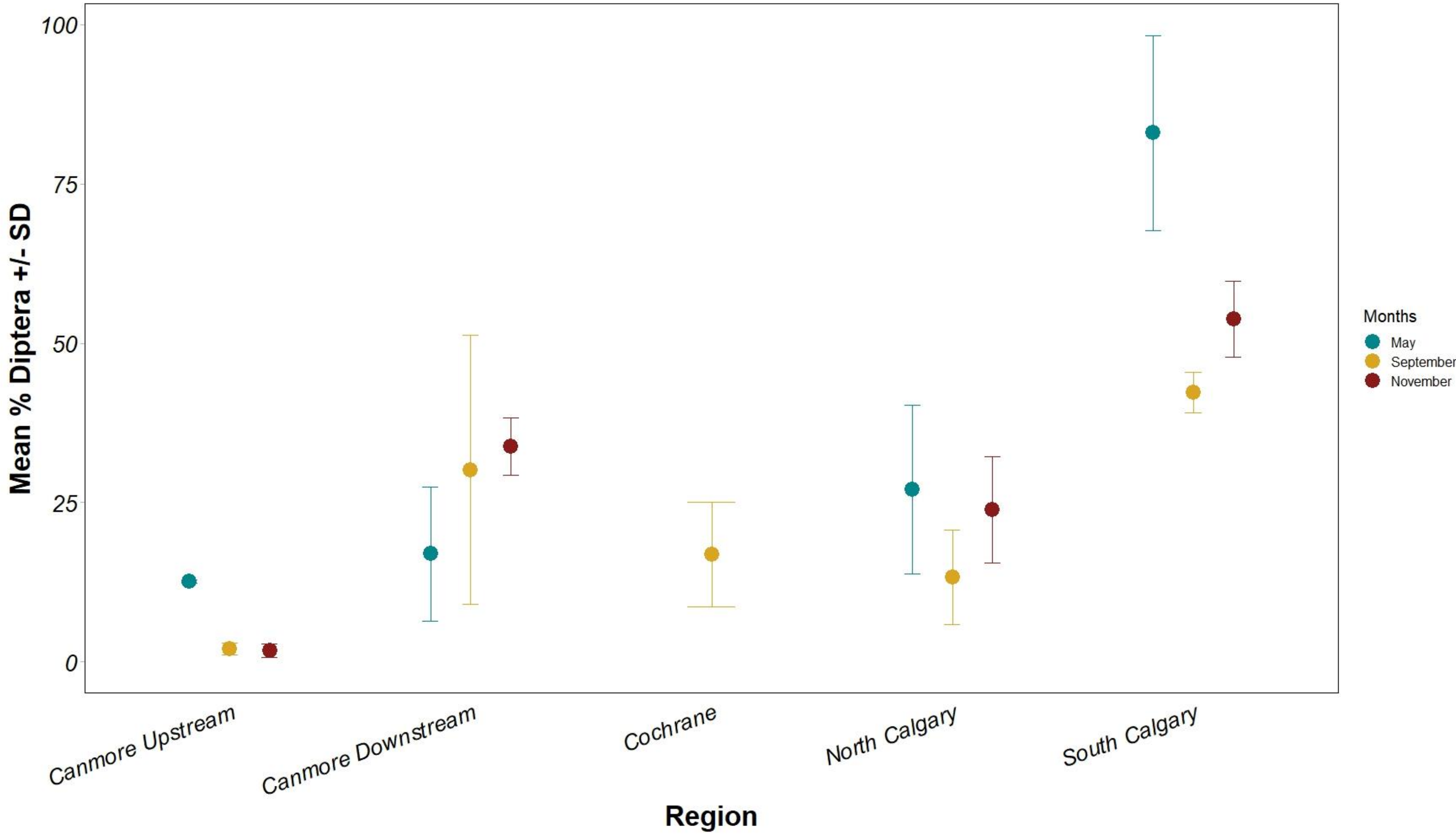


Fig. 2.14. The monthly mean percentage of Diptera taxa in the macroinvertebrate assemblage (% of total community +/- 1 standard deviation). %Diptera was calculated from the abundance of taxa from the Order Diptera divided by the total number of taxa identified in each replicate. All replicates for each site in the defined regions were then averaged. Note that sites sampled for each region differ slightly in different months and the Cochrane region/site was only sampled in September). Regions are arranged by most upstream (Canmore upstream) on the far left and most downstream (South Calgary) on the far right.

The overall abundance of all organisms in each region shows that abundance does not vary largely between regions in any months until the sites in the South Calgary region, which is magnitudes higher than the abundances in upstream regions in both September and November sampling (Fig. 2.15). However, the variation in overall abundance also varies more in these sites. Alternatively, the diversity index in the regions does not vary greatly, apart from a relatively low score in the May sampling in the South Calgary region. There is also a small but consistent increase in diversity in downstream Canmore compared to the nearby upstream site (Fig. 2.16). There is a very slight trend upwards as sites move downstream in the family level richness between regions, varying between a mean of ~8 to 15 families at each site (Fig. 2.17). Dominance and evenness in the regions do not show much of a pattern in regions or months (Fig. 2.18, Fig. 2.19). However, the Hilsenhoff Biotic index (HBI) showed a similar pattern to the abundance measures, with sites being relatively similar (ranging from a rating of excellent to very good at all sites in all months) until the south Calgary region, where sites demonstrate an average rating of Good (in September) to Fair (in May and November) (Fig. 2.20).

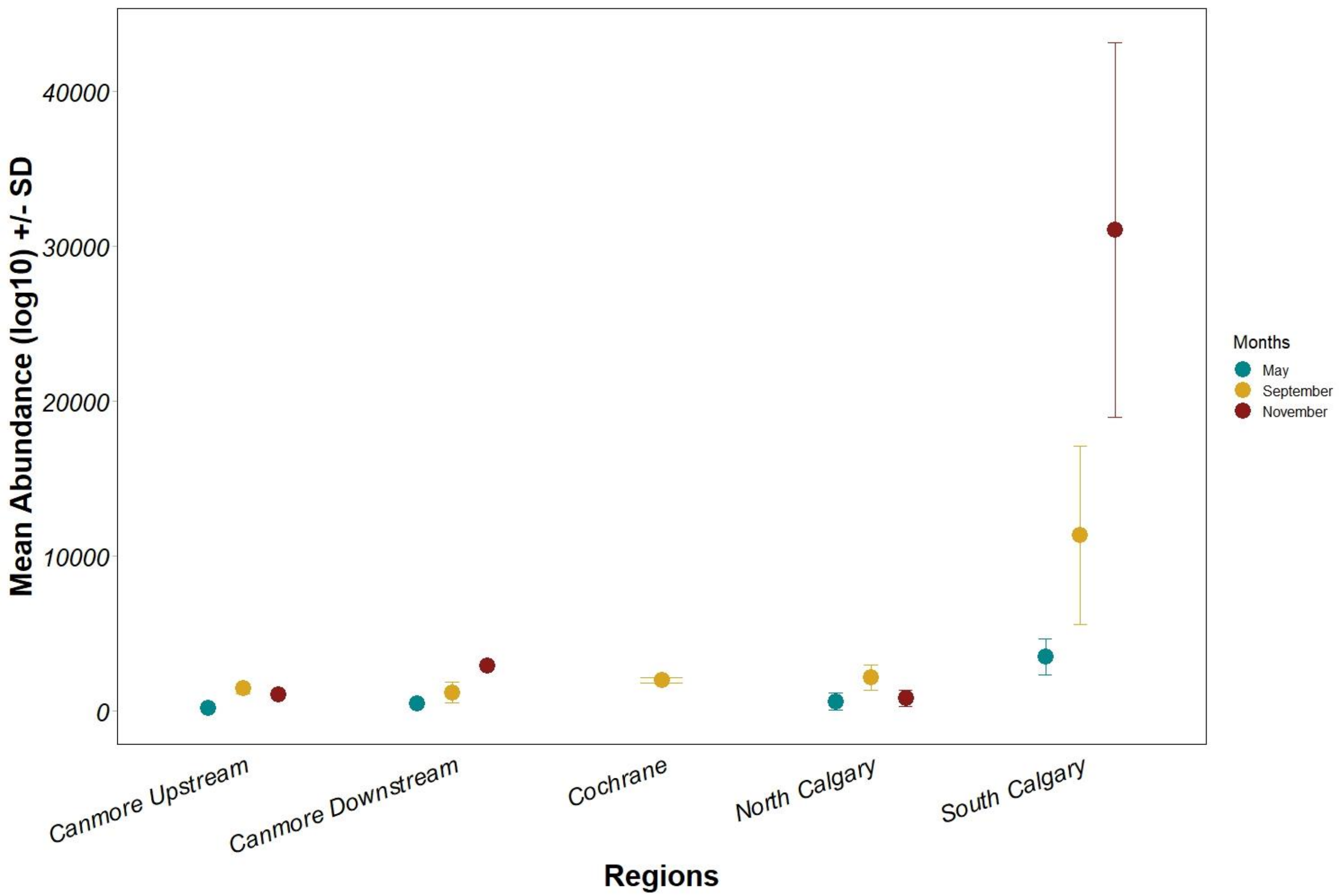


Fig. 2.15. The $\log_{10}$ scale monthly mean abundance of macroinvertebrate taxa (+/- 1 standard deviation). Abundance was calculated from the raw abundance of all taxa in each replicate. All replicates for each site in the defined regions were then averaged. Note that sites sampled for each region differ slightly in different months and the Cochrane region/site was only sampled in September). Regions are arranged by most upstream (Canmore upstream) on the far left and most downstream (South Calgary) on the far right.

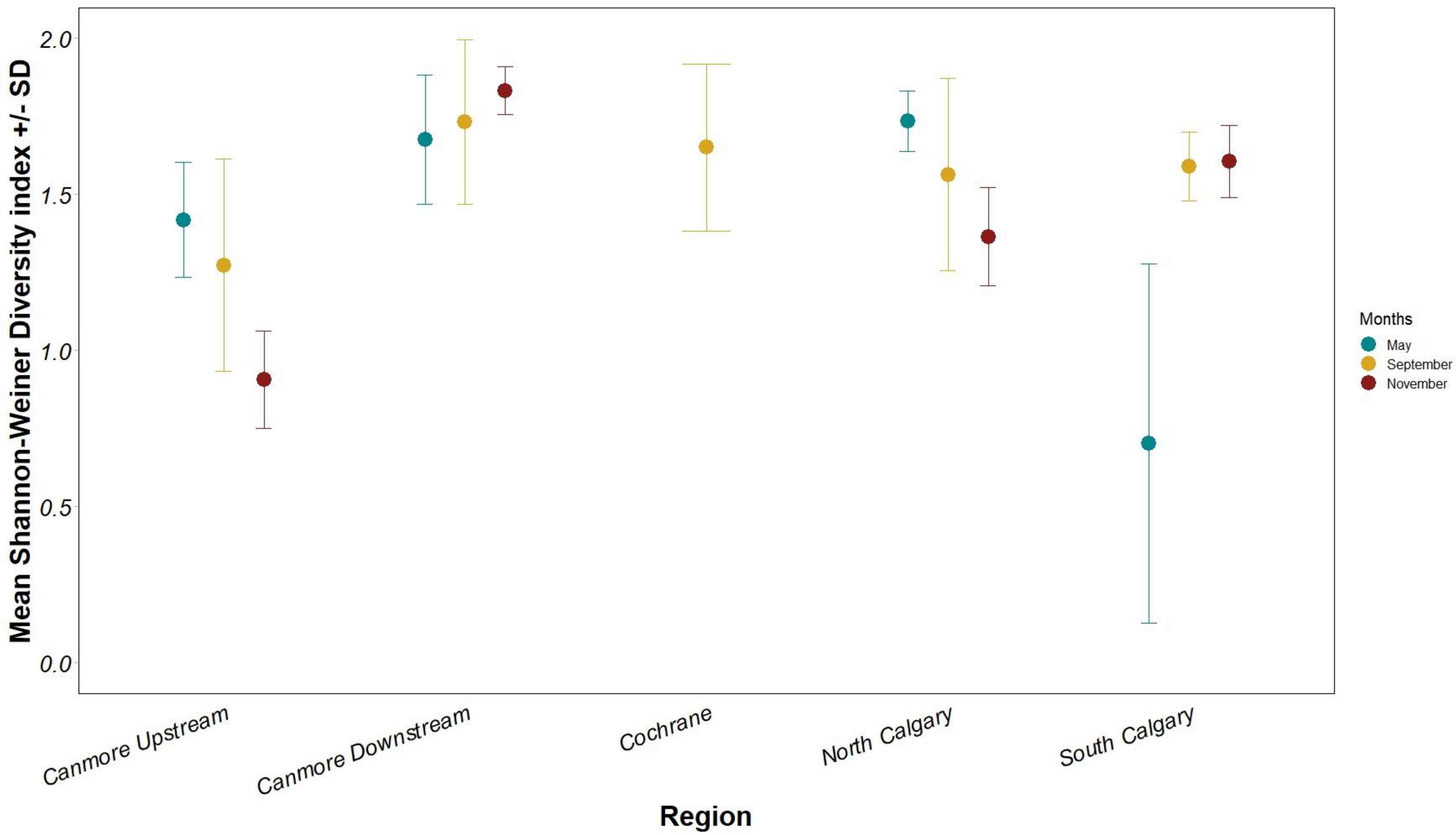


Fig. 2.16. The monthly mean diversity index of macroinvertebrate taxa (+/- 1 standard deviation). Diversity was calculated using the Shannon-Weiner diversity index equation for each replicate. All replicates for each site in the defined regions were then averaged. Note that sites sampled for each region differ slightly in different months and the Cochrane region/site was only sampled in September). Regions are arranged by most upstream (Canmore upstream) on the far left and most downstream (South Calgary) on the far right.

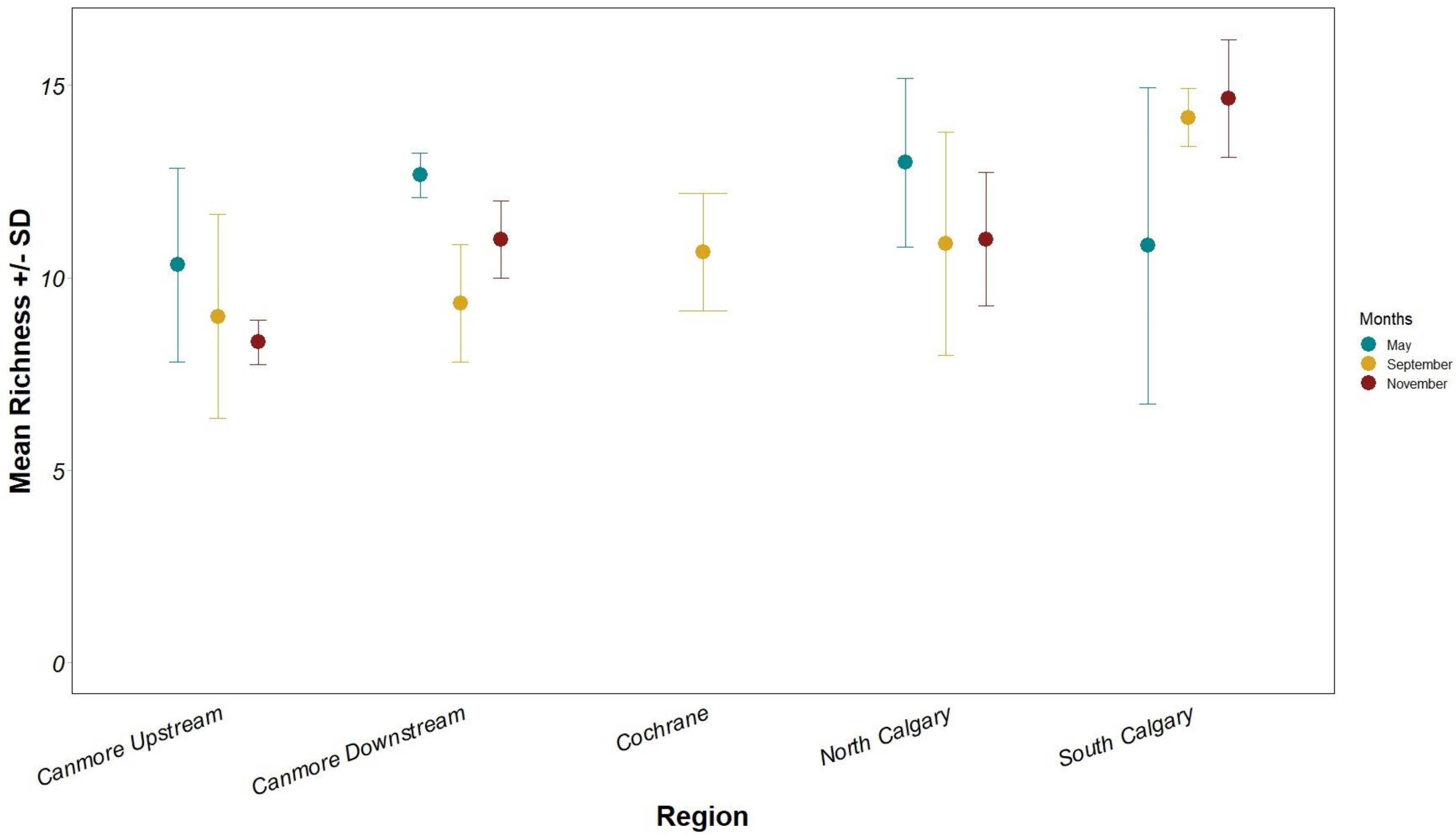


Fig. 2.17. The monthly mean family richness of macroinvertebrate taxa (+/- 1 standard deviation). Richness was calculated by counting unique families for each replicate. All replicates for each site in the defined regions were then averaged. Note that sites sampled for each region differ slightly in different months and the Cochrane region/site was only sampled in September). Regions are arranged by most upstream (Canmore upstream) on the far left and most downstream (South Calgary) on the far right.

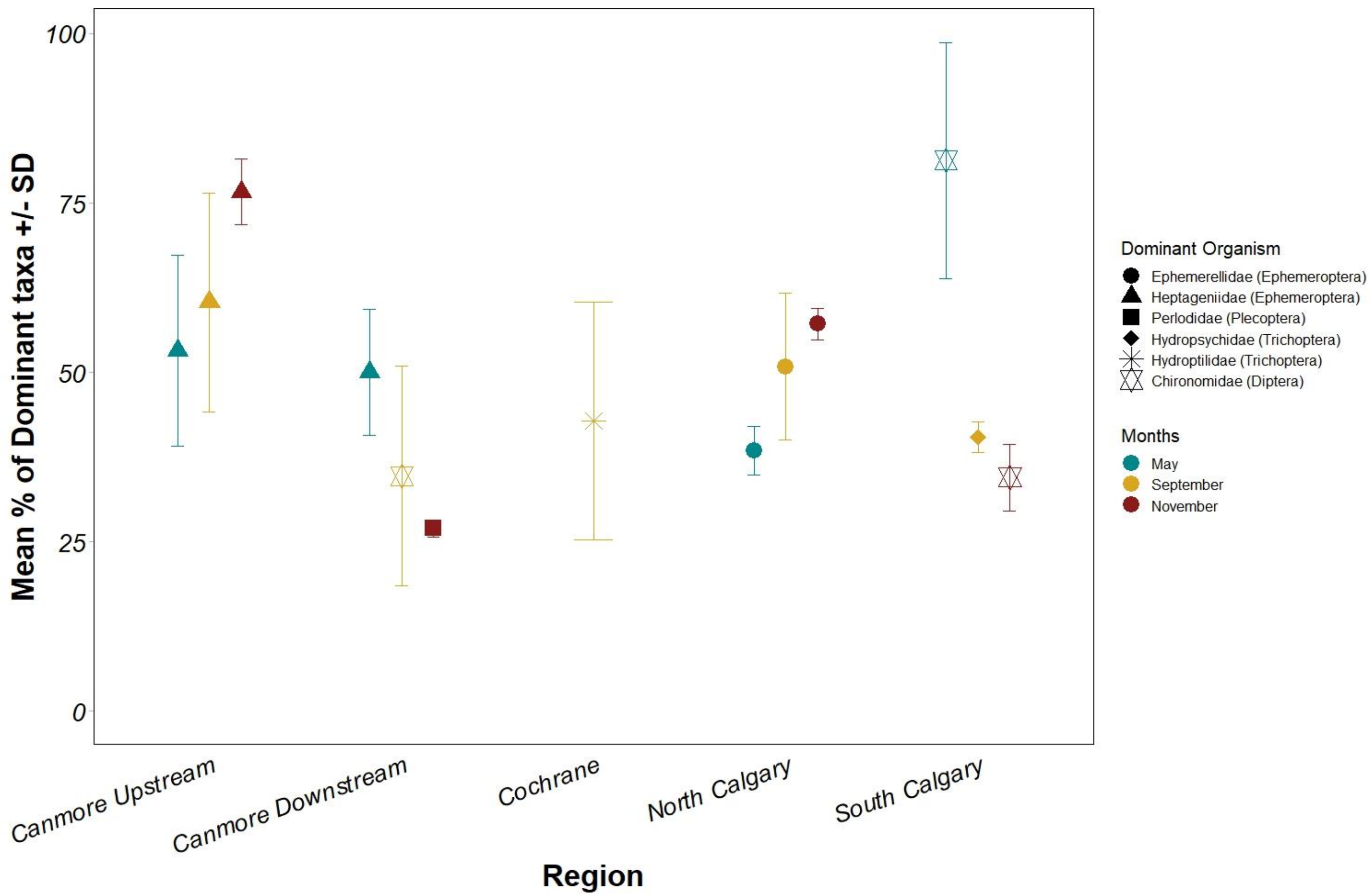


Fig. 2.18. The monthly mean percentage of the dominant taxa in the macroinvertebrate assemblage (% of total community +/- 1 standard deviation). % dominance was calculated from the maximum abundance of a macroinvertebrate family divided by the total number of taxa identified in each replicate. All replicates for each site in the defined regions were then averaged. The most dominant family was calculated from the highest abundance of a macroinvertebrate family in a whole region and is indicated alongside the Order. Note that sites sampled for each region differ slightly in different months and the Cochrane region/site was only sampled in September). Regions are arranged by most upstream (Canmore upstream) on the far left and most downstream (South Calgary) on the far right.

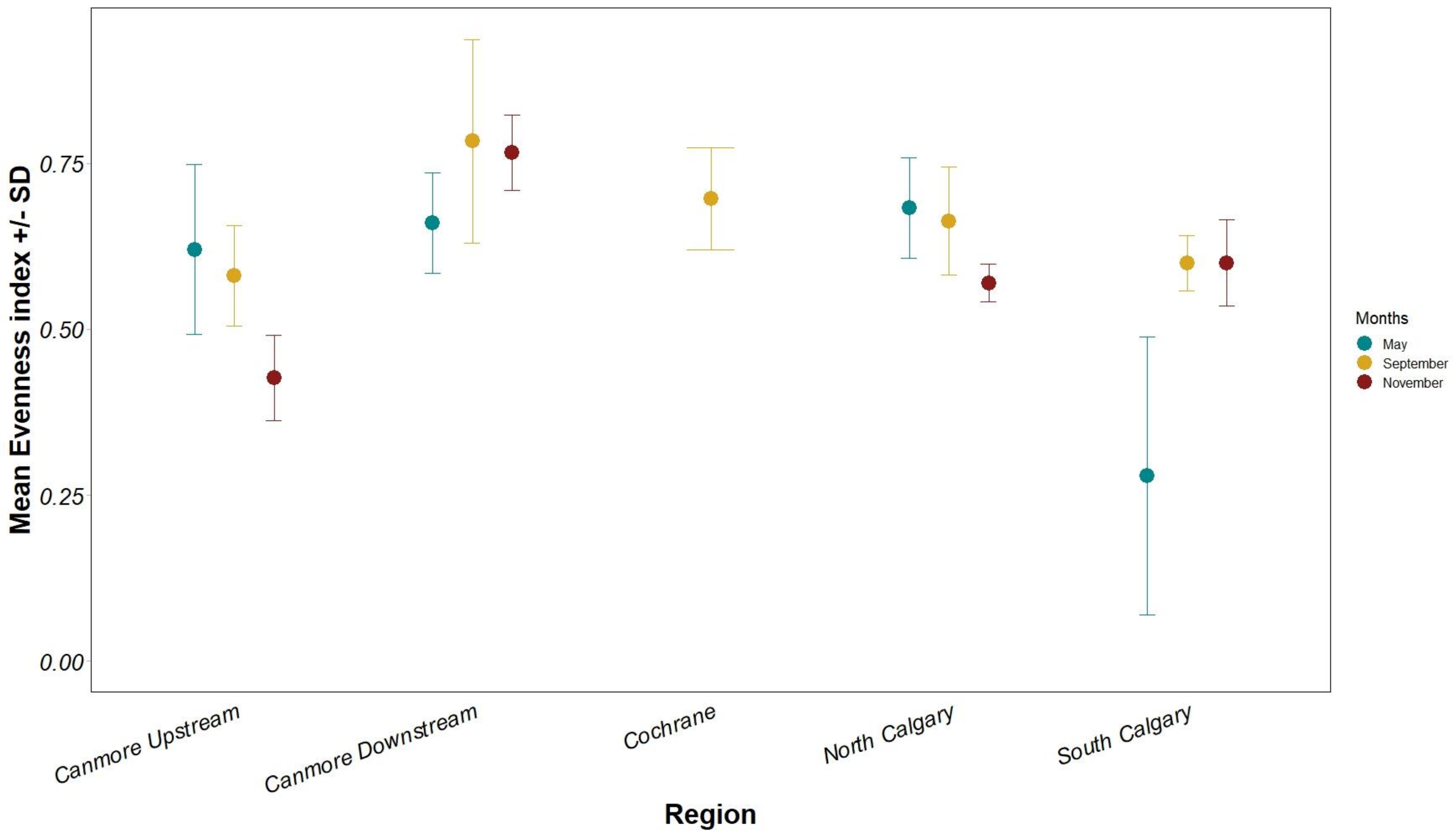


Fig. 2.19. The monthly mean evenness index of macroinvertebrate taxa (+/- 1 standard deviation). Evenness was calculated using the Pielou's evenness index equation for each replicate. All replicates for each site in the defined regions were then averaged. Note that sites sampled for each region differ slightly in different months and the Cochrane region/site was only sampled in September). Regions are arranged by most upstream (Canmore upstream) on the far left and most downstream (South Calgary) on the far right.

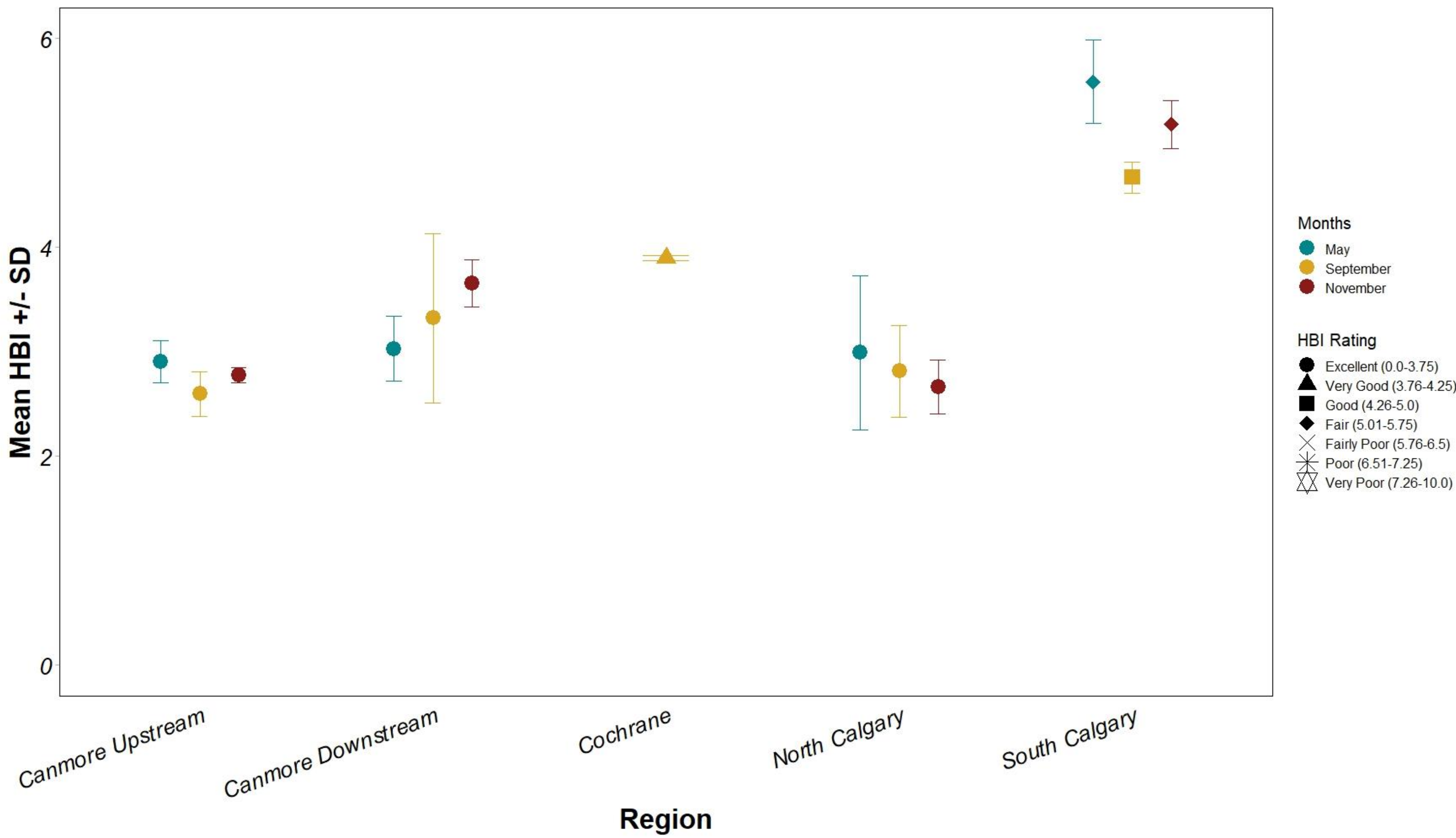


Fig. 2.20. The monthly mean HBI rating of macroinvertebrate taxa (+/- 1 standard deviation). The Hilsenhoff Biotic Index (HBI) was calculated using the HBI equation for each replicate. All replicates for each site in the defined regions were then averaged. The numeric score (between 0 and 10) is also associated with a categorical rating: Excellent (0.0-3.75, Very Good (3.76-4.25), Good (4.26-5.0), Fair (5.01-5.75), Fairly Poor (5.76-6.5), Poor (6.51-7.25), Very Poor (7.26-10.0). Note that sites sampled for each region differ slightly in different months and the Cochrane region/site was only sampled in September). Regions are arranged by most upstream (Canmore upstream) on the far left and most downstream (South Calgary) on the far right.

2.3.3 PERMANOVA & SIMPER

The overall relative abundances of benthic community assemblages differed significantly from each other in May 2021 (Table 2.1). A pairwise comparison showed that this stemmed from a significant difference between North and South Calgary benthic macroinvertebrate communities (Table 2.2). With an increased suite of overall sites sampled and the addition of the Cochrane region in September of 2021, similarly, the overall results of the PERMANOVA showed significant differences among the regions (Table 2.3). Pairwise comparisons of the regions showed significant differences between Canmore Upstream and North Calgary, Canmore Downstream and North Calgary, and North and South Calgary (Table 2.4). In November, the overall regional comparison differed but pairwise comparisons revealed no significant differences between any two regions (Table 2.5, Table 2.6).

Table 2.1. May benthic macroinvertebrate kicknet (log_{10} transformed) PERMANOVA results. * Indicates significant p<0.05.

F-value	df	p-value
11.215	3	0.001*

Table 2.2. May benthic macroinvertebrate kicknet pairwise (log_{10} transformed) PERMANOVA results. * Indicates significant p<0.05.

Pairwise Comparison	df	p-value
Canmore Upstream vs. Canmore Downstream	1	0.60
Canmore Upstream vs. North Calgary	1	0.078
Canmore Upstream vs. South Calgary	1	0.054
Canmore Downstream vs. North Calgary	1	0.054
Canmore Downstream vs. South Calgary	1	0.054
North Calgary vs. South Calgary	1	0.012*

Table 2.3. September benthic macroinvertebrate kicknet (log_{10} transformed) PERMANOVA results. * Indicates significant p<0.05.

F-value	df	p-value
16.095	4	0.001*

Table 2.4. September benthic macroinvertebrate kicknet pairwise (log_{10} transformed) PERMANOVA results. * Indicates significant result.

Pairwise Comparison	df	p-value
Canmore Upstream vs. Canmore Downstream	1	1
Canmore Upstream vs. Cochrane	1	1
Canmore Upstream vs. North Calgary	1	0.02*
Canmore Upstream vs. South Calgary	1	0.06
Canmore Downstream vs. Cochrane	1	1
Canmore Downstream vs. North Calgary	1	0.05*
Canmore Downstream vs. South Calgary	1	0.19
Cochrane vs. North Calgary	1	0.58
Cochrane vs. South Calgary	1	0.17
North Calgary vs. South Calgary	1	0.01*

Table 2.5. November benthic macroinvertebrate kicknet (log_{10} transformed) PERMANOVA results. * Indicates significant p<0.05.

F-value	df	p-value
17.601	3	0.002*

Table 2.6. November benthic macroinvertebrate kicknet pairwise (log_{10} transformed) PERMANOVA results. * Indicates significant $p<0.05$.

Pairwise Comparison	df	p-value
Canmore Upstream vs. Canmore Downstream	1	0.6
Canmore Upstream vs. North Calgary	1	0.6
Canmore Upstream vs. South Calgary	1	0.6
Canmore Downstream vs. North Calgary	1	0.6
Canmore Downstream vs. South Calgary	1	0.6
North Calgary vs. South Calgary	1	0.6

A SIMPER analysis for the May 2021 samples identified between one and five families in each pairwise comparison (with five families total) that contributed to over 5% of the difference between regions (Table 2.7). All comparisons revealed Chironomidae (Diptera) as the primary taxonomic group contributing to the observed regional differences and in three cases this family was the top and only contributor to significant differences between any other region and South Calgary (abundance data shows that South Calgary had high Chironomidae abundance comparatively). The only other Diptera family noted was a smaller contribution from Simuliidae, which differentiated the Canmore sites, as they were largely absent upstream of the Canmore WWTP. Ephemeroptera families Heptageniidae and Ephemerellidae were more significant in the differentiation of Canmore and North Calgary sites, with both Canmore sites having higher abundances of Heptageniidae than North Calgary, which generally had more Ephemerellidae. The Plecoptera family Nemouridae showed a similar pattern of significance between North Calgary and Canmore site comparisons, with the stonefly family only being observed at Canmore sites.

SIMPER analysis in September 2021 identified between three and five families in each pairwise comparison (over eight total families) that contributed to over 5% of the difference between regions (Table 2.8). There was less consensus in highlighted taxa groups in this month, but the Diptera group Chironomidae still occurred in every list other than comparing the Canmore Downstream region to the North Calgary region. However, it was never the most influential taxon for any two sites, unlike in May 2021. Simuliidae, the other Diptera family represented, only showed up to differentiate the South of Calgary from Canmore Downstream, where they were not highly represented. Ephemeroptera was highly influential in differentiating Canmore sites, as the upstream was dominated by Heptageniidae mayflies and the downstream by Ephemerellidae mayflies, which was similar to Canmore upstream and North Calgary as well. Batidae mayflies were additionally more present in Calgary than Canmore and Cochrane, which contributed to significant differences in several of these comparisons. Generally, Cochrane had fewer mayflies than Canmore sites and North Calgary sites and was not differentiated from the South of Calgary or downstream of the Canmore WWTP through mayfly taxa. Canmore downstream was separated from most other sites through the lack of Perlodidae stoneflies (Plecoptera) while two families of Trichoptera separated out Canmore (which had few mayflies) from Cochrane and North Calgary, which were dominated by Hydroptilidae, and from South Calgary, which was dominated by Hydropsychidae.

In November 2021, the SIMPER analysis identified between two and four families (over six total families) responsible for differences between regions (Table 2.9). While the Diptera family Chironomidae was still highly influential between many sites (they were implicated in differentiating every site except the Upstream Calgary and North Calgary site and half the time were the most influential taxa), Simuliidae were more commonly disparate between sites as well.

Simuliids and Chironomids were much more common at the downstream WWTP sites (CDS and PMF), with especially large increases downstream in South Calgary. The dominance of Heptageniids in the upstream Canmore site was notable in its comparisons as well as the similar dominance of Ephemerellids in North Calgary. Baetid mayflies, for the first time, characterized south Calgary. Perlodid stoneflies identified downstream Canmore from other sites, particularly from upstream Canmore and North Calgary.

Table 2.7. SIMPER results for May 2021 kicknet samples comparing regions. The most influential taxa are listed first and % listed is cumulative explanation of difference between regions. Only taxa explaining >5% of region difference are included, and taxa are colour coded by taxonomic group: red (Ephemeroptera), green (Plecoptera), blue (Trichoptera), yellow (Diptera), pink (Coleoptera), or grey (Other taxonomic group). For Canmore regions, n=3, and for all other sites n = 6.

Sites	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**
Canmore Upstream vs. Canmore Downstream	Heptageniidae (38.9%)	Chironomidae (48.6%)	Nemouridae (57.6%)	Ephemerellidae (64.6%)	Simuliidae (71.1%)
Canmore Upstream vs. North Calgary	Ephemerellidae (22.5%)	Heptageniidae (44.8%)	Chironomidae (63.8%)	Nemouridae (74.7%)	
Canmore Upstream vs. South Calgary	Chironomidae (77%)				
Canmore Downstream vs. North Calgary	Heptageniidae (31%)	Chironomidae (47.7%)	Ephemerellidae (63.5%)	Nemouridae (73.2%)	
Canmore Downstream vs. South Calgary	Chironomidae (72.9%)				
North Calgary vs. South Calgary	Chironomidae (74.3%)				

Table 2.8. SIMPER results for September 2021 kicknet samples comparing regions. The most influential taxa are listed first and % listed is cumulative explanation of difference between regions. Only taxa explaining >5% of region difference are included, and taxa are colour coded by taxonomic group: red (Ephemeroptera), green (Plecoptera), blue (Trichoptera), yellow (Diptera), pink (Coleoptera), or grey (Other taxonomic group). For Canmore and Cochrane regions, n=3, for North Calgary, n = 9, and for South Calgary n = 6

Sites	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**
Canmore Upstream vs. Canmore Downstream	Heptageniidae (45.6%)	Chironomidae (63%)	Perlodidae (72.5%)		
Canmore Upstream vs. Cochrane	Heptageniidae (30%)	Hydroptilidae (58.6%)	Ephemerellidae (68.4%)	Chironomidae (76.9%)	
Canmore Upstream vs. North Calgary	Ephemerellidae (30.9%)	Heptageniidae (59.6%)	Chironomidae (67%)	Batidae (74%)	
Canmore Upstream vs. South Calgary	Hydropsychidae (34.1%)	Chironomidae (65.3%)	Heptageniidae (73.3%)		
Canmore Downstream vs. Cochrane	Hydroptilidae (38.2%)	Ephemerellidae (50.4%)	Perlodidae (59.7%)	Heptageniidae (68.4%)	Chironomidae (75.4%)
Canmore Downstream vs. North Calgary	Ephemerellidae (40.4%)	Batidae (49.5%)	Perlodidae (58.5%)	Heptageniidae (66.4%)	Hydroptilidae (72.6%)
Canmore Downstream vs. South Calgary	Hydropsychidae (37.3%)	Chironomidae (68.4%)	Simuliidae (75.3%)		
Cochrane vs. North Calgary	Hydroptilidae (32.7%)	Ephemerellidae (63.3%)	Batidae (72.7%)		
Cochrane vs. South Calgary	Hydropsychidae (35.3%)	Chironomidae (65.2%)	Hydroptilidae (73.7%)		
North Calgary vs. South Calgary	Hydropsychidae (36.1%)	Chironomidae (67.1%)	Ephemerellidae (74.9%)		

Table 2.9. SIMPER results for November 2021 kicknet samples comparing regions. The most influential taxa are listed first and % listed is cumulative explanation of difference between regions. Only taxa explaining >5% of region difference are included, and taxa are colour coded by taxonomic group: red (Ephemeroptera), green (Plecoptera), blue (Trichoptera), yellow (Diptera), pink (Coleoptera), or grey (Other taxonomic group). N = 3 for all regions.

Sites	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**
Canmore Upstream vs. Canmore Downstream	Perlodidae (26.1%)	Chironomidae (50.8%)	Simuliidae (67.2%)	Heptageniidae (76.4%)
Canmore Upstream vs. North Calgary	Heptageniidae (48.6%)	Ephemerellidae (71.7%)		
Canmore Upstream vs. South Calgary	Chironomidae (30.2%)	Batidae (54.8%)	Simuliidae (76.7%)	
Canmore Downstream vs. North Calgary	Perlodidae (25.6%)	Heptageniidae (49%)	Chironomidae (63.4%)	Simuliidae (77.6%)
Canmore Downstream vs. South Calgary	Chironomidae (28.9%)	Batidae (53.4%)	Simuliidae (74.4%)	
North Calgary vs. South Calgary	Chironomidae (29.9%)	Batidae (54.6%)	Simuliidae (76.8%)	

2.3.4 Environmental Variables

Concentrations of a selection of ESOCs sampled from grab samples in the Bow River during this time period were plotted for each month. Across May, September, and November, typically all categories of ESOCs were significantly higher at the site above the Highwood Confluence (at the bottom of the South Calgary region) (Fig. 2.21, Fig. 2.22, Fig. 2.23). This was the only site where all ESOC categories were consistently detected. The next highest concentrations of most ESOC categories, although several magnitudes lower, were generally observed at Highway 22 (analogous to the Cochrane region). Both the Bearspaw Water Treatment Plant Intake and Cushing Bridge (both situated in the North Calgary region) show very low ESOC concentrations in almost all categories.

Typically, the highest concentrations were observed in Artificial Sweeteners (primarily due to sucralose concentrations), Stimulants (caffeine), and Other (Pharmaceuticals) (primarily due to the antidiabetic medication Metformin). These categories were observed in even trace amounts at all sites sampled. Of the remaining pharmaceutical categories, Analgesics and Antidepressants had the next highest concentrations across all three months, although Industrial Compounds (including plasticizers, disinfectants, and others) peaked higher in September. However, since the sites sampled for ESOCs were fewer than the sites sampled for benthic macroinvertebrates and other physicochemical measurements, the data was omitted from the following analysis due to violations of the assumptions associated with the multivariate tests involved.

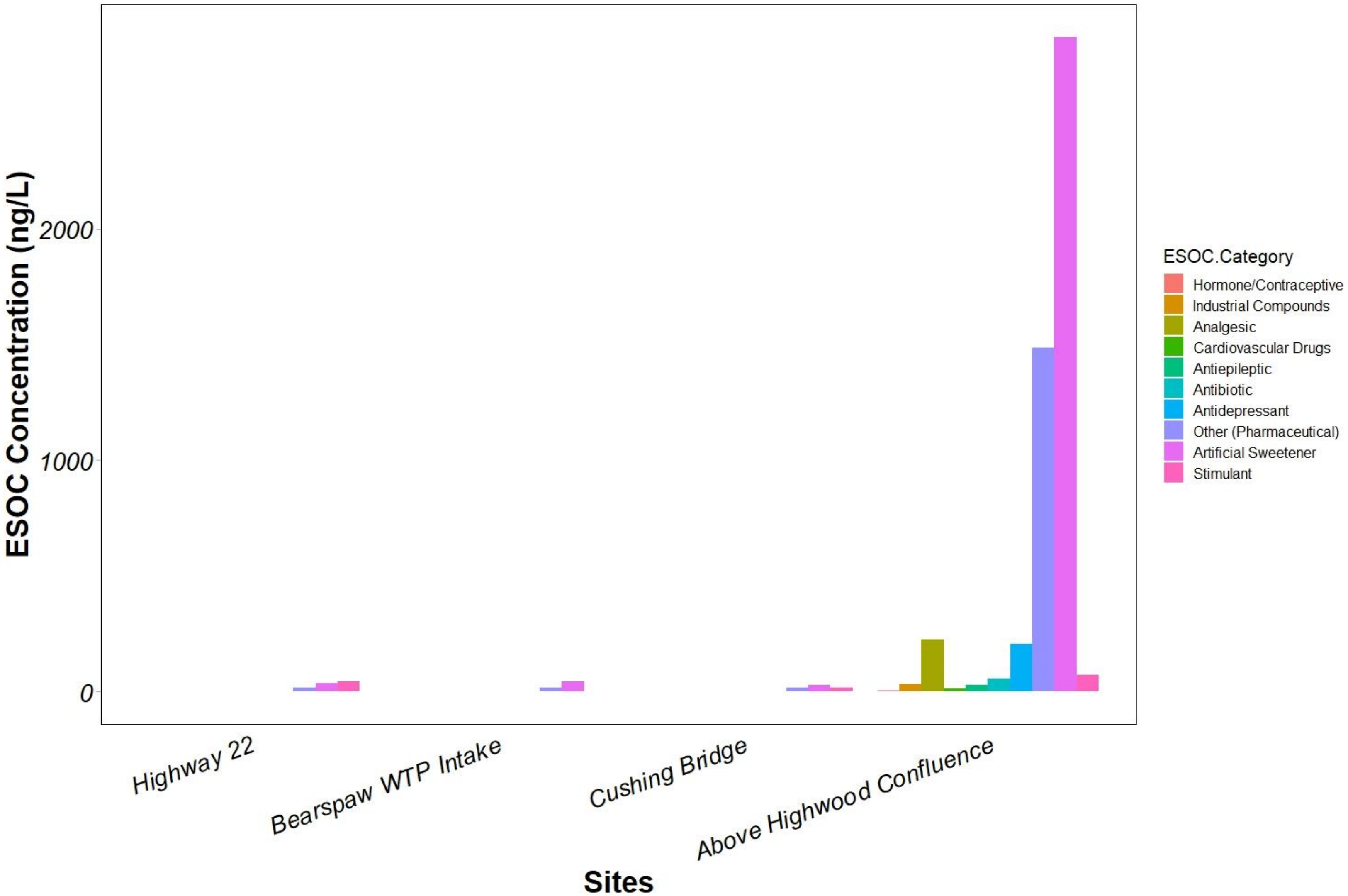


Fig. 2.21. Concentrations of the broader categories of the Emerging Substance of Concern (ESOCs) (ng/L) measured in the Calgary area of the Bow River in May 2021 at sites analogous to regions sampled for biological endpoints (Highway 22 = Cochrane, Bearspaw WTP Intake and Cushing Bridge = North Calgary, Above Highwood Confluence = South Calgary).

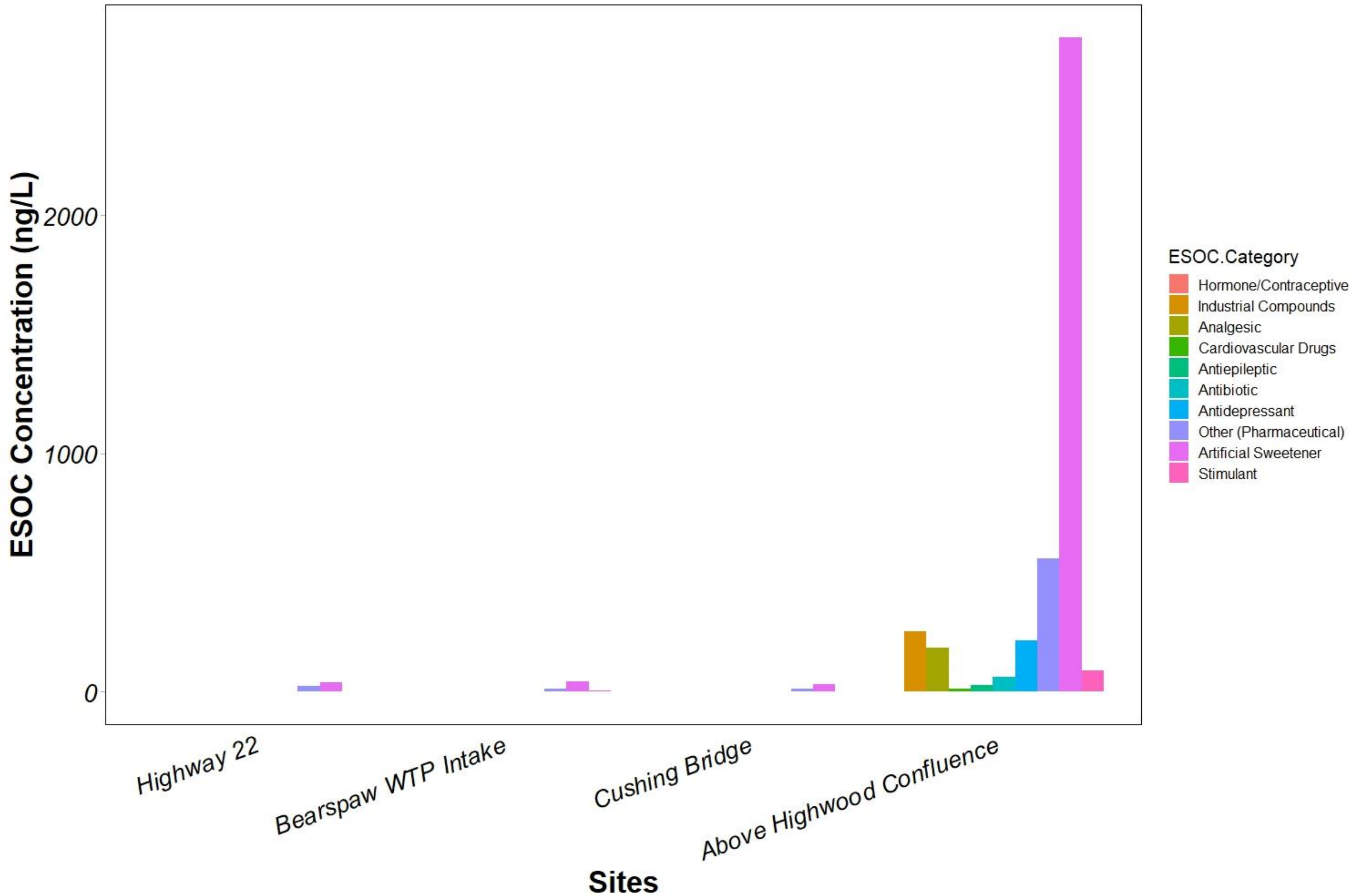


Fig. 2.22. Concentrations of the broader categories of the Emerging Substance of Concern (ESOCs) (ng/L) measured in the Calgary area of the Bow River in September 2021 at sites analogous to regions sampled for biological endpoints (Highway 22 = Cochrane, Bearspaw WTP Intake and Cushing Bridge = North Calgary, Above Highwood Confluence = South Calgary).

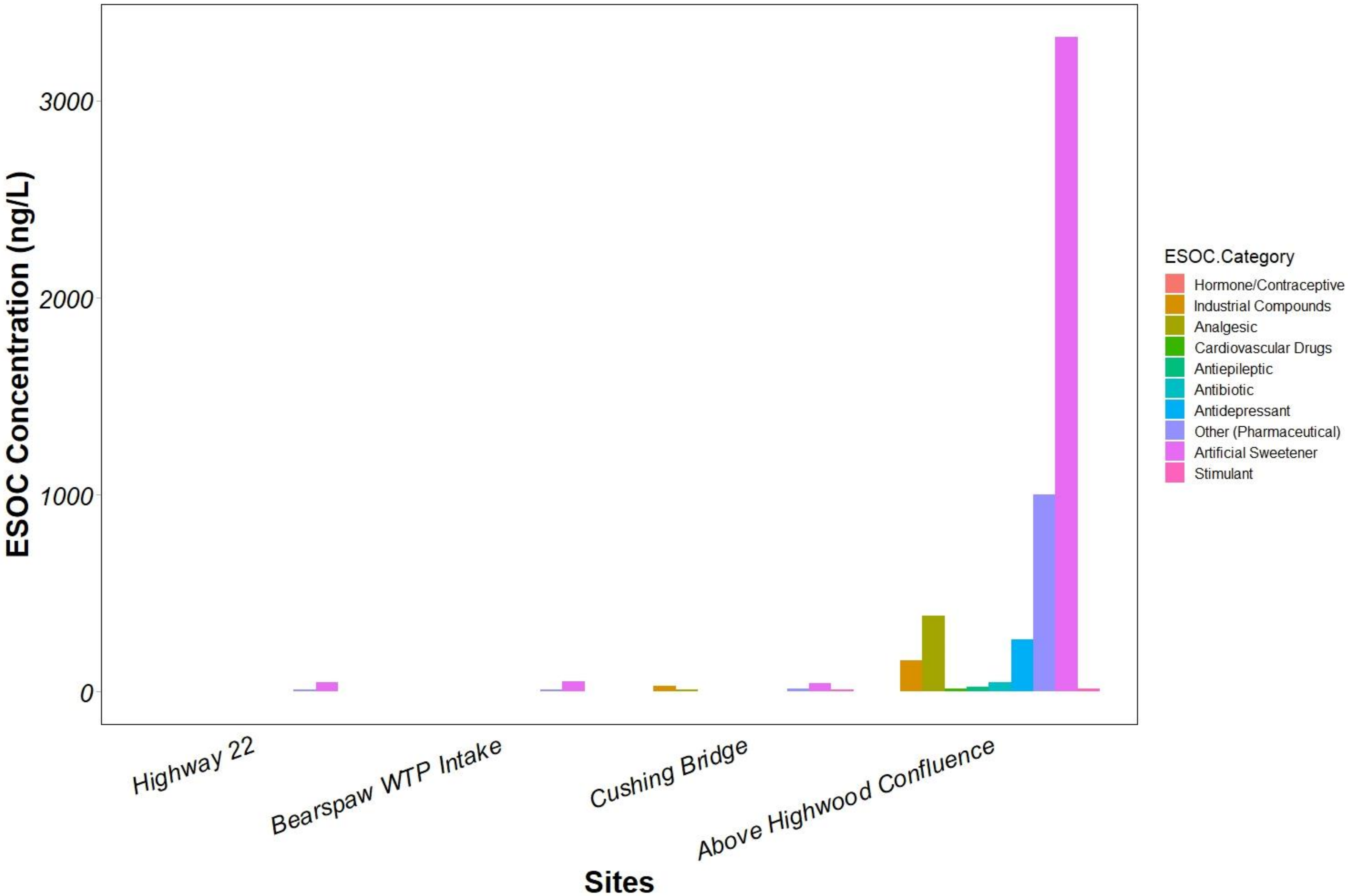


Fig. 2.23. Concentrations of the broader categories of the Emerging Substance of Concern (ESOCs) (ng/L) measured in the Calgary area of the Bow River in November 2021 at sites analogous to regions sampled for biological endpoints (Highway 22 = Cochrane, Bearspaw WTP Intake and Cushing Bridge = North Calgary, Above Highwood Confluence = South Calgary).

A summary of biologically relevant physicochemical parameters to MWWE showed that the largest differences were generally observed between South Calgary and the other regions. Of those parameters that notably changed, the concentration of Total Phosphorus (TP) was approximately ten times higher at the South Calgary sites (Fig. 2.24). Concentrations of Total Kjeldahl Nitrogen (TKN) and Total Organic Carbon (TOC), as well as specific conductance, also tended to increase, although less consistently and to a lesser magnitude (Fig. 2.24, Fig. 2.25). Measured turbidity levels were consistent among the regions, while average substrate size increased at downstream locations (especially South Calgary) (Fig. 2.26). Due to non-normality of some parameters, permutation tests were performed on these variables for the identified regions in the open water season (aside from Cochrane that had only one replicate) showed that TP, TKN, conductivity, and average substrate size differed significantly between regions, while TOC and turbidity did not.

Table 2.10. Significance results from permutation tests on $\log_{10}$ transformed physicochemical data. * Indicates significant $p<0.05$.

Variable	**Chi-squared value**	**p-value**
Total Phosphorus (TP)	15.856	0.00001*
Total Kjeldahl Nitrogen (TKN)	10.355	0.0022*
Specific Conductivity	11.793	1.8×10^{-4}*
Total Organic Carbon (TOC)	4.9927	0.23
Turbidity	0.1702	1.0
Average Substrate Size	80.855	0.0001*

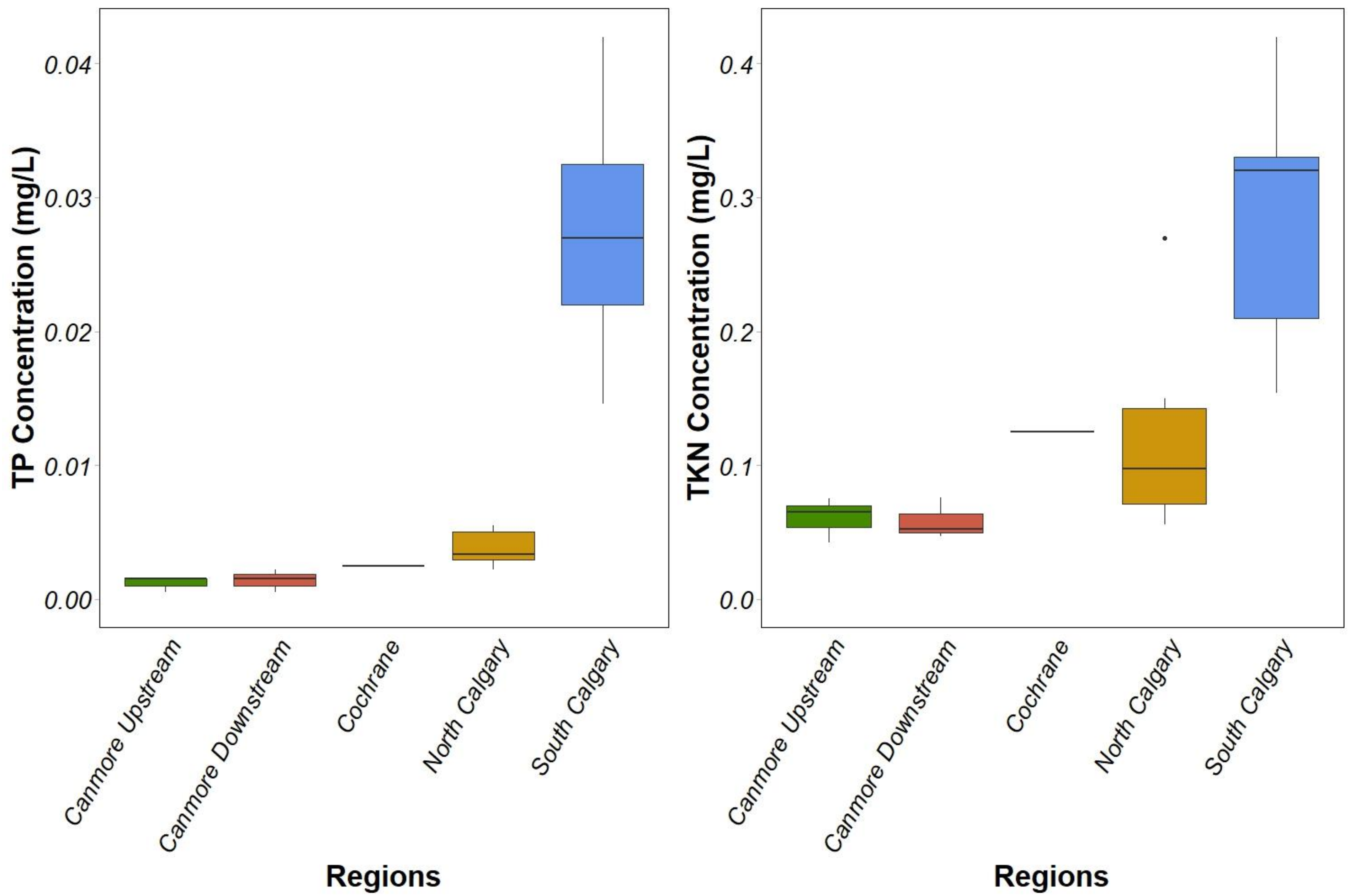


Fig. 2.24 (left) A boxplot showing regional range of Total Phosphorus concentrations (mg/L) over the Bow River open water season (May – November) of 2021 (right) a boxplot showing regional range of Total Kjeldahl Nitrogen concentrations (mg/L) over the Bow River open water season (May – November) of 2021. Boxes represent between the 25th and 75th quartile and the line is the median.

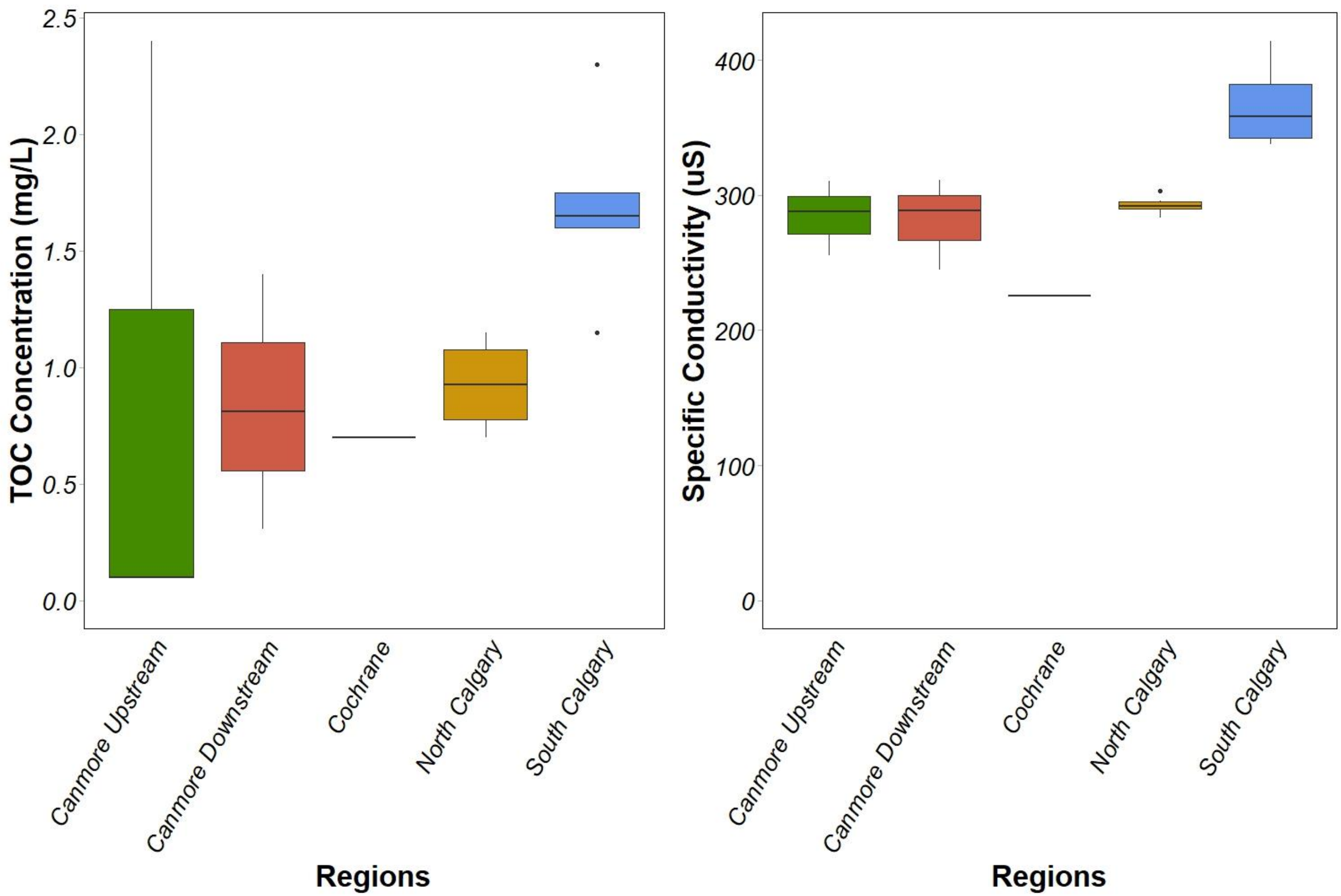


Fig. 2.25 (left) A boxplot showing regional range of Total Organic Carbon concentrations (mg/L) over the Bow River open water season (May – November) of 2021 and (right) a boxplot showing regional range of Specific Conductivity (µS) over the Bow River open water season (May – November) of 2021. Boxes represent between the 25th and 75th quartile and the line is the median.

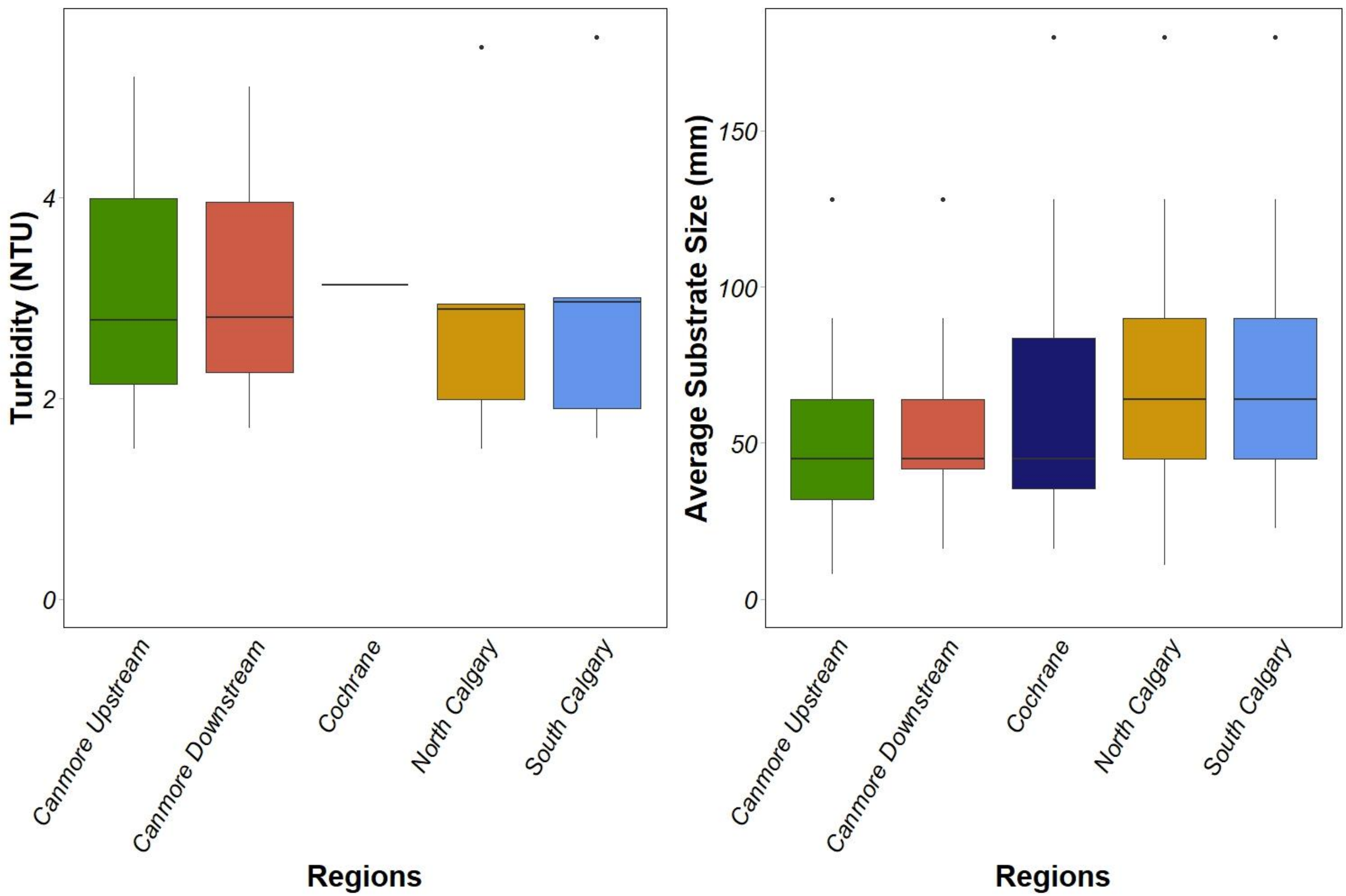


Fig. 2.26 (left) A boxplot showing regional range of Turbidity (NTU) over the Bow River open water season (May – November) of 2021 and (right) a boxplot showing regional range of Average Substrate Size (mm) over the Bow River open water season (May – November) of 2021. Boxes represent between the 25th and 75th quartile and the line is the median.

A distance-based Redundancy Analysis (dbRDA) was performed to examine patterns how physical-chemical parameters influence benthic macroinvertebrate assemblages along the longitudinal sampling gradient. The dbRDA showed that the physicochemical parameters in the model (constrained) explained 69% of the variation in the dissimilarities of the benthic macroinvertebrate assemblages and the Type III ANOVA showed that the model was significant overall (F = 4.1664, p = 0.001) (Table 2.11). The first three axes (dbRDA1, dbRDA2, dbRDA3) respectively explained 28%, 24% and 9% of the constrained inertia and also were also significant (Table 2.12). Of the individual parameters tested, TP, Conductivity, and substrate size were identified as significant parameters (Table 2.13).

Table 2.11. The constrained and unconstrained proportion of inertia explained by the dbRDA.

	Inertia	**Proportion explained**
Total	5.44	1.00
Constrained	3.78	0.69
Unconstrained	1.66	0.31

Table. 2.12. The proportion of constrained inertia explained by each dbRDA, and the results of the Type III ANOVA performed on the dbRDA model. * Indicates significant p<0.05.

	Proportion explained	**F-Statistic**	**p-value**
dbRDA1	0.28	10.03	0.001*
dbRDA2	0.24	8.53	0.001*
dbRDA3	0.092	3.33	0.031*
dbRDA4	0.044	1.59	0.55
dbRDA5	0.026	0.95	0.79
dbRDA6	0.016	0.58	0.81

Table 2.13. The results of the Type-III ANOVA performed on the dbRDA model. * Indicates significant p<0.05.

Physicochemical Parameter	**F-statistic**	**P-value**
TKN	1.40	0.22
TP	3.04	0.006*
TOC	1.51	0.18
Conductivity	3.34	0.002*
Turbidity	1.76	0.113
Average Substrate Size	4.44	0.0001*

Stepwise building of the model assessed by Akaike's Information Criterion (AIC) done through both forward and backward selection suggested different parsimonious models. A backwards selection model indicated that the highest rated model kept four of the six physicochemical parameters: TP, Conductivity, TOC, and average substrate size (Table. 2.15). This model (constrained) explained 62% of the total inertia (Table 2.14). A forward stepwise model indicated that the highest rated model made use of only one physiochemical parameter: conductivity (Table 2.16). This model (constrained) explained 50% of the total inertia (Table 2.14).

Table 2.14. The constrained and unconstrained proportion of inertia explained by the highest rated forward and backward stepwise selection models.

	Inertia (backward)	**Proportion explained (backward)**	**Inertia (forward)**	**Proportion explained (forward)**
Total	5.44	1.00	143100000	1.00
Constrained	3.37	0.62	71430000	0.50
Unconstrained	2.07	0.38	71680000	0.50

Table 2.15. The AIC best ranked backward stepwise dbRDA model. * Indicates significant $p<0.05$.

Physiochemical Parameter	**AIC**	**F-Statistic**	**p-value**
TP	23.93	3.11	0.01*
Conductivity	24.40	3.53	0.005*
TOC	24.71	3.82	0.005*
Average Substrate Size	26.01	5.08	0.005*

Table 2.16. The AIC best ranked forward stepwise dbRDA model* Indicates significant $p<0.05$.-

Physiochemical Parameter	**AIC**	**F-Statistic**	**p-value**
Conductivity	339	15.943	0.005*

A biplot of the dbRDA showed clear clustering of sites, where South Calgary sites, North Calgary and Cochrane sites, and Canmore sites clustered in clear spatial patterns (Fig. 2.27). The only site that did not cluster well was one May North Calgary site, which clustered more closely to the Canmore Upstream and Downstream sites. Similarly, one Canmore Downstream site was less closely clustered and was equidistant from the North Calgary and Cochrane cluster. There was no clear pattern associated with the month of the open water season that sites were sampled during. The arrows of greatest magnitude were average substrate size and specific conductivity, closely followed by TP. The clearest observable pattern is that nutrient parameters (TP, TKN, TOC) are correlated with specific conductivity and these parameters were generally high in South Calgary sites. Secondarily, Canmore sites were also generally negatively associated with average substrate size.

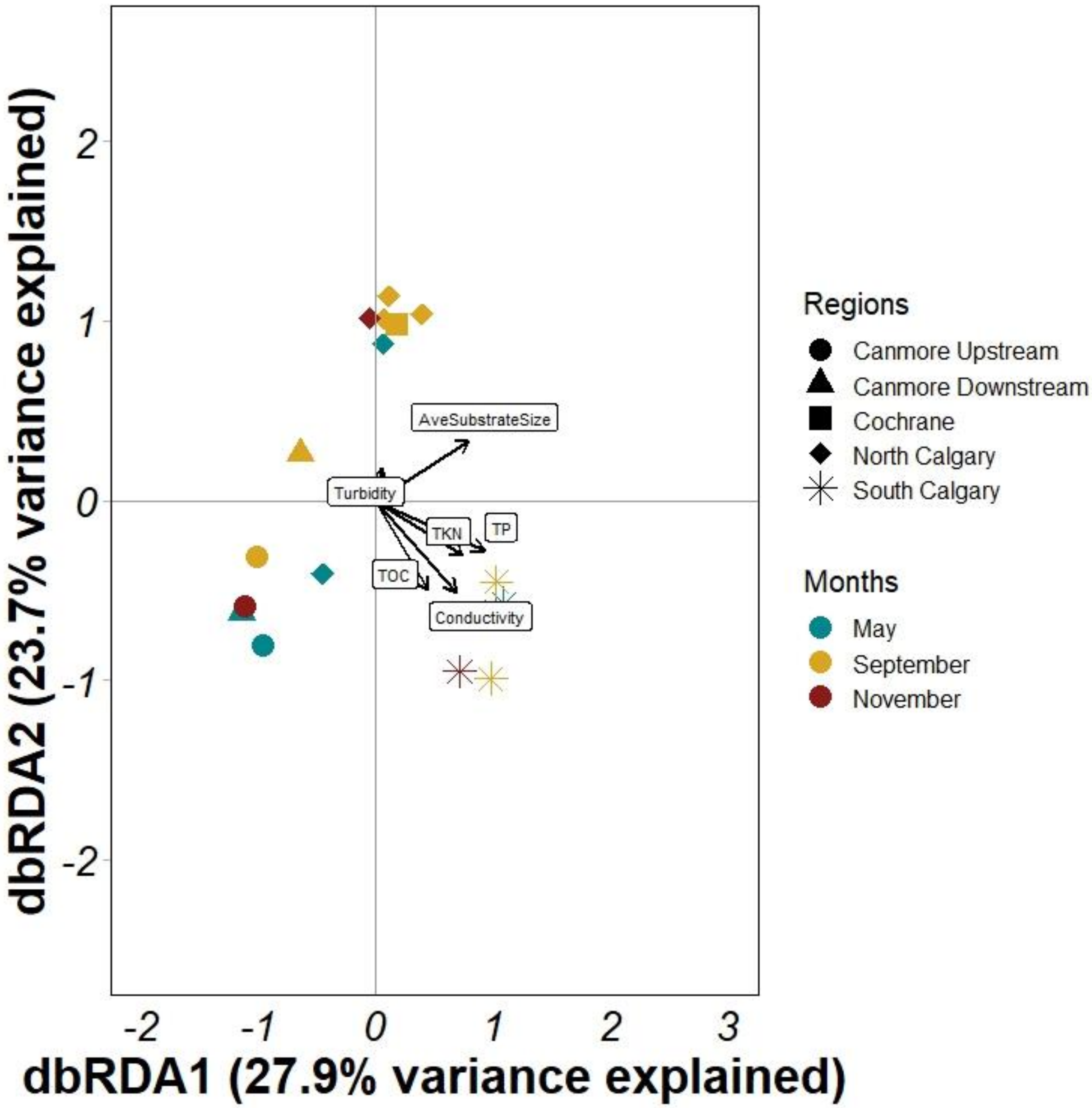


Fig. 2.27. A dbRDA biplot of physicochemical parameters gathered from sites within the pre-determined Bow River regions where benthic macroinvertebrate assemblages were sampled over three months (May, September, November) of the 2021 open water season.

2.4 Discussion

2.4.1 Patterns in benthic macroinvertebrate assemblages and community indices

Community-based metrics of benthic invertebrate community assemblages demonstrated inconsistent assessments on the impact of MWWE in urban areas of the upper Bow River. The relative values of regional diversity (Shannon's diversity index), family richness, % dominance of the dominant family, and family evenness all remained relatively constant along the river gradient, despite the sampling sites spanning long distances through two distinct ecozones and flowing through a large urban area with three WWTPs (Calgary). Previous work in the Bow River yielded similar results when assessing benthic macroinvertebrate communities at a landscape ecology scale; even when focusing on changes over distance rather than MWWE exposed sites, diversity metrics did not reliably indicate change occurring within the assemblages of the Bow River (Powell 2008). Similarly, a review of benthic macroinvertebrate communities in the Bow River using sites bracketing the CoC at Cochrane and Policeman's Flats (formerly Stier's Ranch) showed that richness, diversity (Shannon's diversity index), and % dominance did not significantly differ (evenness was not measured) (Saffran et al. 2009). As these sites bracket Calgary, at a larger scale, they capture a snapshot of the overall community level response to notable exposure to MWWE in Calgary at this time. However, other studies in other locations have indicated that diversity may be reduced by MWWE exposure, particularly in the presence of WWTPs that contribute high volumes of effluent to the receiving environment (Peralta et al. 2020, Aristone et al. 2022). It is possible that the concentration of MWWE released from WWTPs in Canmore and Calgary is not high enough to impact metrics of community diversity, but this would require closer tracking of MWWE concentrations associated with benthic macroinvertebrate communities over time. Overall, diversity indices have been previously demonstrated to poorly characterize the health of benthic macroinvertebrate communities exposed to MWWE, indicating that communities were less diverse following the improvement of WWTP treatment protocols in two American rivers, when pollution tolerant organisms had instead largely been replaced by more sensitive taxa (Lydy et al. 2000).

Alternatively, % EPT and % Diptera were observed to be a sensitive metric for considering the effects of MWWE in the Bow River. The relative contribution of EPT to the total community decreased dramatically below WWTP exposures in Southern Calgary and % Diptera correspondingly increased. Breakdowns of the community at the Order level supported these conclusions, particularly showing a reduction in Ephemeroptera (of the EPT taxa) below WWTPs both in Calgary and Canmore where Diptera increased. There were some notable changes in what components of EPT were dominant, with Plecoptera becoming far less common downstream of Canmore and Trichoptera families spiking as a contributor in September samples. However, the overall % EPT was relatively stable between regions up to South Calgary, with these changes likely being related to water temperature and lifecycle timing (Haidekker and Hering 2008). Reduced relative downstream presence of EPT has been observed previously in the Bow River above and below the city of Calgary (Saffran et al. 2009). This has also been observed more widely, including one multi-river study in the United Kingdom that concluded % EPT was the most sensitive metric to the presence of MWWE over alternative measures including taxa richness (Morrissey et al. 2013).

Similarly, the Hilsenhoff Biotic Index (HBI) demonstrated consistent degradation of sites immediately downstream of WWTP in Calgary, although similarly to other analysis results, the difference between upstream and downstream Canmore was not notable (both sites rated as "Excellent" quality in all months). Overall, the HBI rating of sites on the Bow River reflects previous results showing that while the communities further downstream, and especially those immediately downstream of WWTPs, have lower ratings, the general quality of benthic macroinvertebrate communities in these urban areas of the upper Bow River are generally high quality. The lowest HBI rating observed was in South Calgary and had a mean Good to Fair rating across the seasons sampled here and a historical sample (Saffran et al. 2009). No single replicate in any month of sampling rated lower than Fair and most rated Very Good or Excellent, suggesting that benthic macroinvertebrate communities support assessments of the Bow River as generally healthy.

The observed patterns and lack of differences in community-based metric values among the defined regions in the Bow River collectively reveal a positive assessment of the Bow River's overall ecological condition. Many studies of urban streams treat stressors associated specifically with elevated presence of human populations, such as MWWE, stormwater effluent, runoff from impervious surfaces, and legacy pollutants from industrial applications as a united stressor under the umbrella of urban stream syndrome (Walsh et al. 2005). Urban stream syndrome most often is associated with a number of physicochemical changes, but particularly reduced biotic richness, which is not observed in this study (Paul and Meyer 2001)). While MWWE is clearly playing a role in the anthropogenic stressors of an urban river, such as the Bow River, richness, based on previous analysis and metrics, does not indicate concern at this time. However, this also potentially suggests that these metrics are not particularly sensitive to the changes that are observed with MWWE exposure and are, therefore, poor early predictors for a MWWE monitoring program (Arciszewski et al. 2017).

The overall abundance of benthic macroinvertebrates showed the most dramatic change, which is supported by previous Bow River studies as well as more general reviews of MWWE in aquatic systems (Saffran et al. 2009, Grantham et al. 2012). However, it is notable that the changes in relative abundances clarify that this transition is not occurring equally across all components of the whole assemblage. This aligns with other studies in the Bow River around Calgary, particularly in increases of taxa that were low trophic level (herbivorous) taxa (Askey et al. 2007). While abundance patterns did respond strongly to MWWE in Calgary (and to a lesser extent in Canmore), these results are indicative of nutrient driven change and should be contextualized with information regarding what types of taxa are contributing most to the increases.

In this vein, results from the SIMPER analysis show that the differences between communities were largely related to few dominant families, which were also often consistent between sites and seasons. While family richness typically ranged up to an average of fifteen families in a region, SIMPER usually selected only three to four families as characterizing more than 5% each of the overall differences between any two sites. Additionally, the families chosen were also drawn from a pool of only nine total families across the entire study. This corresponds to other assessments of benthic macroinvertebrate community composition that have also found that regardless of level of organization (taxonomic or functional), a few discrete groups tend to influence most heavily the observed differences observed between communities (Feijó-Lima et al. 2023). This suggests that in the Bow River, a more targeted approach at monitoring the responses of select taxonomic groups might be more efficient to assess changes related to MWWE exposure than assessments of the whole community.

For the Bow River, the presence of Diptera families Simuliidae and Chironomidae were strongly associated with the presence of a WWTP and often were identified as the influential taxonomic group differentiating between upstream and downstream sites around WWTPs, particularly in separating South Calgary from other regions. Diptera have been well characterized as a pollution tolerant family and their dominance is commonly observed with organic pollution. Their contribution to the dissimilarity of sites, particularly South Calgary from upstream sites, is strong evidence that the nutrient inputs associated with MWWE are driving observed changes in community composition.

Notably, Trichoptera families were only influential in differentiating between sites in September and had much lower relative abundance in May and November as well, suggesting that seasonality and life cycle stages play an important role in using taxon-specific approaches to differentiate sites. Additionally, of the two numerically dominant families of Trichoptera, one was the family Hydropsychidae, which are often classed as relatively tolerant of nutrient pollution. This family was rarely observed in Canmore, somewhat observed in North Calgary, and often the dominant family observed in September South Calgary samples. Comparatively, another Trichoptera family Hydroptilidae were common only in North Calgary and were often the dominant taxon at these sites. Hydroptilidae disappear entirely downstream of any WWTP in Calgary. Like Chironomidae, Hydropsychidae are filtering collectors, while Hydroptilidae have unique mouthparts that allow them to pierce into filamentous algal cells (Merritt et al. 2017, Cummins 2018). Both families have the same HBI tolerance value. When considering effective groups for rapid assessment of benthic macroinvertebrates in the Bow River to build a toolbox, observations like this of taxa presence may be helpful in developing early warning systems of concern for MWWE exposure. One possible avenue of explanation for this change would be to examine the bacterial and fungal communities associated with various food sources for ESOC related impacts. For example, in ecotoxicological studies on a common antibiotic in MWWE demonstrated low toxicity on shredder organisms, but exposure of the antibiotic to their leaf material food source dramatically changed the leaf decomposing fungal communities and increased growth of the shredder organisms overall (Bundschuh et al. 2020, Konschak et al. 2020). It is possible that MWWE is exerting an indirect physiological impact on some benthic macroinvertebrate taxa through an ESOC related exposure, although more specific research on Hydroptilidae food sources and their associated microbial communities is necessary.

Differences in common benthic macroinvertebrate community indices between sites and regions are important on two counts: firstly, in assessing whether MWWE is demonstrating an effect on the community and secondly, which indices are demonstrating differences overall, which is crucial to the development of future monitoring programs (Walker et al. 2003). These results do differ somewhat from the recommendations stemming from EEM frameworks for other types of anthropogenic effluents. Pulp mill effluents are particularly relevant to the development of monitoring systems for MWWE due to their similarities as nutrient dominant complex mixtures with additional chemical components. However, the recommended endpoints for pulp mill effluents under an EEM framework include, as mentioned, total & taxa densities, taxa richness, evenness (Simpson's), similarity (Bray-Curtis), and taxa presence/absence (Environment and Climate Change Canada 2010). However, in the Bow River system, some community-based metrics do not perform well as candidates as indicators for MWWE monitoring. When compared to other metrics that do show large changes at key points of MWWE exposure, metrics that demonstrate consistent values across all sites in this study are unlikely to be adequately sensitive to MWWE exposure. Particularly, any metric not specific to the type of taxa (such as richness and diversity) tended to not show any difference between sites and regions, while metrics that included this information (such as % EPT) appeared more sensitive to MWWE inputs. When tracking nutrient enrichment in pulp mill effluent, similar observations have been recorded on the efficacy of Bray-Curtis clustering over other benthic macroinvertebrate community endpoints (Culp et al. 2003).

Studies that aim to isolate the effects of MWWE using mesocosm-based exposure have demonstrated that even 5% effluents may demonstrate notable impacts on benthic macroinvertebrate communities, which is approximately what most Bow River WWTPs are currently releasing (Grantham et al. 2012). However, the use of many common descriptive metrics of benthic macroinvertebrate communities such as richness and diversity have been established to poorly represent community change in the presence of MWWE (Enns et al. 2023). This study, previous Bow River studies, and other local studies of MWWE have shown more taxon-specific metrics such as % EPT to strongly respond to MWWE exposure, but these patterns are not observed to be universal in MWWE and benthic macroinvertebrate community composition studies (Enns et al. 2023). A large study of European WWTPs suggests that observation of individual species, through the process of calculating taxa turnover on a temporal or exposure basis mostly consistently reveals patterns in the effects of MWWE; however, the process was most strongly observed the smaller the size of the waterway (Enns et al. 2023). Generally, these results suggest that future monitoring of benthic macroinvertebrate communities and MWWE should focus on understanding how community assemblages specifically change on a taxa level, such as the broad classification of Orders in EPT compared to Diptera, which should help to detangle the contributions of components of complex MWWE mixtures.

2.4.2 Patterns of difference in benthic macroinvertebrate assemblages

Based on patterns observed in the relative similarities of different sites (using Bray-Curtis differences) and the clear regional clusters that emerged in all three seasonal samplings, MWWE is consistently an environmental driver influencing changes to benthic macroinvertebrate community assemblages in urbanized areas of the Bow River above the Highwood confluence. Crucially, the calculation of Bray-Curtis dissimilarities arranges site similarity almost entirely along the linear riverine gradient, but the strength of the similarity between sites was not consistent with relative spatial separation or shifts in ecozones. Across samples from spring (May), early autumn (September), and late autumn (November) sampling, the analysis of sites in the regions of Canmore, North Calgary, and South Calgary ranked both Canmore regions and North Calgary (and Cochrane in September) as more similar than South Calgary. This relationship persists despite North and South Calgary sites being closer together and both in a prairie ecozone, while Canmore is a part of the montane cordillera ecozone. Similarly, when the clusters observed were separated into regions representing Canmore upstream of the WWTP (CUS), Canmore downstream of the WWTP (CDS), Cochrane (COCH), North Calgary (BWP, SGI, NCA, and CUB), and South Calgary (GRB and PMF), the clearest clusters appearing in all NMDS were South Calgary sites, North Calgary sites, and Canmore sites.

However, not all observed shifts in community assemblages were supported as being significantly different when compared using PERMANOVA significance testing. Generally, the region that was most often significantly different from other regions was North Calgary, which was significantly different from South Calgary in May and from Canmore Upstream, Canmore Downstream, and South Calgary in September (no regions were significantly different in November samples). Interestingly, South Calgary was not significantly different from either Canmore site. However, what does emerge from these results is that North Calgary (sites ranging from Bowness in the NW of the city to Cushing Bridge in the upper SE) is a distinct region of the Bow River from other locations along the river gradient sampled. Particularly, there is most consistently a significant difference between North Calgary and South Calgary sites, which are primarily different from one another not through large spatial distance but in terms of land-use through exposure to at least one WWTP.

Both results occur in opposition to predictions from models such as the RCC, that suggest that change in benthic macroinvertebrate communities occurs more gradually over a longitudinal gradient that matches continuous change in environmental conditions, suggesting that sites far upstream would be most distinct in both physicochemical parameters and, correspondingly, benthic macroinvertebrate community composition (Vannote et al. 1980). Instead, the most dramatic shift in benthic community assemblage composition occurs directly below Calgary's highest WWTP (Bonnybrook). While not surprising, as many studies have characterized the strong influence of MWWE on benthic macroinvertebrate communities, it is important to understand how strong of an environmental driver anthropogenic influence may be in relatively pristine rivers such as the Bow River and what elements of urbanization should be prioritized in monitoring (Gücker et al. 2006).

Generally, the sampling sites chosen for this study were located at locations on the river to disentangle the potential influence of anthropogenic stressors on benthic community assemblages (the confluence of other tributaries, sites also having stormwater discharges, etc.) However, due to minimal disturbance along the river below Canmore and upstream of Calgary, sampling was also minimal in this area and assessment of the variation in communities in this area is coarse. This project did not assess the local effects of the presence of a cement plant, several large tributaries (some, like the Kananaskis River having their own MWWE footprint), and some agricultural land-use along the Bow River in this area, but the comparative similarity of Canmore and North Calgary in the cluster analysis suggests that while there is a change through this area, it is a less apparent and possibly more ephemeral change than that seen in the communities downstream of Calgary's WWTPs. This is supported by these regions only being significantly different in the September sampling. It is also difficult to attribute this change to specifically any specific anthropogenic stressor when paired with change in the surrounding landscape (such as the transition from a mountain cordillera ecozone to a prairie ecozone). However, a notable point source input of MWWE, such as that represented by Calgary's three WWTPs (and especially Bonnybrook), appears to dramatically shift benthic macroinvertebrate communities in a longer lasting and distinctive manner than some alternative stressors: heavy presence of stormwater outflows through the northwest quadrant of Calgary, the presence of tributaries, or the presence of historic logging sites in the inner city. This is observed in the consistent separation of South Calgary sites from other sites in both the cluster analysis, PERMANOVA, and NMDS for this study. This is supported in previous benthic macroinvertebrate community studies on the Bow River that demonstrate that communities even further downstream from PMF (the most downstream site studied here) show strong similarities to South Calgary communities, particularly through their dominance of pollution tolerant taxa (Saffran et al. 2009).

However, it is somewhat notable that Canmore upstream and Canmore downstream (sites ~1km apart) do not show the same relative strength of difference as sites upstream and downstream of the Bonnybrook WWTP (NCA or CUB upstream and GRB and downstream) in all analyses. Canmore sites on either side of Canmore's WWTP were not fully distinguishable through cluster analysis in May of 2021 and in all other months, were still more similar to one another than to any other site. This suggests that MWWE is not a strong enough driver, at current volumes, of benthic macroinvertebrate community composition to have a MWWE exposed site in a montane cordillera ecozone resemble that of a MWWE exposed site in a prairie ecozone. In addition, both larger volume and lower treatment quality of MWWE dispensed from WWTPs are associated with more dramatic increases in benthic macroinvertebrate abundances, decreases in diversity, and relative increases of tolerant taxa (Aristone et al. 2022). Since the outflow from the Canmore WWTP is much smaller than that from Calgary's WWTP (especially Bonnybrook), it is possible that the community is less affected downstream in Canmore. Also, investigating landscape as a driver of community composition in previous benthic macroinvertebrate community composition research in the Bow River has found that mountainous areas of the Bow River did have distinct community composition compared to other areas downstream (Powell 2008). That overall pattern was significant despite observed variation within the defined ecoregions, which is also observed here. However, this more local variation was examined only in relation to a small selection of anthropogenic influences that did not include WWTPs (Powell 2008).

Notably for this study, replicates at all 2021 Canmore upstream or downstream sites (outside of the spring May samples) were clearly distinct from one another in community similarity, suggesting that within this area, MWWE is at least responsible for a noticeable local effect. In a somewhat similarly sampling regime, one study with two sites immediately upstream and downstream of a WWTP associated with a ski resort (in a higher elevation, alpine area) and a site downstream of a larger city, some distance away (at a lower elevation), similarly found that although there was some evidence of degradation at the higher elevation site downstream of a WWTP, the two higher elevation sites were more similar to one another than the further downstream, more urban site. Also interestingly, the differences between the two mountainous sites seemed to differ in magnitude based on seasonality and anthropogenic pressure at the related ski resort, supporting the role of volume of MWWE in driving observed responses in the benthic macroinvertebrate communities. In this study, the sites were more similar during the summer months, when anthropogenic presences were lower, and the flow was generally higher (Lencioni et al. 2020). While this tracks opposite to the pattern of the Canmore sites, it is worth noting that differences between the sites were more apparent during the summer and less apparent immediately following the initial snow melt at the beginning of May, meaning that some degree of seasonality, related to environmental changes (such as flow) or changes to anthropogenic pressures, was present. This is also demonstrated in a study of an eastern Canadian river system with a large and small WWTP that noted that the communities were more distinctively affected by seasons at a smaller WWTP (Aristone et al. 2022). Noting seasonal patterns of stress on the ecosystem may be of importance when developing future monitoring programs.

Moving to assessing sites further downstream on the Bow River, one explanation for Cochrane's outlying position in the cluster analysis, despite being spatially close, is identified in the most recent biomonitoring assessment in the Bow River, which chose Cochrane as the most upstream site but acknowledged that this site has also been suspected to be somewhat degraded from the effects of the nearby upstream dam (Saffran et al. 2009). Since the impacts of various impoundments have been shown to affect downstream areas for a significant amount of time in the Bow River these studies suggest that Cochrane is not necessarily a high-quality baseline site for representing the river as it flows into Calgary (Powell 2008).

Within the City of Calgary, a particular reason for the focus on sites surrounding tributaries immediately upstream of the Bonnybrook WWTP is in response to previous studies of biotic factors response to MWWE in the Bow River. One fish study demonstrated reproductive impairment at sites upstream of Bonnybrook, suggesting that at higher levels of the food web, detrimental effects on biota not necessarily linked to direct MWWE inputs are occurring (Patel 2018). However, this was not reflected in the benthic macroinvertebrate community examined in this project, as sites bracketing the Elbow River and Nose Creek, including a shared site with the fish study at Cushing Bridge (CUB), were almost indistinguishable in the cluster analysis and NMDS, while the next site downstream below Bonnybrook saw relatively high dissimilarity in community composition. Given that impacts on fish were related to reproductive impairment, with synthetic estrogens as a suspected culprit (a common endocrine-disrupting chemical), this provides additional clarity on what particular components of anthropogenic river inputs are important in the context of overall community function for benthic macroinvertebrates (Patel 2018). While the effects of ESOCs, including synthetic estrogens, are not well studied in benthic macroinvertebrates, this does correspond to a study performed on fish and benthic macroinvertebrates in the experimental lakes area that found similarly that there was no evidence of direct toxicological impact on benthic macroinvertebrates from endocrine-disrupting chemicals (Kidd et al. 2014).

For this study overall, the lack of significant difference in the PERMANOVA between Canmore sites and sites in South Calgary is possibly the result of the unbalanced design suggested from grouping sites based on the results of the cluster analysis (Anderson 2017). While replication at sites was minimal ($n = 3$), the low variance between sites that is typified by the consistent relationship of replicates in the cluster analysis suggests that it is more likely that more balanced sampling within the now defined regions would be advantageous in clarifying these differences. Ultimately, however, given that the Bow River is a relatively pristine system with world-class WWTP processes, lack of significance in changes in communities generally is likely indicating that stressors in the system are not causing dramatic enough shifts in the communities to register significant differences. While cluster analysis provides a sense of what sites are most similar, the differences between the actual sites may not be as meaningful, from a significance testing point of view.

Generally, these results are important in context of attempting to reconcile the relative significance of anthropogenic pressures when applied to a large, naturally varying system. The paradigms observed here in the Bow River are well described by companion models to the RCC that treat flowing water systems as a mosaic system with “patches,” that are similar in biotic or abiotic characterizations and distinct from other patches (Naiman et al. 1988, Winemiller et al. 2010). However, the way these patches are defined is integral to understanding what is driving changes in benthic macroinvertebrate communities in the Bow River, and, by proxy, other changes to the ecosystem. The concept of patches has been applied on radically different scales and many studies of community composition define patches as areas that can be kilometres in length and are separated by boundaries that can have various sources, including a point-source disturbance, which matches the scale of this project (Naiman et al. 1988).

The apparent existence of North and South Calgary patches from a perspective of Bray-Curtis dissimilarity matrices, and to a lesser extent patches for Cochrane, Canmore Upstream, and Canmore downstream, is matched largely by a similar clustering process for environmental parameters measured at the same sites, supporting biotic and abiotic differences between patches. While changes to Bow River benthic macroinvertebrate communities have not been clearly linked to the presence of WWTPs before, previous work on the Bow River’s benthic macroinvertebrate communities has largely focused on the use of descriptive community metrics over significance testing or cluster analysis of the overall community, despite some evidence that

Bray-Curtis differences tend to be most sensitive to other anthropogenic effluents such as pulp mill effluent in other EEM programs in Canada (Walker et al. 2003). Furthermore, the presence of a significantly unique community composition in North Calgary sites is not measured at a fine enough resolution in previous studies, that typically bracket the CoC, to be clearly compared to historical studies, but does represent a snapshot of the river's recent condition that may be crucial in developing monitoring programs for MWWE. Lastly, the lack of results in comparing the Canmore sites to South Calgary sites suggests significance testing is not necessarily observing the differences seen previously with the cluster analyses. However, when sampling is focused around WWTPs and addressed with properly sensitive metrics, there are clear disruptions to community composition that suggest that in the Bow River, the release of MWWE from WWTPs on the Bow River are responsible for the creation of boundaries between patches, due to the degree of similarity reflecting the presence of WWTPs on both a larger river scale and a local scale.

2.4.3 Patterns in Environmental Conditions

As a less studied component of MWWE, ESOC concentrations, combined into larger functional categories, were examined at sites representative of the regions indicated by the cluster analysis but were not used in further analysis as only limited sites were available. However, qualitative observation of the data suggests that it is unlikely that ESOCs are present in the Bow River in high enough concentrations to have significant effects on the biological endpoints in this project. By several magnitudes, the highest ESOC categories observed included Caffeine (Stimulants) and Sucralose (Artificial Sweeteners), typically have very high relative concentrations, often being used as WWTP tracers (Arlos et al. 2023). However, neither has been consistently indicated to affect benthic macroinvertebrate community assemblages, although caffeine was associated with some loss of average biomass through indirect effects (Moore et al. 2008, Lencioni et al. 2020, Marshall et al. 2022). In literature, ESOCs that have been indicated to have potential to affect benthic macroinvertebrate community assemblages include antibiotics and analgesics (Lencioni et al. 2020) In the Bow River, antibiotics were generally low in concentration compared to other chemical categories and in relation to literature results where they were able to exert pressures on benthic macroinvertebrate assemblages (Konschak et al. 2020). Analgesics, while still lower than concentrations with observed invertebrate toxicity, were consistently the highest category of pharmaceuticals across all three months (Geiger et al. 2016). From an assemblage standpoint, analgesics are associated with higher toxicity for grazers and detritivores and while grazers were low abundance in the Bow River overall, especially in South Calgary, detritivores (in the form of collectors) were extremely dominant (Lencioni et al. 2020). This also suggests that while ESOC concentrations in the river are not at demonstrably relevant laboratory levels, chronic effects from consistent MWWE exposure are not occurring either or are occurring in ways that are not observable from the ecological endpoints measured. Notably also, estrogenic compounds (in the Hormone/Contraception category), which have been of interest in the Bow River previously related to impacts on fish, were usually not detected or detected in very small amounts, even at the South Calgary site downstream of all three WWTPs (Patel 2018).

A recent study of ESOCs in the Bow River, using the same data set, observed that while most concentrations of measured ESOCs were well below detection limits in most areas, the anti-inflammatory drug Diclofenac is consistently over recommended EU concentrations (Arlos et al. 2023). However, diclofenac and other NSAIDs have not been shown to have strong toxicological effects on benthic macroinvertebrates in laboratories and so either may have little effect on the environment or benthic macroinvertebrates may be an ineffective endpoint (Cleuvers 2004). Meanwhile, the Bow River's exposure to ESOCs seems to be primarily related to the treatment process in WWTP; all three treatment plants employ tertiary treatment, but the treatment processes vary somewhat, with the newest and southmost WWTP (Pine Creek) contributing the least to loadings of measured ESOCs. This suggests that effects from ESOCs in MWWE may be more locally tied to particular treatment plants and would require increased observation around WWTPs on the Bow River to disentangle. Overall, it is worth noting that some additional wastewater treatment processes, such as activated charcoal, intended to capture ESOCs have failed to result in any tangible shift to benthic macroinvertebrate assemblages (Johnson et al. 2019).

MWWE related parameters that consistently showed patterns of change in explorations of visualizations of data were assessed for significance using a permutation test. While there was some degree of difference between sampling events in different seasons, the patterns observed were consistent and the analysis was performed on the data set to be representative of the whole open water season (May-November) in 2021. Total Phosphorus (TP) was significantly different between regions, as was Total Kjeldahl Nitrogen (TKN), specific conductivity, and average substrate size. The differences in Total Organic Carbon (TOC) and turbidity were not significant. Generally, this suggests that while nutrient concentrations would increase naturally in the river due to landscape-fluvial interactions, the differences associated with the presence of MWWE (as suggested by TP and TKN concentration changes) are far more notable over the distance observed in the Bow River for this project. Visualizations of the data generally indicate that differences are likely to be related to substantial changes in physicochemical values at South Calgary sites. Additionally, there is a slight bias towards larger substrate in downstream habitats, particularly in Calgary, which could also be associated with changes to benthic macroinvertebrate assemblages (Erman and Erman 1984, Duan et al. 2008). However, while the heterogeneity of substrate size was greater in Calgary sites, the median between the North and South Calgary region was similar, which is more associated with changes to the assemblage than the amount of heterogeneity (Erman and Erman 1984). This might have more association with differences between Canmore and Calgary but the overall change in median substrate size may be biologically more relevant to sites far upstream (such as Canmore) than to nearby sites in Calgary with different MWWE exposures, even if statistically significantly different (Duan et al. 2008).

A distance-based Redundancy Analysis (dbRDA) using environmental conditions in the Bow River, as represented by a suite of physicochemical parameters, to constrain the patterns of relationships in SIMPER reduced benthic macroinvertebrate assemblages, did not consistently identify a single water quality parameter or group of parameters associated with MWWE to explain the observed inter-site variation in community composition across all sites and times of sampling. Two broad classes of water quality parameters most strongly associated with WWTPs (nutrients and conductivity) were highly correlated positively with each other and South Calgary benthic macroinvertebrate assemblages in all months. This supports the previous results in identifying three major clusters of sites (primarily Canmore Upstream and Downstream, Cochrane and North Calgary, and South Calgary) but with the added context that increased nutrients are primarily correlated with these changes in South Calgary, although the other two sites appear more uncorrelated with nutrients, rather than negatively correlated. This suggests that there are alternative drivers, likely associated with changes in the landscape ecology, that may be major drivers for sites not exposed to WWTP effluents. Average substrate size, unrelated to WWTP, does appears to have some role here, as it is negatively associated with the Canmore sites, suggesting that these sites have smaller than average substrate size and correspondingly dissimilar benthic macroinvertebrate assemblages.

The dbRDA model is significant, overall, and testing the individual significance of model parameters and the use of stepwise selection models revealed that measured levels of TOC and Turbidity did not significantly explain the variation in the benthic macroinvertebrate assemblages, which is supported by the permutation tests performed on the physicochemical parameters independently of this model. In contrast, regional (site) differences were associated with changes in levels of in TP, TKN, and conductivity as a group, collectively, but TKN was generally not significant overall. The forward selection model only identified specific conductivity as a primary explanatory variable. This, alongside the correlation scores of the variables, could suggest that conductivity in the Bow River may be a candidate water quality parameter to trace MWWE impacts. The backward selection model retained a larger selection of parameters, but they largely supported the previous results, in that TP, conductivity, and average substrate size were kept but, surprisingly, so was TOC, which typically was not identified as a significant parameter. Overall, substrate size tended to have some effect, primarily between Canmore and Calgary sites, while site differences in Calgary were primarily differentiated through a suite of parameters associated with more directly with MWWE exposure.

Therefore, that all expected physicochemical parameters chosen seemed to relate well to MWWE exposure, based on the cluster analysis of the sites, suggests that the broader environmental changes wrought by exposure are cumulatively important. However, the consistent importance of TP throughout the analysis supports literature results regarding the role nutrients have demonstrated in influencing benthic macroinvertebrate community composition.

For instance, in tropical streams, up to 50% of variation in benthic macroinvertebrate assemblages has been predicted to be explained by patterns of anthropogenically sourced nutrient and organic pollution from a variety of sources (Peralta et al. 2020). While temperate rivers likely differ in the role of various physicochemical drivers, the consistently high ranking of TP in responsibility for between site variation supports that TP may be a significant driver in driving the physiochemical and biological aspects of sites around WWTPs. As mountain streams are often P-limited, it is likely that changes in TP are, firstly, changing substantially with MWWE exposure, and secondly, benthic macroinvertebrate assemblages are changing because of changes to producer growth (Bowman et al. 2007).

Previous work on the Bow River that incorporated multiple historical datasets to assign stressor patterns (both environmental and anthropogenic) to changes in attributes of the river system similarly found that benthic macroinvertebrate communities (specifically density and proportion of EPT) failed to significantly differ in relation to environmental factors (chlorophyll-α) in the river (Sinnatamby et al. 2020). In this case, it was suspected that a higher sampling resolution was necessary or that the Bow River was too large to accommodate very notable benthic macroinvertebrate in-stream migration. Overall, the Bow River has not had an exhaustive assessment of the role of environmental variables in driving community assemblages previously or within this study but results from similar rivers can provide insight. Tributaries of the Athabasca River in Northern Alberta, for a similar analysis, showed that the environmental drivers of benthic macroinvertebrates varied widely from upstream to downstream and from stream to stream (Suzanne 2015). In the mainstem however, TP was also one of a set of physicochemical parameters strongly and consistently associated with shifts in benthic macroinvertebrate assemblages (Culp et al. 2003).

Other studies have observed similarly that while communities vary significantly around WWTPs, this change does not always track well with environmental parameters such as nutrient loads or BOD (Enns et al. 2023). One large analysis of European Rivers observed overall that most studies showed more pressing effects from poorly treated wastewater over wastewater with micropollutants (Berger et al. 2016). Therefore, the Bow River, having relatively high-quality treatment processes, may not represent drivers that shift any environmental physicochemical measurement to levels that can be independently responsible for driving dissimilarity in benthic macroinvertebrate communities. Similarly, many physicochemical parameters are not important drivers of benthic macroinvertebrate communities until they reach certain biologically relevant levels, which may explain the sudden shift in benthic macroinvertebrate assemblages in South Calgary. In this case, for the Bow River, while some parameters were consistently detectably measured, they may not have been at levels that are environmentally significant, such as turbidity, which was relatively low and did not increase by amounts associated with change to ecosystem function at sampled timepoints (Henley et al. 2000).

To further improve the identification of physicochemical drivers of benthic macroinvertebrate assemblages in the Bow River, it is likely necessary to increase resolution of samples of the benthic macroinvertebrate communities and associated physicochemical parameters, especially of ESOCs, around WWTPs in the City of Calgary. Alternatively, it is important to recognize that there may be no single parameter (or missing important parameters) associated with MWWE responsible for driving the community in a particular direction, especially in a relatively pristine river with high-quality WWTPs. While MWWE appears to drive community change, detangling the specific drivers in MWWE complex mixtures may need to make use of high frequency sampling and comparisons to areas where MWWE exposures are more significant.

2.5 Conclusions

The role of MWWE from point-source WWTPs in affecting the health of Bow River aquatic ecosystems, specifically through benthic macroinvertebrate community assemblages, is assessed in this chapter through a weight of evidence approach. As a complex mixture of nutrients, ESOCs, and unique physical characteristics compared to the receiving environment, MWWE effects are difficult to quantify for *in-situ* water ecosystems. Additionally, many biomonitoring approaches will fail to identify clear changes in the ecosystem when exposed to multiple impacts that have potential to degrade biological organisms in river systems (Enns et al. 2023). Therefore, observations in systems such as the Bow River, which is relatively pristine aside from the high-quality effluent inputs from WWTPs in urban centres up to and including Calgary, are crucially important in isolating community composition effects for benthic macroinvertebrate communities.

The first and third guiding questions for this project were related and attempted to identify if there were observable regions, if they were significantly different, and what the differences were in benthic macroinvertebrate assemblages among sites at different points on the longitudinal river gradient, primarily reference sites and potentially impacted sites below point source MWWE exposures. The role of WWTPs and MWWE in contributing to major shifts in the benthic macroinvertebrate assemblages was supported. Cluster analysis and NMDS showed that the most prominent outliers in terms of community assemblage composition were sites immediately below Calgary's WWTPs and distinct regions emerged from assemblages in relation to both placement along the longitudinal gradient and exposure of MWWE. Additionally, PERMANOVA analysis found significant differences between regions, and these differences were related to changes in taxonomic composition in sites with reduced water quality.

The second guiding question for this project was to examine the current status of key endpoints associated with benthic macroinvertebrate assemblages at sites along the longitudinal river gradient in the Bow River. While community-based metrics that did not consider the types of taxa observed did not demonstrate much change over the longitudinal gradient, those that did show changes in community composition were associated significantly with MWWE exposure from the City of Calgary. Typically, benthic macroinvertebrate community assemblages changed with MWWE exposure, for example, increasing pollution tolerant Diptera and reducing sensitive EPT families. The distribution of different EPT orders did differ between regions but in ways that appeared to be primarily related to life cycles of organisms and ecozone related shifts. Overall, this difference was mostly related to a few dominant taxonomic families, which mostly fit into the above categories. The most dissimilar sites to all others were those exposed to large Calgary WWTPs, even though other sites were separated by much larger distances with clear changes in the surrounding ecozone.

Lastly, this project was guided by the question of whether there exist predictive relationships that link system drivers (MWWE related nutrients or ESOCs) to benthic assemblage responses. ESOC concentrations were observed, overall, to be lower than concentrations demonstrated to affect benthic macroinvertebrate assemblages. Furthermore, patterns of change in assemblages that are associated with particular ESOC categories, such as Analgesics, were not observed. Permutation tests showed that TKN and TP concentrations, alongside overall specific conductivity in the river, differed significantly between river-reach regions. Multivariate analyses using dbRDA showed differences were strongly correlated to MWWE associated parameters, which were consistently responsible for differentiating variation in environmental conditions between sites across the seasonal sampling. This supports the general findings of previous studies that nutrients are primarily responsible for driving differences in benthic macroinvertebrate assemblages. The inclusion of a qualitative assessment of ESOC concentrations in the Bow River was used to assess an alternative hypothesis for the role of nutrient enrichment in driving any potential observed changes in benthic macroinvertebrate community composition, which was that communities could be strongly affected by ESOCs in the complex MWWE mixtures. However, there was little evidence to support this prediction. While acute toxicity was an unlikely outcome, based on historically observed levels of ESOCs in the rivers, the significant change in overall benthic macroinvertebrate abundances downstream of WWTPs in both Calgary and Canmore do not provide any evidence of direct ESOC-related toxicity to the overall community. More detailed *in situ* and controlled exposure studies would be required to ascertain the potential role of nutrient/ESOC interactions in affecting benthic invertebrate community responses.

Chapter 3: Assessing Environmental Effects of Municipal Wastewater Effluent Exposure in on Benthic Macroinvertebrates Assemblages in ACWA Experimental Streams

3.1 General Background

3.1.1 MWWE & Mesocosm Approaches

Large, flowing, freshwater systems have complex relationships with the surrounding landscape, which influences ecosystem characteristics from physicochemical parameters to biological communities (Allan 2004). Lotic systems can be heavily impacted by anthropogenic influences, with industrial, agricultural, and urban pressures (Malmqvist and Rundle 2002)). Combined, varied landscapes and increasing anthropogenic pressures are even more likely to shift, although also more unpredictably, key physicochemical parameters and affect biological communities (Malmqvist and Rundle 2002, Townsend et al. 2008, Berger et al. 2016). This is observed particularly in areas that are quickly urbanizing. Pollutants associated with urban environments are frequently complex, and it can be difficult to identify ecosystem drivers, especially when the effects of contaminants are often heavily mediated through the surrounding landscape (Booth et al. 2016). Urban areas where a lotic system is not already impacted by diffuse pollutants, are crucial in developing specific understandings of urban contaminant drivers. Additionally, these conditions are particularly appropriate for understanding the role that point source pollutants may exert on a lotic system (Berger et al. 2016).

This type of study provides the opportunity to characterize various contaminants in such a way that can inform broad policy and management decisions in the future. For instance, characterizing differences between impacted and unimpacted sites for a certain pollutant is the basis of large-scale environmental monitoring programs in Canada known as Environmental

Effects Monitoring (EEM) (Walker et al. 2003). These frameworks have been developed to address major pollutants in Canadian systems on the basis of characterizing and then monitoring for signs of ecosystem impairment, generally using biological indicators. EEM programs are primarily predicated on determining if there is quantifiable impact on valued components of ecosystems prior to dedicating resources to determining specific causal stressors (Kilgour et al. 2007). In early stages, this often may mean that minimal environmental data is assessed alongside biological endpoints, as causal relationships are more difficult to establish and may require higher investment into experiments. Therefore, symptoms of impairment from anthropogenic stressors must be well understood to adequately assess and manage the ecosystems and stressors being monitored.

In urban areas, one common point source anthropogenic stressor is MWWE, for which no EEM framework has yet been developed. However, interpreting the role of various environmental drivers and their interactions on the overall ecological system can be difficult for *in situ* experiments. Additionally, MWWE exposures can occur multiple locations in a lotic system, as they are generally associated with any large urban area. This underlines the need to understand not just exposure outcomes but also any associated cumulative effects associated with incremental discharges. It is particularly complicated to identify the chemical constituents of MWWE producing the observed effects because it is a complex mixture where nutrients and ESOCs (including pharmaceuticals and personal care products) are combined in the released effluent and the combined interactions of the components are not well known (Holeton et al. 2011). Due to this complexity, an EEM approach based on first quantifying observed changes in biological and ecological endpoints in areas of MWWE exposure can help to develop techniques and responses that improve and validate future research (Kilgour et al. 2007).

This approach to detangling the potential ability of complex components of MWWE to drive ecological patterns in systems exposed to MWWE mixtures may include laboratory studies that assess endpoints such as toxicity through bioassay experiments (Alexander et al. 2020). However, while the two types of studies are complimentary in that laboratory studies may identify patterns that can potentially be observed in biomonitoring larger systems, laboratory studies are correspondingly limited in their ability to integrate a full complement of contaminants and lack the complexity of an *in-situ* study with a varying environment. Crucially, this simplicity may not allow experiments to capture interactions between potential contaminants and the environmental condition of the receiving environment. An additional type of experiment to augment information provided in both of these studies is through the use of mesocosm-type experiments, such as the use of artificial streams for lotic systems (Alexander et al. 2020). For MWWE, often released into flowing river ecosystems, mesocosms and similar types of experiments (such as artificial streams) can fill a crucial gap between field and laboratory studies and have been used to assist in the development of EEM frameworks before (Dubé et al. 2002, Liber et al. 2009).

Simulated outdoor streams allow many environmental parameters and stressors to be controlled while still in the presence of natural environments (Alexander et al. 2020). A key aspect of artificial streams in ecological studies is the ability to integrate but control ecological processes and environmental conditions while manipulating a stressor. For example, flow rates of lotic systems can be controlled to an approximately constant rate in an artificial stream, unlike a river with natural and changing geomorphic features. The significance of this type of control is observed in one artificial stream study addressing flow that found that variation in flow velocity was a significant driver in affecting both community structure metrics and trait-based metrics for benthic aquatic macroinvertebrates (Juvigny-Khenafou et al. 2021). Generally, the reproducibility of conditions between replicates in mesocosm-type experiments has been observed to be relatively high in outdoor artificial streams, which is crucial in understanding ecological patterns caused by contaminants, environmental conditions, and their interactions (Harris et al. 2007).

This reproducibility is important to the utilization of mesocosm studies alongside larger *in-situ* studies. Similar to large scale *in-situ* biomonitoring programs, such as EEM programs in Canada, assessing the impacts of MWWE exposure in artificial can be done through sampling of biotic endpoints. Particularly, benthic macroinvertebrates have long been established as a biological endpoint through which ecological health can be measured. Patterns of change in benthic macroinvertebrate assemblages can provide information essential to identify drivers of system change (Hroch 2022). Benthic macroinvertebrate assemblages also have a significant history of use in identifying effects from MWWE, as many types of benthic macroinvertebrates are sensitive to common environmental changes associated with MWWE exposure, such as increases in total suspended sediment and sedimentation patterns, increases in growth of producers through nutrient enrichment, and related shifts to oxygenation (Holeton et al. 2011, Dos Reis Oliveira et al. 2019).

The effects of ESOCs from MWWE are less well understood than the nutrient and physical changes described above, as the physiological differences of benthic macroinvertebrates are associated with different outcomes for many pharmaceuticals and personal care products, ranging from toxicological impacts to no notable effects (Kidd et al. 2014). However, some specific effects from notable ESOC groups, such as analgesics, have demonstrated direct and indirect effects in laboratory experiments that may be relevant in disentangling complex MWWE in controlled (Konschak et al. 2020, Lencioni et al. 2020). Additionally, benthic macroinvertebrates are good candidates for artificial stream level assessment due to their ubiquity across aquatic ecosystems, small size, and various roles as consumers in the basal food web (Alexander et al. 2020). Particularly, shifts to the composition of benthic macroinvertebrate assemblages, as represented through the whole assemblage or various representative indices such as a diversity or abundance, can demonstrate impacts to the overall system (Buss et al. 2014).

These identified endpoints, studied through observation of benthic macroinvertebrate assemblages, can be achieved through a variety of sampling techniques. For larger systems, active methods such as kicknetting, which utilizes an (often timed) travelling substrate disturbance with a net, are common and increasingly have been written into formal sampling systems, such as the Canadian Aquatic Biomonitoring Network (CABiN) and the National Rivers and Streams Assessment (NARS) (Carter and Resh 2001, Environment and Climate Change Canada 2017, US EPA 2023). For a smaller mesocosm system that is frequently utilized, an area limited type of sampler, such as a Surber sampler may be more appropriate to avoid excessive disturbance. However, both these types of samplers provide “snapshots” of stream conditions. Comparatively, passive samplers that are deployed for a longer time period and allow for benthic macroinvertebrate colonization can provide a more integrated summary of the environmental conditions driving the ecosystem (Yeardley et al. 2020). The use of varied sampling methods in an artificial stream setting with controlled physicochemical conditions for assessing a complex mixture, such as MWWE, could help elucidate further differences in how macroinvertebrate assemblages are shaped by MWWE exposure. Additionally, investigation of different techniques can inform the improvement of rapid assessment methods, which can lead to more efficient monitoring tools.

Furthermore, mesocosms and artificial streams with benthic macroinvertebrates have successfully been utilized to test a number of nutrient-related stressors similar to and including MWWE. Nutrient enrichment mesocosm experiments have demonstrated changes to benthic macroinvertebrate assemblage metrics such as evenness as well as changes to physiological endpoints such as body size, which increased. Additionally, controlled changes to physicochemical conditions, such as flow, often interacted with nutrient addition in the artificial streams (Juvigny-Khenafou et al. 2021). This emphasizes the importance of limiting or manipulating environmental parameters when attempting to identify the role of stressors on biological endpoints such as benthic macroinvertebrates. Artificial streams have also been used to identify change to benthic macroinvertebrate community composition from pulp mill effluent. This demonstrated that pulp mill related nutrient enrichment was evident in periphyton, and benthic macroinvertebrate communities exposed to lower pulp mill effluent concentrations, but while higher concentrations showed similar patterns for benthic macroinvertebrates, periphyton community composition dramatically changed. The ability to manipulate the stressor concentration was key in identifying potential impacts in larger receiving environments and the development of a Canadian environmental effects monitoring program (EEM) (Culp et al. 2003).

When MWWE specifically is manipulated in artificial stream experiments, exposures have demonstrated clear impacts on systems. One study that utilized several levels of MWWE exposure, with the effluent making up 5%, 15%, 30% of total stream volume (alongside a control), showed that all mesocosms exposed to MWWE were substantially different from the control in terms of benthic macroinvertebrate community composition. Indices of ecological health were generally negatively affected by MWWE, most affected by higher concentrations, and exacerbated by additional stressors, such as drought (Grantham et al. 2012, Loskotová et al.

2022). However, as MWWE is a complex effluent, the contribution of various components to the overall composition can vary. Artificial streams that can better control and replicate different characteristics of MWWE mixtures have also shown that the composition and the overall concentration clearly affect biological endpoints (Pereda et al. 2019, Peralta et al. 2020). A study controlling major physicochemical water quality parameters while manipulating exposure to a common chemical found in MWWE from personal care products (triclosan) was found to depress periphyton growth at high concentrations but did not strongly impact benthic macroinvertebrate communities (Nietch et al. 2013). Another study observing the effects of titanium dioxide nanoparticles (TiO_2NP) from WWTPs showed that periphyton biomass, a common food source for benthic macroinvertebrates, declined significantly in high exposures (Wright et al. 2018).

Additionally, stream experimental systems provide an important approach in disentangling the complex interactions and resulting water quality and ecological impacts of nutrients and ESOCs in MWWE. Depending on their length, and if they are colonized with various biological assemblages, including algae and macrophytes, it is possible discriminate an environmental gradient of effects on the aquatic food web based on the relative exposure of different MWWE components (von Schilller et al. 2017). For example, one experimental stream system 152 m long showed a significant gradient in nutrient removal (via % nutrients in harvested biomass) along its length. The MWWE input to the stream had been secondarily treated but over the course of the gradient, the stream removed enough nutrients to reach standards considered appropriate for tertiarily treated wastewater (Craggs et al. 1996). However, relationships of nutrient uptake and corresponding changes in primary production and benthic macroinvertebrate assemblages has not been assessed where the potential confounding effects of exposure to ESOCs is also considered.

The Pine Creek WWTP in Calgary is associated with a set of constructed, replicated streams that are attached to the main facility, providing access to treated MWWE for experimental purposes. These streams are part of the Advancing Canadian Water Assets (ACWA) initiative's facility, which aims to advance knowledge of MWWE effects and the effectiveness of alternate treatment technologies. The streams are outdoors, and streamside vegetation, benthic macroinvertebrates, and fish were permitted to colonize naturally from the surrounding system, as well as through the intake of Bow River water for the benthic macroinvertebrates. Maintenance on the streams is minimized to allow natural function; however, they are flushed at a 10x increased volume every spring to simulate a freshet and streamside vegetation is sometimes removed. Streams are not observed to freeze over.

Given that similar experiments have established capacity to identify the role that anthropogenic contaminants, like MWWE, play in ecosystems through control of environmental parameters that cannot be controlled for *in-situ* experiments, identifying responses from a ubiquitous and easy to sample biological community is an important step in understanding the larger Bow River system. The Bow River itself is an important step in developing better programs for monitoring MWWE in aquatic systems. Additionally, the Pine Creek WWTP represents a high-quality effluent source associated with a WWTP that is intended to eventually serve a much larger population size. Overall, the experimental streams at ACWA provide a key opportunity to understand how this quality of effluent may affect components of the basal aquatic food web in the Bow River in the future.

3.1.3 Objectives and Hypotheses

The Pine Creek WWTP where ACWA is located was completed in 2010 and was the third WWTP constructed to support the City of Calgary (CoC) and is the most downstream plant at the south edge of the city. The discharge area around this WWTP in the Bow River is already exposed to other cumulative impacts from the CoC, including point-source effluents, such as stormwater, and non-point sources inputs (industrial areas, urban and recreational uses). Pine Creek WWTP produces extremely high-quality effluent, having more advanced treatment processes than either of Calgary's other WWTPs: Bonnybrook and Fish Creek). It also currently releases a much smaller total volume of WWTP in the CoC than Bonnybrook and serves a smaller overall population but has potential to significantly increase capacity, unlike Fish Creek, which has a similar capacity but is eventually intended to be replaced by Pine Creek. The experimental stream facility associated with Pine Creek provides a standardized, *in-situ* study system for MWWE exposure in aquatic systems.

The objective of this chapter is to examine responses of benthic macroinvertebrate assemblages when exposure to Bow River water (control) and Pine Creek MWWE treated effluent in experimental streams at the ACWA facility in Calgary using and Environmental Effects Monitoring assessment approach. I hypothesize that the incremental addition of 5% (by dilution volume) of Pine Creek MWWE will produce a significant change in the macroinvertebrate community assemblages between the MWWE treatment versus the Bow River control streams. It is further postulated that a gradient effect will be observed between upstream and downstream sites within the streams. A final objective is to compare the compatibility of two benthic macroinvertebrate sampling methods (conventional area-limited Surber sampling versus artificial substrates), examining the relative strengths and weaknesses of the two approaches.

This broad hypothesis will be addressed through several key guiding questions:

- What is the current status of key ecological endpoints associated with benthic macroinvertebrate assemblages in the control (Bow River) and Pine Creek Effluent treatment streams?
- Are there significant differences in benthic macroinvertebrate assemblages (i.e. community composition metrics) between the Pine Creek MWWE and the Bow River (control) streams?

3.2 Methods

3.2.1 The Advancing Canadian Water Assets Facility

ACWA's experimental stream facility is composed of twelve ~320m long, hydraulically isolated streams with an alternating riffle (10m) and pool (20m) structure. The streams are inspired by the nearby Alberta stream, Jumpingpound Creek, which, similarly to the Bow River, arises in the Rock Mountains and eventually joins the Bow River just upstream of Calgary in Canmore. On a regular basis, the experimental streams are run as triplicates for four MWWE treatment types: a Bow River control, tertiary-treated Pine Creek effluent, Pine Creek effluent with ozonation, and Pine Creek effluent with reverse osmosis. Notably, the Bow River (control) streams represent water quality conditions of source water that is already downstream of two of Calgary's three WWTPs, thereby providing the unique opportunity to assess incremental cumulative impacts associated with the addition of the various types of Pine Creek treated effluent. All effluent treatments, when added, make up 5% of the total flow (13.4 L/s). However, the % of total flow coming from treated effluent can also be experimentally increased and treatment types can be mixed. Each of the twelve replicate constructed streams were randomly assigned to receive the above MWWE treatments. For this project, only the Bow River streams (control) and the Pine Creek effluent stream were included (no streams utilizing additional experimental treatments for MWWE). An initial study of the constructed streams found that between the control and treatment streams, temperature and dissolved oxygen regimes were generally consistent among all the replicate streams; however, upstream to downstream gradient differences in pH were observed (Jackson 2020).

3.2.2 MWWE Exposure Experiment (Field and Laboratory Methods)

Using an EEM-based, weight of evidence approach, the potential impact of the addition tertiary-treated Pine Creek effluent was assessed by comparing changes in a suite of measured and derived macroinvertebrate community assemblage endpoints in the Bow River (control) and 5% Pine Creek MWWE experimental streams.

Characterization of the chemical and physical attributes of the streams was researched to confirm that the underlying abiotic conditions of the replicate streams were similar, other than the addition of Pine Creek effluent. Assessment of background water quality over the experimental period (Sept-Oct 2020) involved examination of monthly water chemistry data collected from the head pond feeding Bow River water into the experimental streams, and included temperature, pH, total organic carbon (TOC), total nitrogen (TN), and total phosphorus (TP). Water quality samples were taken by ACWA technical staff and were processed on-site in the water quality laboratory (Table 3.1). Physical characteristics of the streams (average width, depth, substrate size) were assessed at both the upstream (riffle 2) and downstream (riffle 10) sampling sites in each stream. Final measurements were based on an average of three measurements taken at the top, middle, and bottom of the riffle. The substrate was characterized using an adaptation of the 100-pebble count from the Substrate Characteristics section of the CABiN field manual for wadable streams (Environment and Climate Change Canada 2017). Fifty rocks were randomly chosen by taking the boot-end rock every 2 steps. Rocks were classified using Wentworth substrate classes of the length of the longest axis, rather than measured precisely. Flow was standardized in each replicate stream through a regulated discharge from the ACWA facility (Table 3.1).

Benthic macroinvertebrate assemblages were quantified in the control and PC treatment streams using artificial substrate rock baskets and area-limited Surber (12” x 12”) sampling. Standardized artificial substrate rock baskets were placed in the experimental streams on September 14th, 2020 and allowed to colonize for a period of six weeks, The rock baskets were constructed of wire cages (30cm x 24cm x 5.5cm) and consisted of a bottom layer of inert plastic scour pads to collect fine sediment covered with 40 identical ceramic briquettes (two rows of 20 briquettes arranged in a 5x4 pattern) (Fig. A.1). Triplicate rock baskets were placed in both the upstream and downstream riffles in a randomly selected Bow River (control) and Pine Creek MWWE stream. After six weeks, the rock baskets were removed during the same time Surber sampling was conducted (Suzanne 2015). The baskets were dissembled on site and all components and associated biological material was frozen at -20C until processing. Surber samples were taken from each of the three replicate Bow River (control) and Pine Creek effluent experimental streams, one from upstream (riffle 2) and one from the downstream (riffle 10) locations for each stream, yielding 12 samples in total. All samples were frozen at -20°C until processed. It is to be noted that the Surber sample at BRR1Riff10 was lost in transit and is excluded from all further processing and analysis.

Macroinvertebrates in rock baskets were removed by rinsing briquettes and picking individuals off the scour pads before subsampling. Both rock basket samples and Surber samples were subsampled using an Imhoff cone (Wrona et al. 1982). For both sampling methods, subsamples ranged from 25% to 100%, depending on the overall abundance of taxa. A subsample was required to contain a minimum of 100 organisms for identification and was resampled until the sample either met that threshold or was 100% identified to establish distribution of taxa (Hickley 1975, Wrona et al. 1982). Samples were multiplied by the coefficient of subsampling fraction to standardize sorting effort before analysis. Taxa were identified to the lowest practical unit, usually family, primarily following *Aquatic Invertebrates of Alberta* (Clifford 1991). Sites and dates of benthic samples are identified in Table A.1.

Table 3.1. Summary of background abiotic parameters in the ACWA Bow River (control) (BR) and Pine Creek (PC) effluent streams, including water chemistry of the Bow River source head pond during the experimental period (September-October 2020). Physical attributes (flow, width, depth) of each stream are based on an average from three measurements in each riffle (Upstream riffle 2 and Downstream riffle 10). Average substrate size was determined by taking 50 random stone samples at each riffle site.

Site	Temperature (°C)	pH	TOC (mg/L)	TN (mg/L)	TP (mg/L)	Flow (L/s)	Mean Width (cm)	Mean Depth (cm)	Mean Substrate size (mm)
Head pond (September)	13.75	8.03	1.65	0.85	0.03	n/a	n/a	n/a	n/a
Head pond (October)	7.55	8.19	1.4	0.75	0.015	n/a	n/a	n/a	n/a
BRR1Riff2	n/a	n/a	n/a	n/a	n/a	13.4	150.1	10.5	60.9
BRR1Riff10	n/a	n/a	n/a	n/a	n/a	13.4	148.1	8.8	n/a
BRR2Riff2	n/a	n/a	n/a	n/a	n/a	13.4	153.3	7	67.1
BRR2Riff10	n/a	n/a	n/a	n/a	n/a	13.4	133.7	9.2	57.0
BRR3Riff2	n/a	n/a	n/a	n/a	n/a	13.4	157.7	6.2	65.0
BRR3Riff10	n/a	n/a	n/a	n/a	n/a	13.4	118.3	8	53.2
PCR1Riff2	n/a	n/a	n/a	n/a	n/a	13.4	151.3	8.7	65.5
PCR1Riff10	n/a	n/a	n/a	n/a	n/a	13.4	143.7	8.5	55.3
PCR2Riff2	n/a	n/a	n/a	n/a	n/a	13.4	156.7	8	64.8
PCR2Riff10	n/a	n/a	n/a	n/a	n/a	13.4	137.2	7.4	54.8
PCR3Riff2	n/a	n/a	n/a	n/a	n/a	13.4	150.7	8.5	57.3
PCRRiff10	n/a	n/a	n/a	n/a	n/a	13.4	131	7.2	56.5

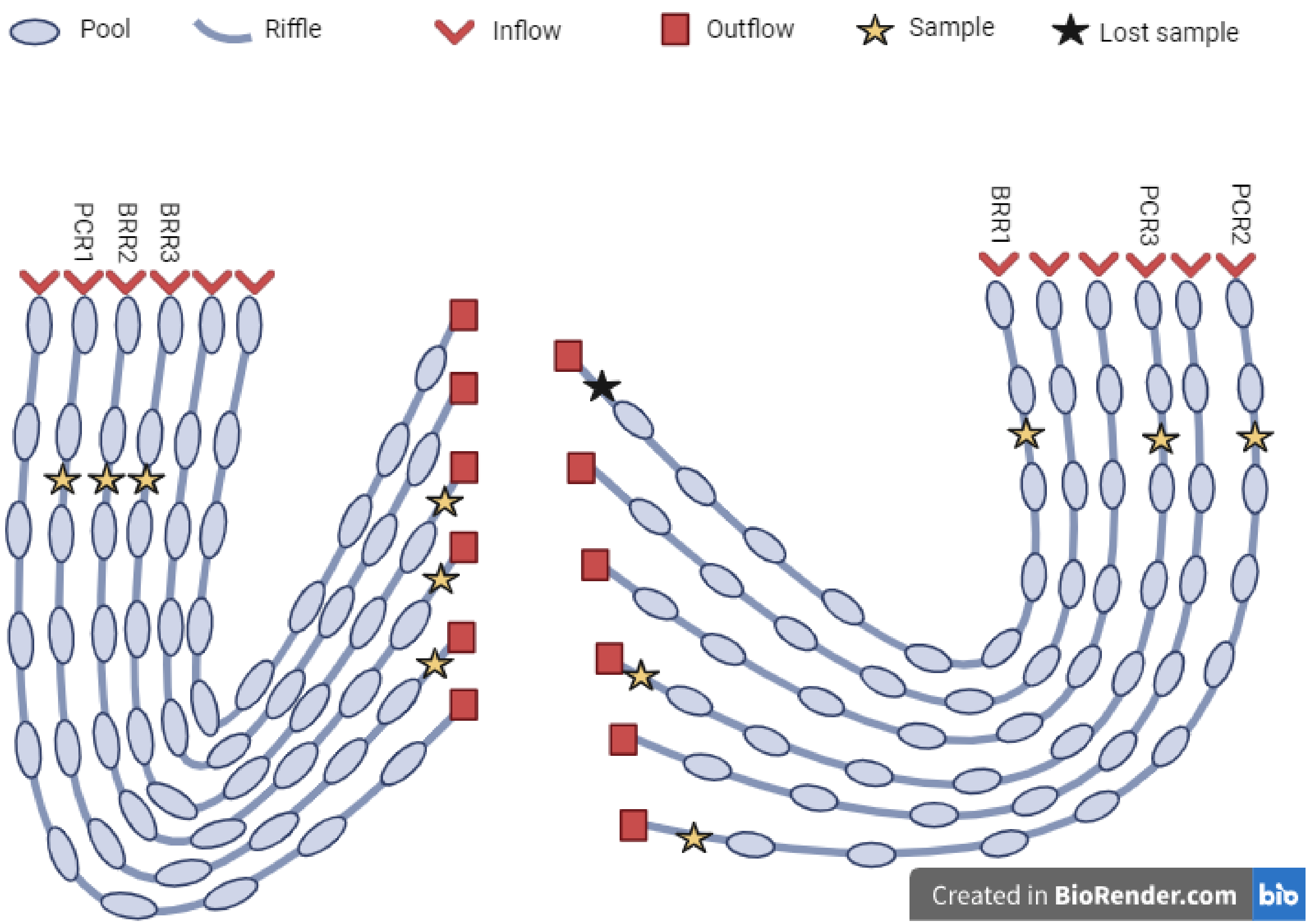


Fig. 3.1. Advancing Canadian Water Assets experimental streams sampling locations. BR – Bow River (control) streams replicates; PC – Pine Creek MWWE streams replicates.

3.2.3 Data Analyses

Objective 1: The status of key endpoints associated with benthic macroinvertebrate assemblages in the Bow River (control) and the Pine Creek MWWE treatment streams was assessed using multivariate analyses performed using RStudio (R version 4.2.3). Alpha-diversity was characterized for macroinvertebrate assemblages for both sampling methods used. Comparisons between upstream and downstream sampling locations were assessed using a range of ecological metrics. This included: total abundance (using the `rowSums` function), taxonomic richness (using the `specnumber` function from the vegan package), Shannon-Weiner diversity (using the `diversity` function from the vegan package), evenness, and % dominance of the dominant taxa. The percent composition of dominant taxonomic groups was also calculated, including the total percentage of Orders known for low pollution tolerance, typically referred to as EPT (Ephemeroptera, Plecoptera, Trichoptera) (Buss et al. 2014). The proportion of Diptera, an Order known for high pollution tolerance, was also calculated. Lastly, the Hilsenhoff Biotic Index for each defined region was calculated and a categorical rating of stream quality was assigned (Haney 2013).

Objective 2: Only prior to significance testing was raw benthic macroinvertebrate data $\log_{10}$ transformed to reduce the influence of zeroes in a the data set (Chin et al. 2016). Transformed data was used for all analyses from this point on. For each sampling method, significance testing was performed to identify differences between sites using matrices of benthic macroinvertebrate assemblages. After assessing for assumptions, this was done using the `adonis2` function from the vegan package in R to perform one-way PERMANOVA (999 permutations) and, where appropriate, pairwise comparisons with a Bonferroni correction, using the `pairwise.adonis` function ($p < 0.05$) (Anderson 2017, Arbizu 2020, Oksanen et al. 2022). A follow-up SIMPER analysis determined which taxa were most responsible for differences between sites using the `simper` function from the vegan package (Oksanen et al. 2022, Hroch 2022). Bray-Curtis distances for sites within regions were used to calculate non-metric multidimensional scaling (nMDS), using the `metaMDS` function from the vegan package, which spatially represented replicate similarity between sampling sites in streams for both sample types (Oksanen et al. 2022).

3.3 Results

3.3.1 Biotic Indices

In general the largest proportions of the macroinvertebrate community composition in Surber samples taken from the Bow River (control) and Pine Creek MWWE streams were from the Orders Diptera and Trichoptera, comprising >50% of the represented taxa (Fig. 3.2). However, differing community composition patterns were observed between the two treatment streams, with both Diptera and Trichoptera being consistently the two most prominent taxonomic groups in both the upstream and downstream sites of the Bow River control streams, together comprising >60% of the total relative abundance. In contrast, in the Pine Creek streams, just members of the Order Trichoptera were the most dominant taxonomic group in the upstream site (69%), but these decreased in prominance in the downstream site to relative equivalence with Diptera and Coleoptera taxa. Unlike the mainsteam Bow River, Plecoptera were almost entirely absent and Ephemeroptera were rare in both ACWA treatment streams. The category containing all additional taxonomic groups, Other (which primarily consisted of amphipods and leeches) was generally a less dominant category with some streams demonstrating larger groups (mostly based on amphipod abundance) but in inconsistent patterns across stream exposure types. The clearest pattern observed was an increase in relative abundance of Coleoptera (both larval and adult) in the downstream compared to upstream riffles in both the BRC and PC streams.

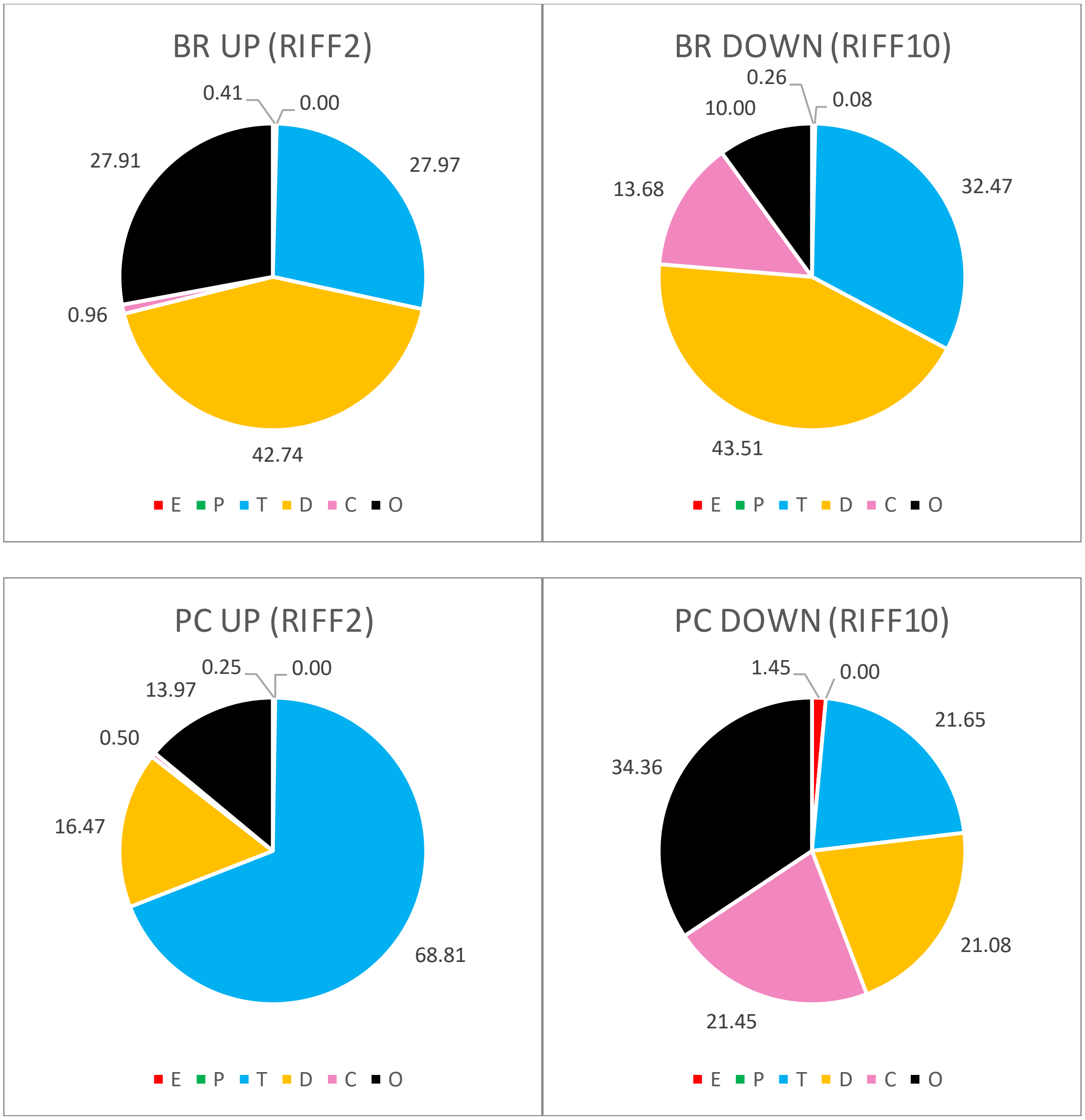


Fig. 3.2. In Surber samples of October 2020, the relative contribution (%) in the overall benthic macroinvertebrate community assemblage of key, coarse (mainly Order) taxonomic groups including EPT (Ephemeroptera (E), Plecoptera (P), Trichoptera (T)) taxa, Coleoptera (C), and Diptera (D) taxa, and cumulative other organisms (O). Values are averaged across three triplicate streams for the Bow River (control) and Pine Creek 5% MWWE treatment streams sampled at upstream (riff2) and downstream (riff10) locations.

After a 6-week colonozation period, the rock basket artificial substrates displayed very different patterns in macroinvertbrate assemblage composition compared to Surber samples (Fig. 3.3). Diptera and Trichoptera taxa combined comprised >75% of the total community composition in all streams, but their relative contributions changed dramatically in the any Pine Creek treatment stream, where Diptera alone represented 87% and 70% of the total community composition in the upstream and downstream sites, respectively. Similar to what was observed in the Surber samples, Plecoptera were entirely absent and Ephemeroptera were extremely rare in both the BCR and PC streams. The Other category that contained all remaining taxonomic groups, which was primarily made up of amphipods and now snails, was generally a less dominant category. However, there was consistently more of these additional taxa in the control stream sites, most of which was related to an increase in relative snail abundance, coupled with a relative increase in amphipods in the upstream sampling location.

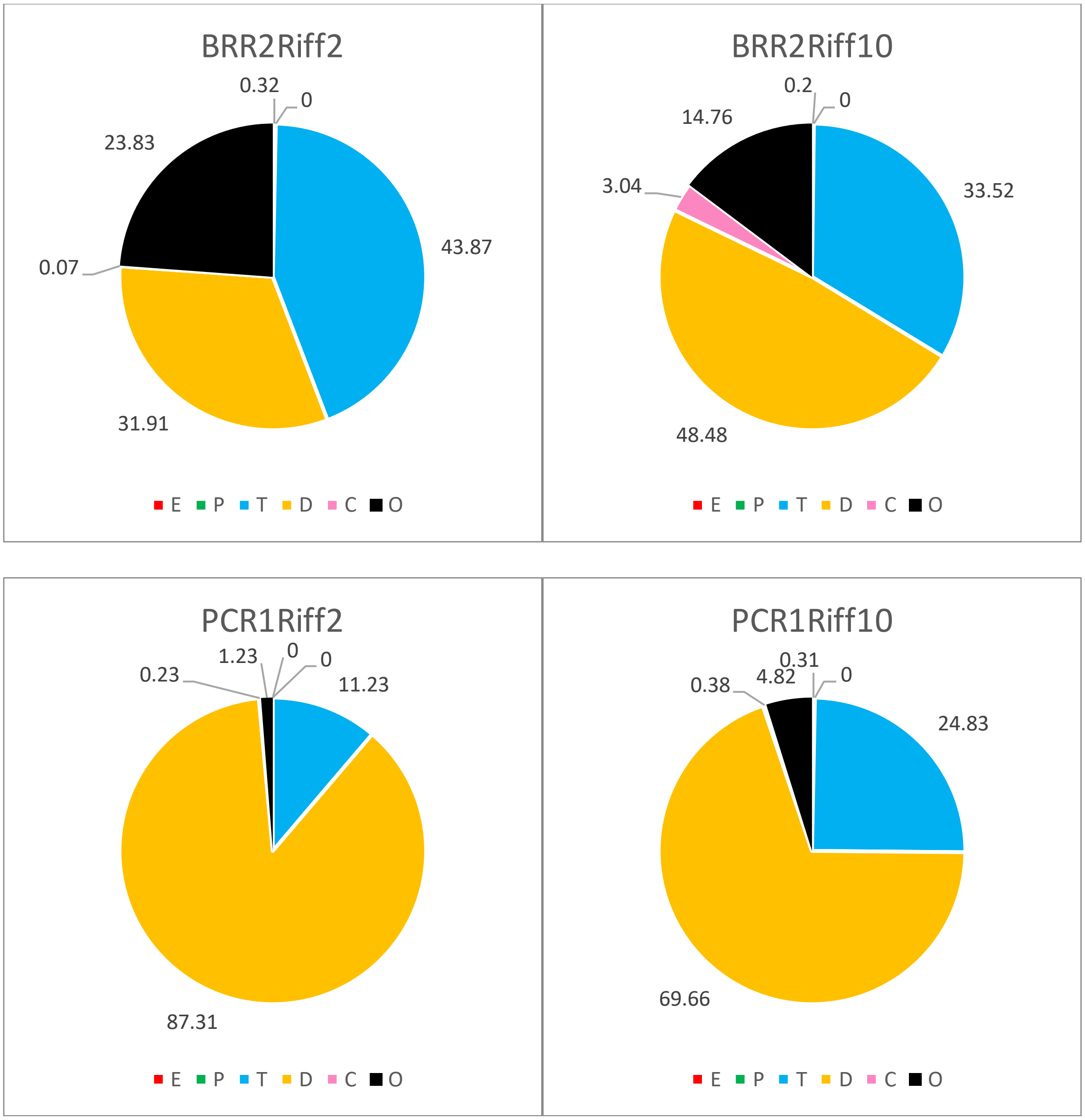


Fig. 3.3. In artificial substrate rock basket samples of October 2020, the relative contribution (%) in the overall benthic macroinvertebrate community assemblage of key, coarse (mainly Order) taxonomic groups including EPT (Ephemeroptera (E), Plecoptera (P), Trichoptera (T)) taxa, Coleoptera (C), and Diptera (D) taxa, and cumulative other organisms (O).. Values are averaged across triplicate samples taken from one representative control and treatment stream sampled at upstream (riff2) and downstream (riff10) locations.

Overall, for the Surber samples, the % EPT metric was primarily composed of the Order Trichoptera, of which the main representative was the Family Hydropsychidae (Fig. 3.4). The changes observed in % EPT at sample sites did not correspond to stream treatment, varying from an average of 25 – 75% of the sampled population. The highest % EPT was observed from the upstream sampling sites in the Pine Creek treatment streams, which had very high relative EPT and were dominated by Hydropsychidae. The % of Diptera in each sample was generally higher in the Bow River (control) than Pine Creek treatment streams, without much variation in relation to upstream or downstream locations (approximately 10-20%) (Fig. 3.6). Where the % Diptera was lower, there usually were more Coleoptera or Hydropsychidae.

The EPT benthic community assemblages in rock baskets were also primarily composed of the Order Trichoptera and Family Hydropsychidae (Fig. 3.5). The % EPT was generally higher in Bow River streams than in the Pine Creek streams but was notably lower in the upstream site in the Pine Creek stream. The % Diptera was correspondingly higher in the Pine Creek stream than in the control stream and highest in the upstream location of the Pine Creek stream (Fig. 3.7). Diptera consisted of least 30% of the total relative abundance of all taxa on average for all streams and sampling locations.

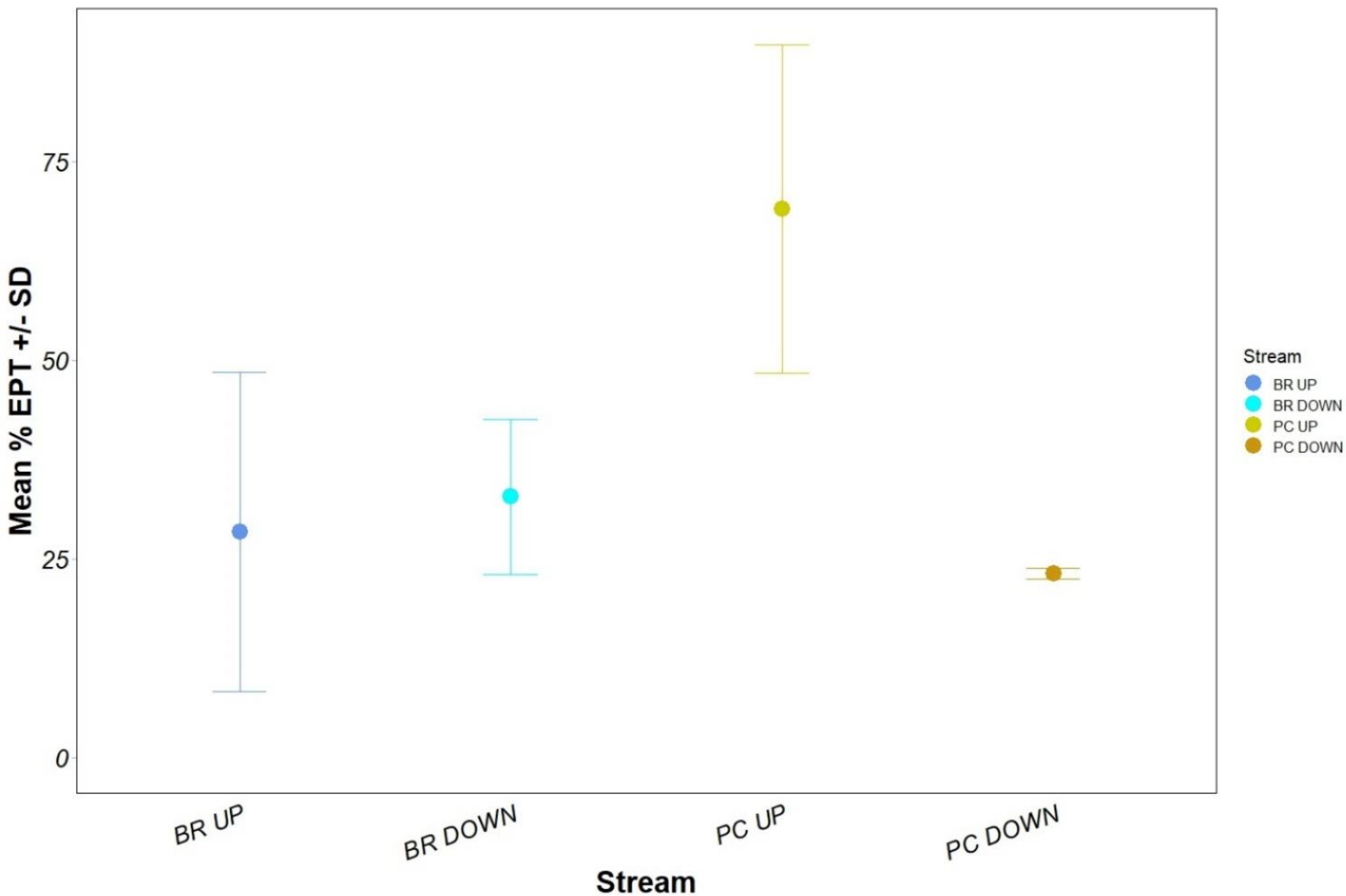


Fig. 3.4. The mean percentage of EPT taxa in the Surber sample macroinvertebrate assemblage (% of total community +/- 1 standard deviation). %EPT was calculated from the abundance of taxa from the Orders Ephemeroptera, Plecoptera, and Trichoptera divided by the total number of taxa identified in each sample from each stream at each location. All individual samples for each site in the defined treatment and location combinations were averaged (n=3, aside from BR DOWN, for which n=2).

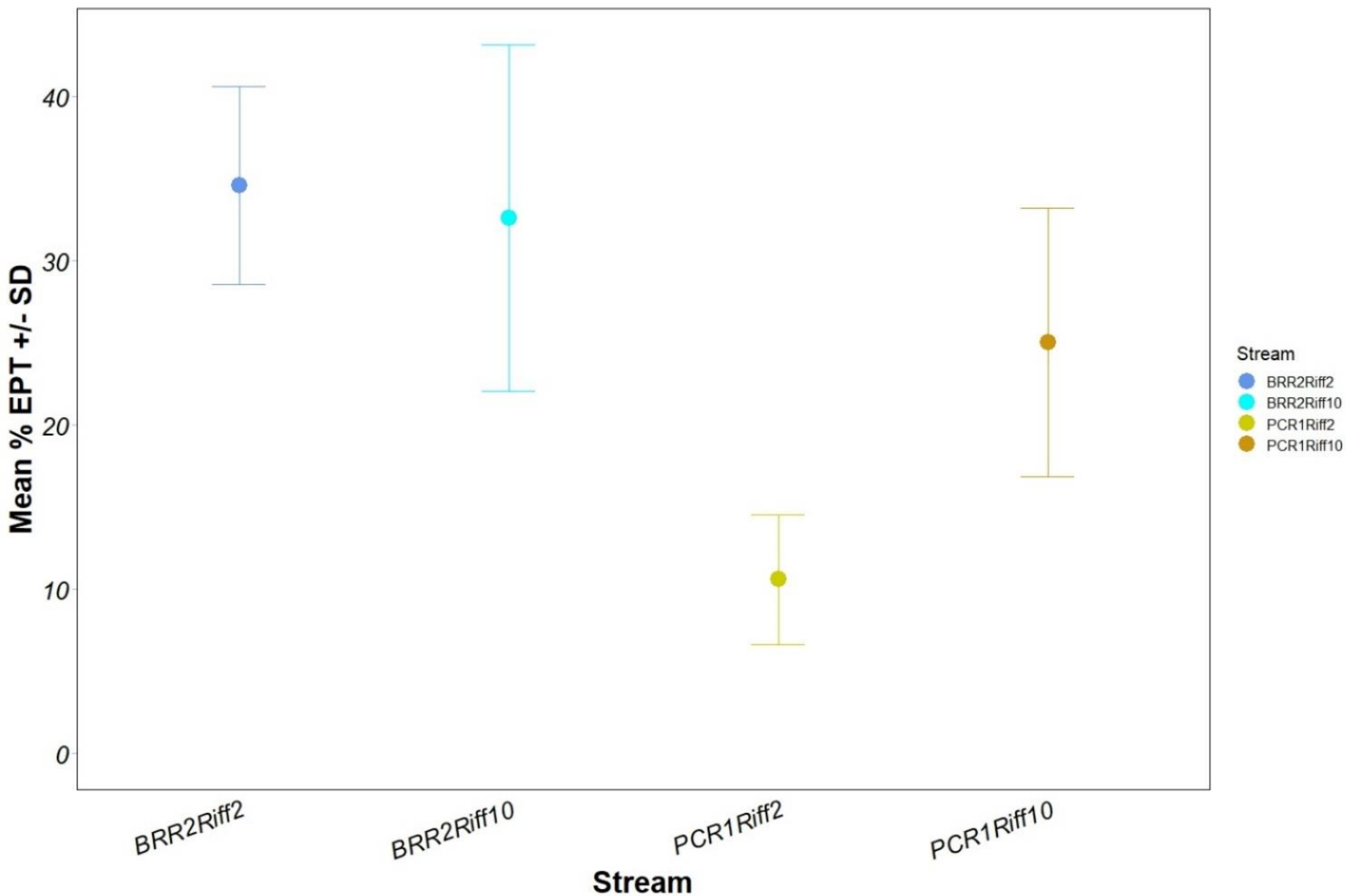


Fig. 3.5. The mean percentage of EPT taxa in the rock basket sample macroinvertebrate assemblage (% of total community +/- 1 standard deviation). %EPT was calculated from the abundance of taxa from the Orders Ephemeroptera, Plecoptera, and Trichoptera divided by the total number of taxa identified in each sample from the representative treatment and control stream at each location. All individual samples for each site in the defined treatment and location combinations were averaged (n=3).

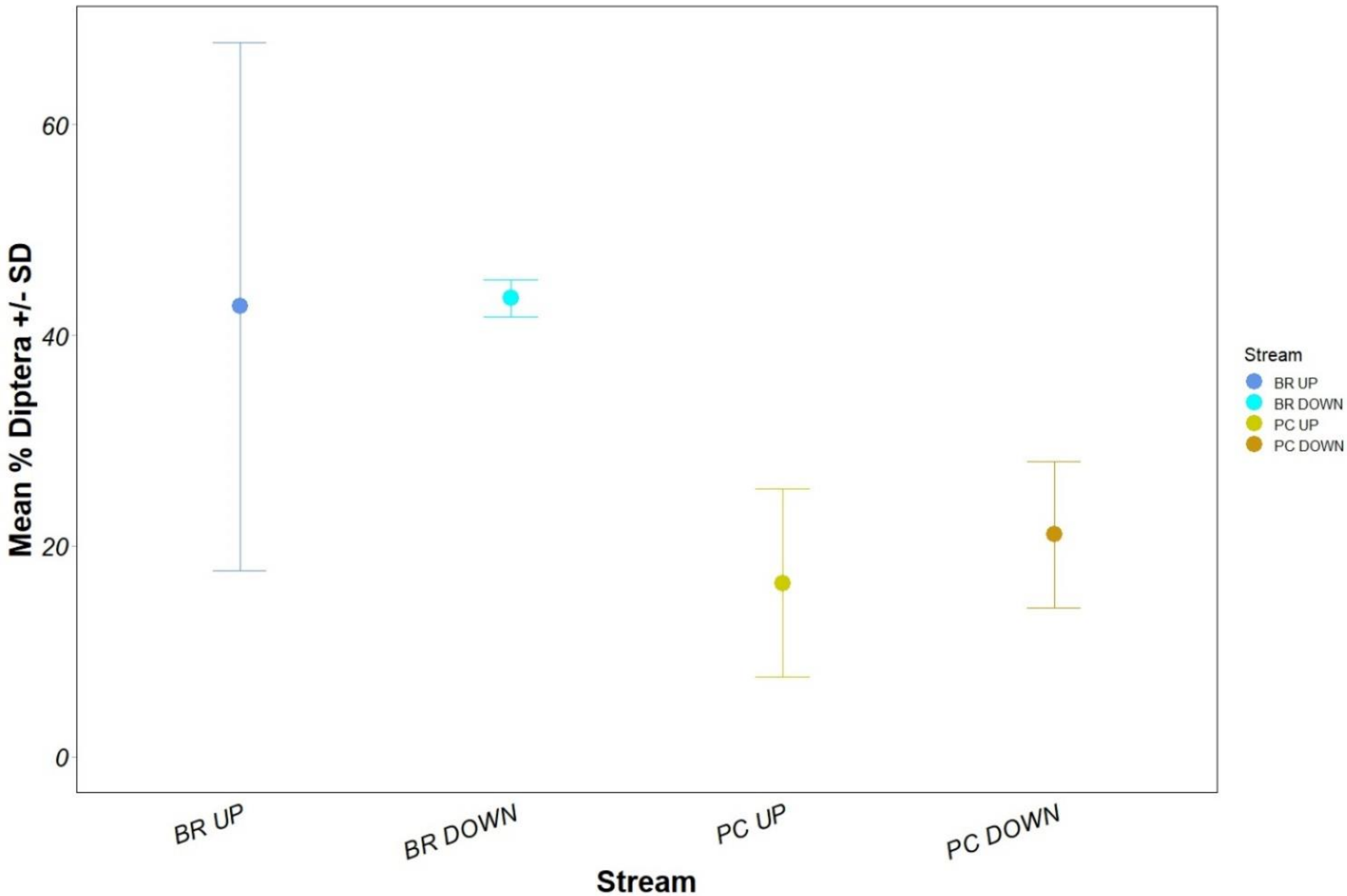


Fig. 3.6. The mean percentage of Diptera taxa in the Surber sample macroinvertebrate assemblage (% of total community +/- 1 standard deviation). %Diptera was calculated from the abundance of taxa from the Order Diptera divided by the total number of taxa identified in each sample from each stream at each location. All individual samples for each site in the defined treatment and location combinations were averaged (n=3, aside from BR DOWN, for which n=2).

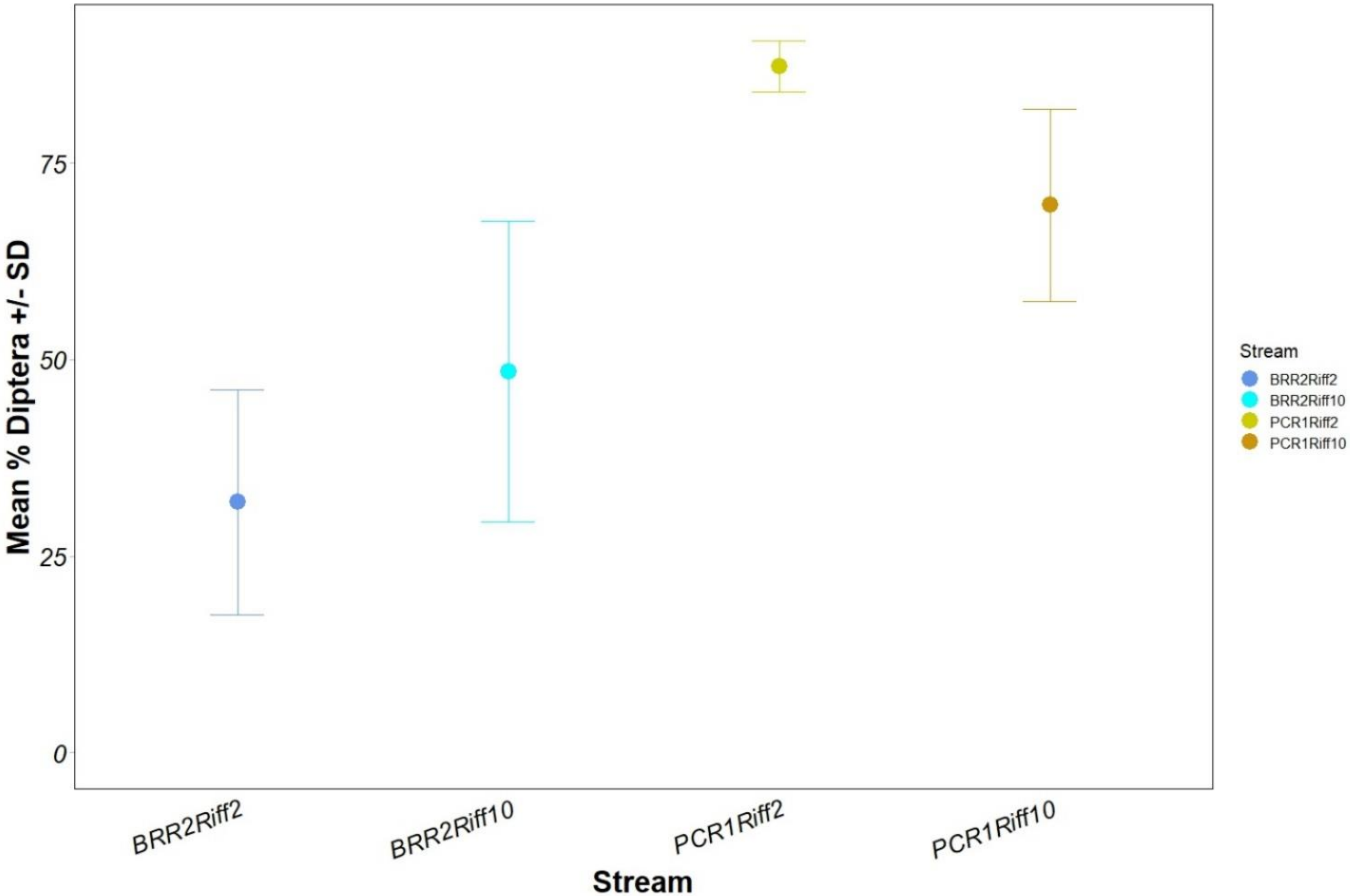


Fig. 3.7. The mean percentage of Diptera taxa in the rock basket sample macroinvertebrate assemblage (% of total community +/- 1 standard deviation). %Diptera was calculated from the abundance of taxa from the Order Diptera divided by the total number of taxa identified in each sample from the representative treatment and control stream at each location. All individual samples for each site in the defined treatment and location combinations were averaged (n=3).

For the Surber samples, total macroinvertebrate abundance was generally lower in the upstream sites, for both the Bow River and Pine Creek streams (Fig. 3.8). Upstream sites had a mean abundance of approximately 300, while the downstream sites had a mean abundance of 1200-1500. Family level richness did not vary notably between sites, averaging between 8-13 Families in each sample for each site (Fig. 3.10). The upstream site in the Pine Creek stream had a lower mean Shannon-Weiner diversity index value compared to the other sites, which appeared to be largely due to one particularly low replicate (Fig. 3.12). The evenness of the taxonomic Families was consistent across the treatment streams and locations (Fig. 3.14). The % dominant taxonomic group for each stream varied somewhat but with no consistent pattern (Fig. 3.16). The dominant organism was different for each site, with a Diptera family being the dominant organism at both control stream locations. Hydropsychidae was most dominant in the upstream sites of the Pine Creek streams, which was additionally the highest dominant taxon (~75% of the assemblage). Downstream sites in the Pine Creek treatment streams were dominated by amphipod *Hyallela azteca*. Lastly, the Hilsenhoff Biotic Index (HBI) rated both the Bow River (control) and Pine Creek treatment streams and sampling sites as “Fair”, showing no discernable differences associated with stream treatment or location (Fig. 3.18).

For the rock baskets, total mean abundance was higher in both the upstream and downstream sampling sites of the treatment stream than the control stream (Fig. 3.9). However, both control stream sites demonstrated abundances of ~2000 taxa, while the abundance of the downstream treatment stream was ~4000 and the upstream treatment stream was ~6000, so the downstream treatment stream site was notably higher than all other stream sites. Family level richness across rock basket sites was relatively even, with upstream and downstream sites having a richness of approximately 9-10 families (Fig. 3.11). Mean diversity (Shannon-Weiner diversity index) between streams and locations that the diversity in the control streams, and particularly the upstream control stream, was higher in the upstream region of the Bow than the treatment streams (Fig. 3.13). However, the evenness of taxa was clearly higher in the control streams at upstream and downstream sites (Fig. 3.15). Dominant organisms were more dominant in the treatment streams, which were both dominated by Diptera families (Fig. 3.17). However, the downstream sites for control and treatment streams were both dominated by the Diptera family Simuliidae. The only non-Diptera dominated stream was the upstream control stream, which was dominated by the Trichoptera family Hydropsychidae. Lastly, the HBI score rated each stream as having a Fair rating except for the downstream treatment stream, which was Fairly Poor (Fig. 3.19). However, all sites scored similarly overall.

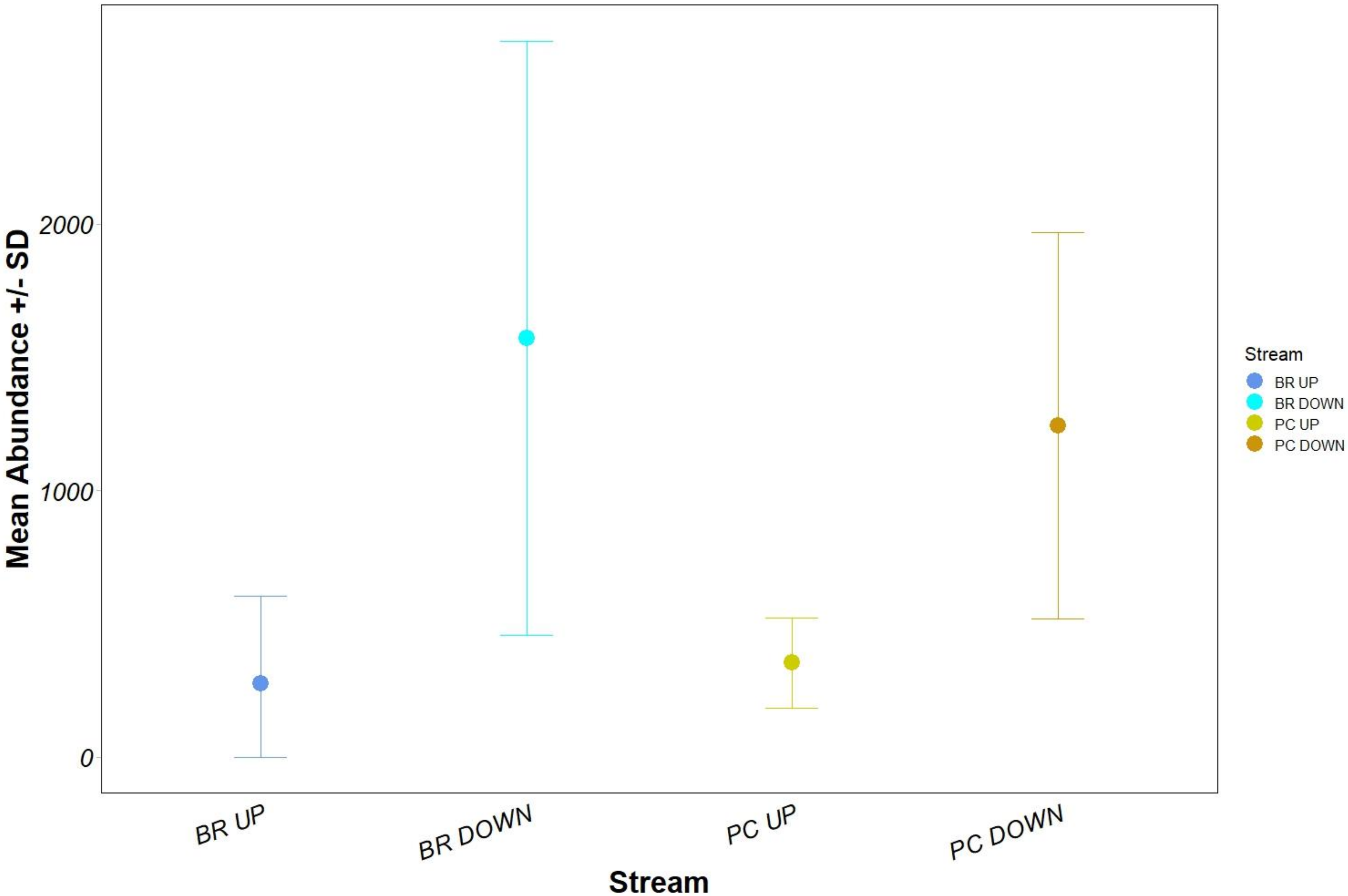


Fig. 3.8. The mean abundance of macroinvertebrate taxa in Surber samples (+/- 1 standard deviation). Abundance was calculated from the raw abundance of all taxa in each sample from each stream at each location. All individual samples for each site in the defined treatment and location combinations were averaged (n=3, aside from BR DOWN, for which n=2).

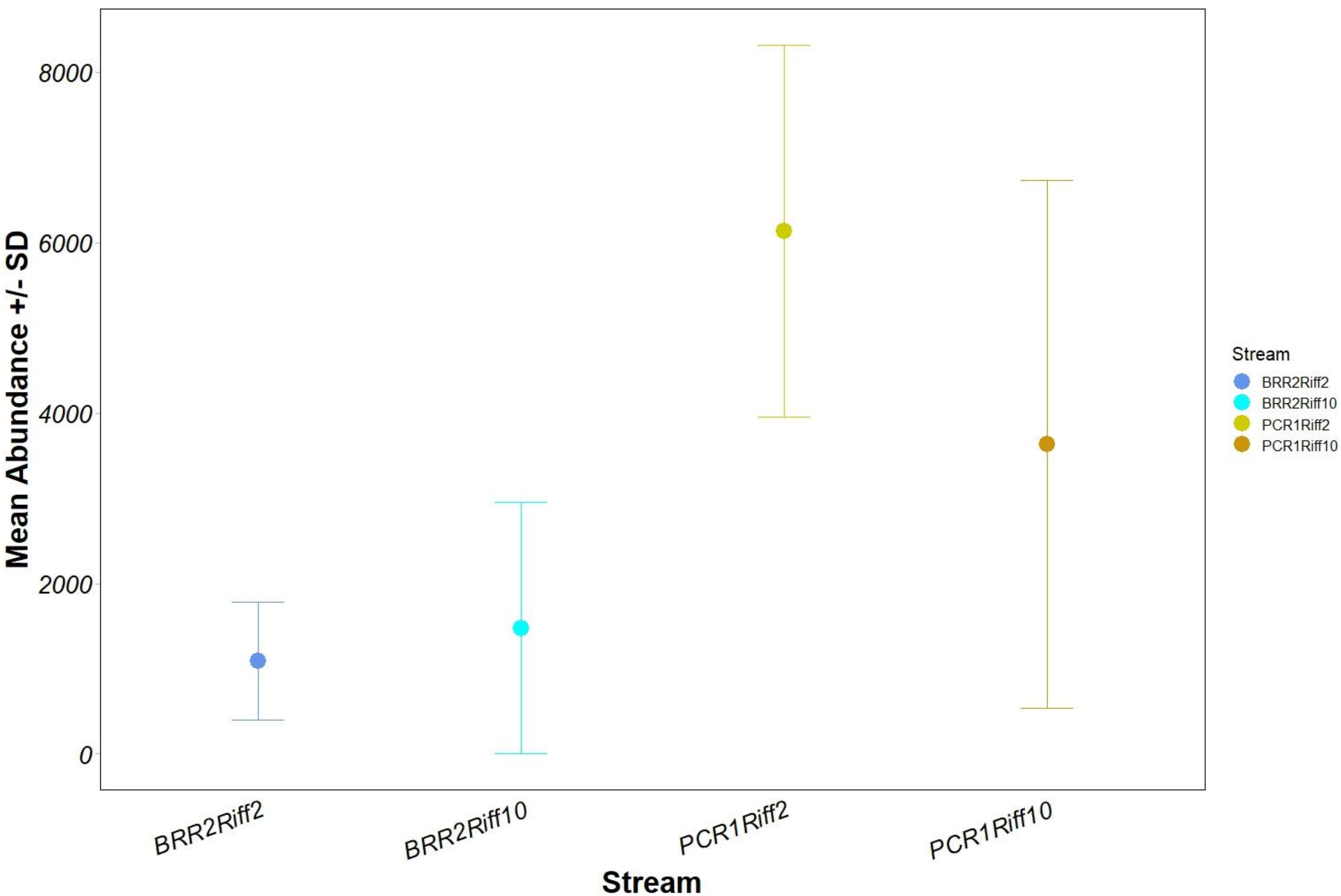


Fig. 3.9. The mean abundance of macroinvertebrate taxa in rock basket samples (+/- 1 standard deviation). Abundance was calculated from the raw abundance of all taxa in each sample from the representative treatment and control stream at each location. All individual samples for each site in the defined treatment and location combinations were averaged (n=3).

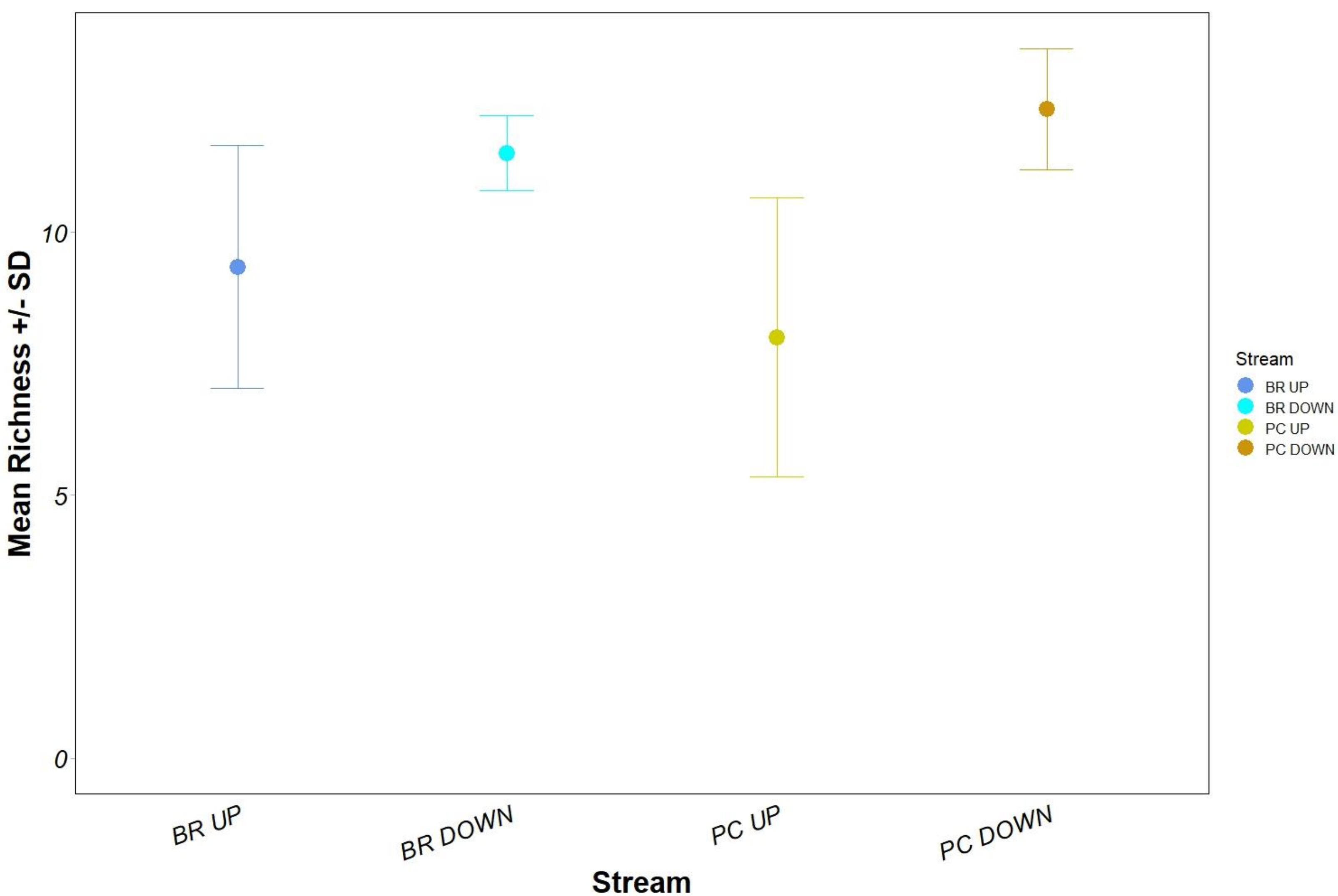


Fig. 3.10. The mean family richness for Surber samples of macroinvertebrate taxa (+/- 1 standard deviation). Richness was calculated by counting unique families in a sample from each stream at each location. All individual samples for each site in the defined treatment and location combinations were averaged (n=3, aside from BR DOWN, for which n=2).

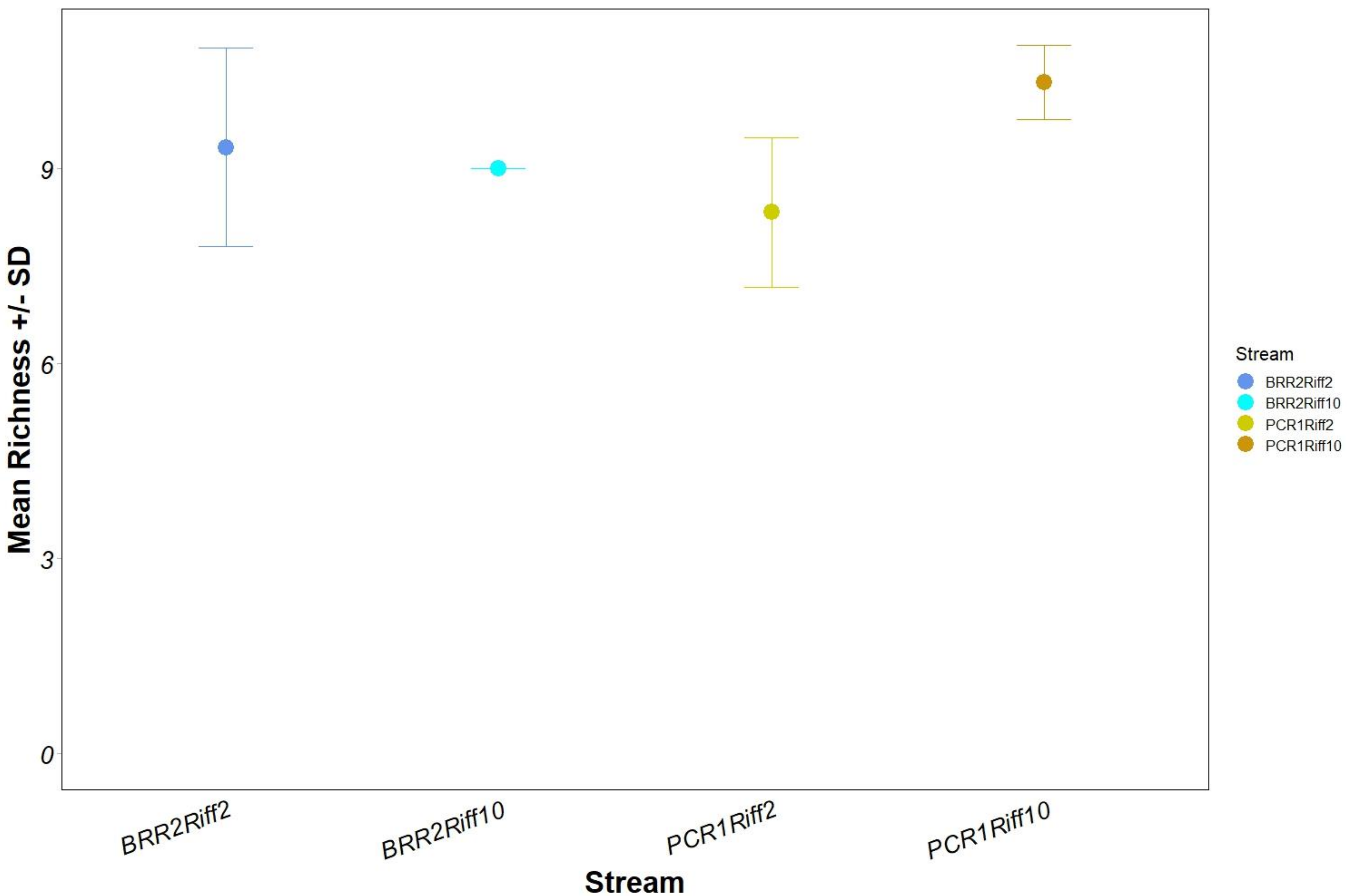


Fig. 3.11. The mean family richness for rock basket samples of macroinvertebrate taxa (+/- 1 standard deviation). Richness was calculated by counting unique families in a sample from the representative treatment and control stream at each location. All individual samples for each site in the defined treatment and location combinations were averaged (n=3).

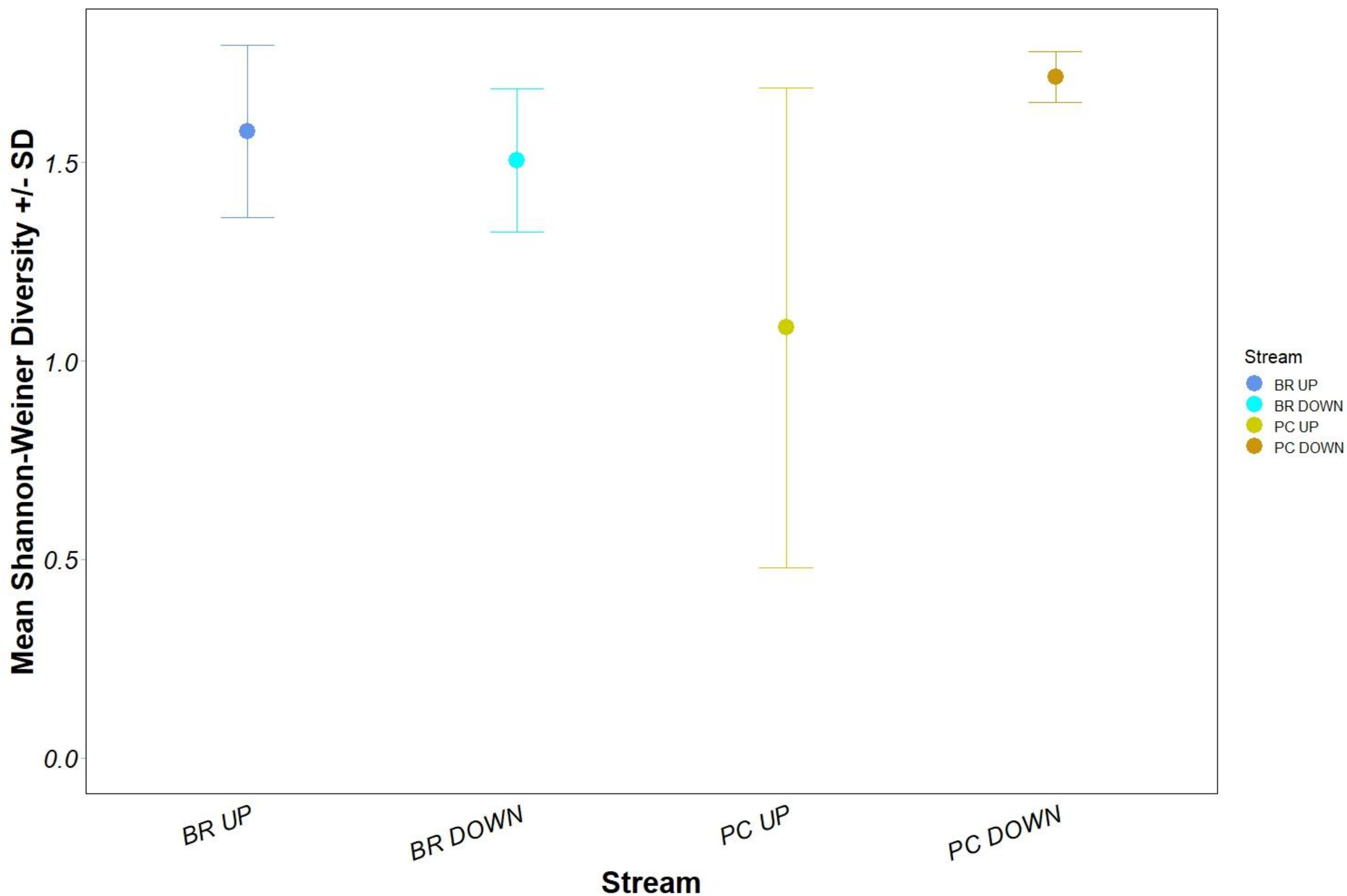


Fig. 3.12. The mean diversity index of Surber sample macroinvertebrate taxa (+/- 1 standard deviation). Diversity was calculated using the Shannon-Weiner diversity index equation for each stream at each location. All individual samples for each site in the defined treatment and location combinations were averaged (n=3, aside from BR DOWN, for which n=2).

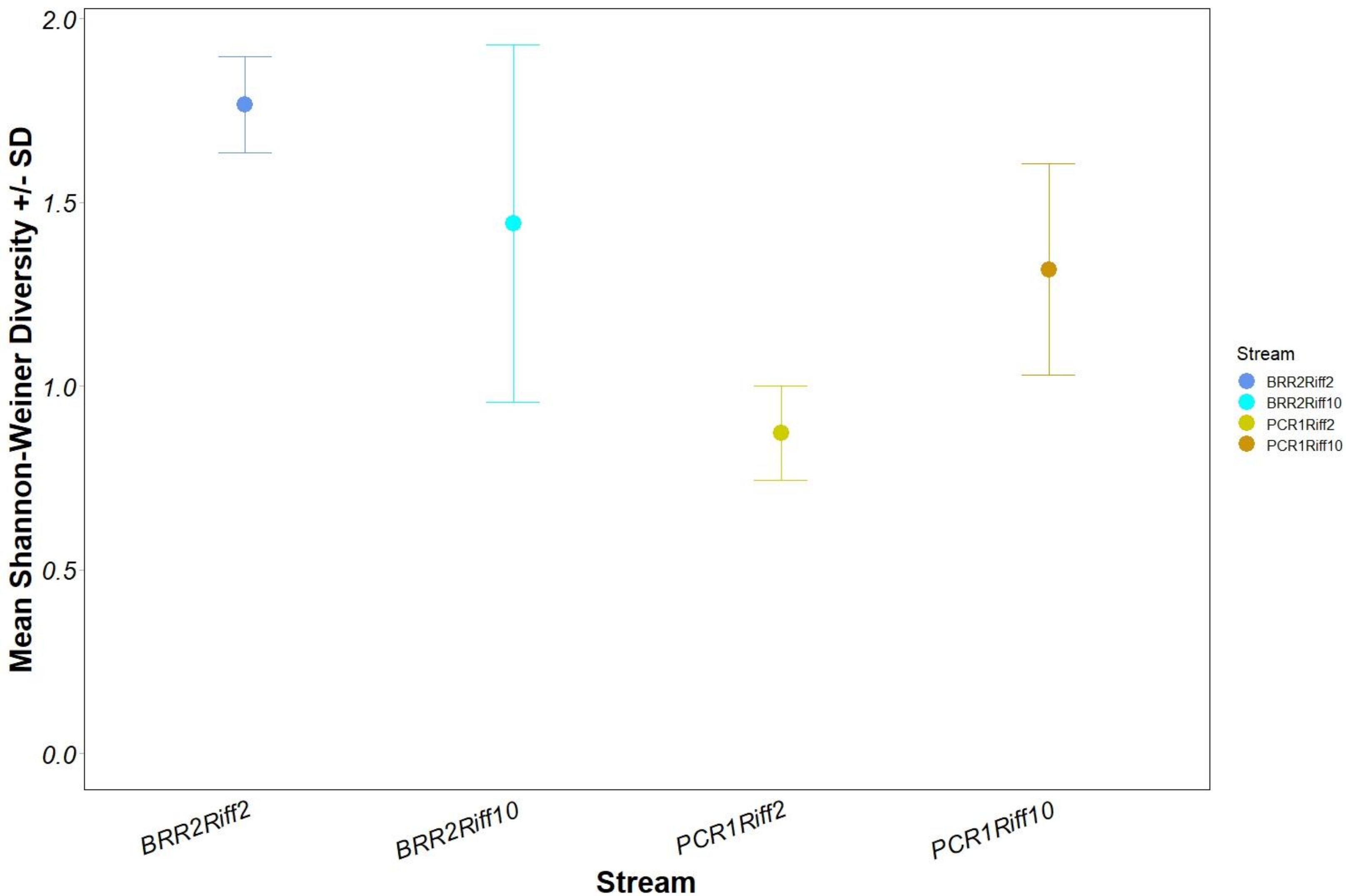


Fig. 3.13. The mean diversity index of rock basket macroinvertebrate taxa (+/- 1 standard deviation). Diversity was calculated using the Shannon-Weiner diversity index equation for each sample from the representative treatment and control stream at each location. All individual samples for each site in the defined treatment and location combinations were averaged (n=3).

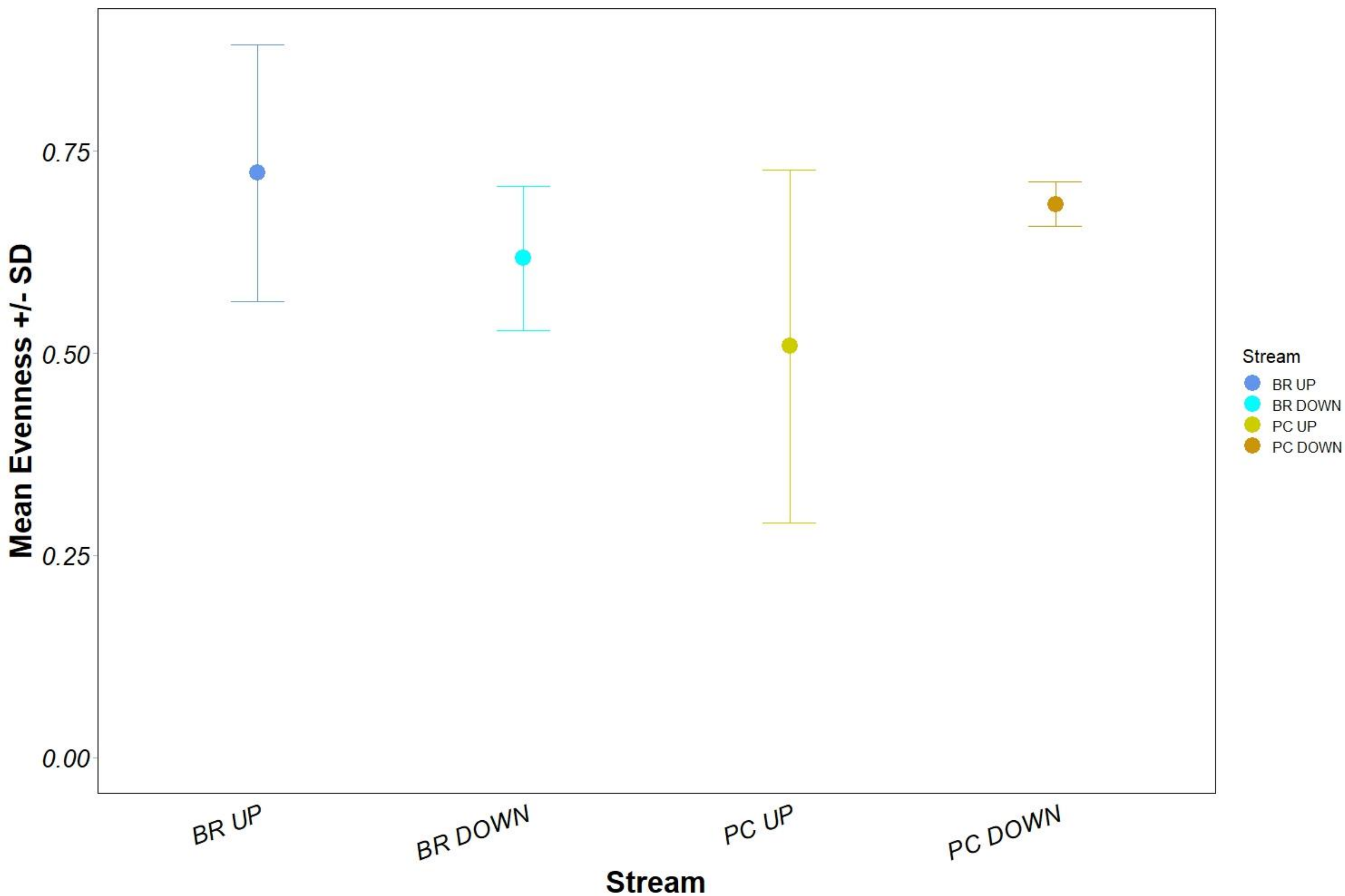


Fig. 3.14. The mean evenness index of Surber sample macroinvertebrate taxa (+/- 1 standard deviation). Evenness was calculated using the Pielou's evenness index equation for each stream at each location. All individual samples for each site in the defined treatment and location combinations were averaged (n=3, aside from BR DOWN, for which n=2).

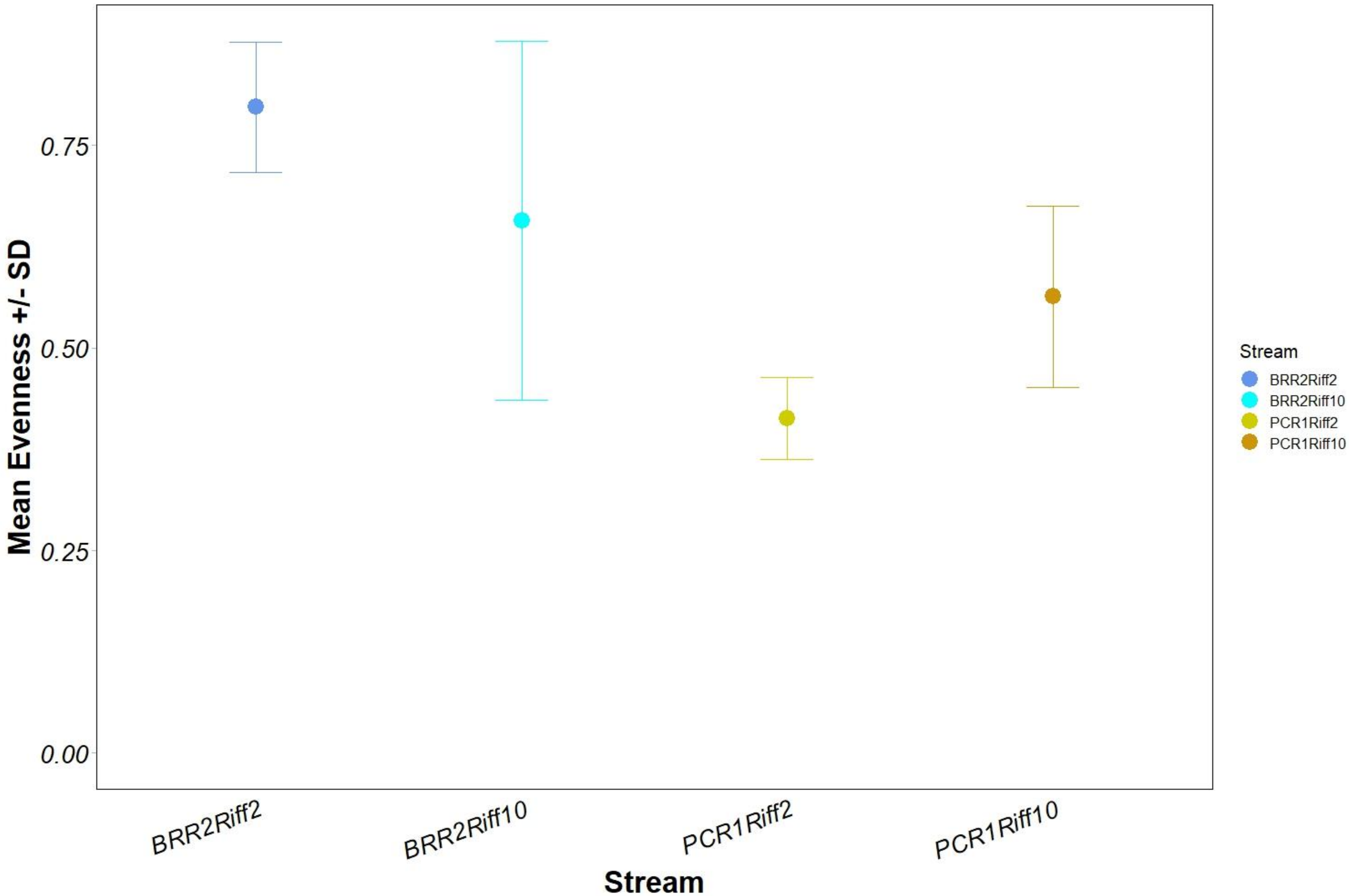


Fig. 3.15. The mean evenness index of rock basket sample macroinvertebrate taxa (+/- 1 standard deviation). Evenness was calculated using the Pielou's evenness index equation for each sample from the representative treatment and control stream at each location. All individual samples for each site in the defined treatment and location combinations were averaged (n=3).

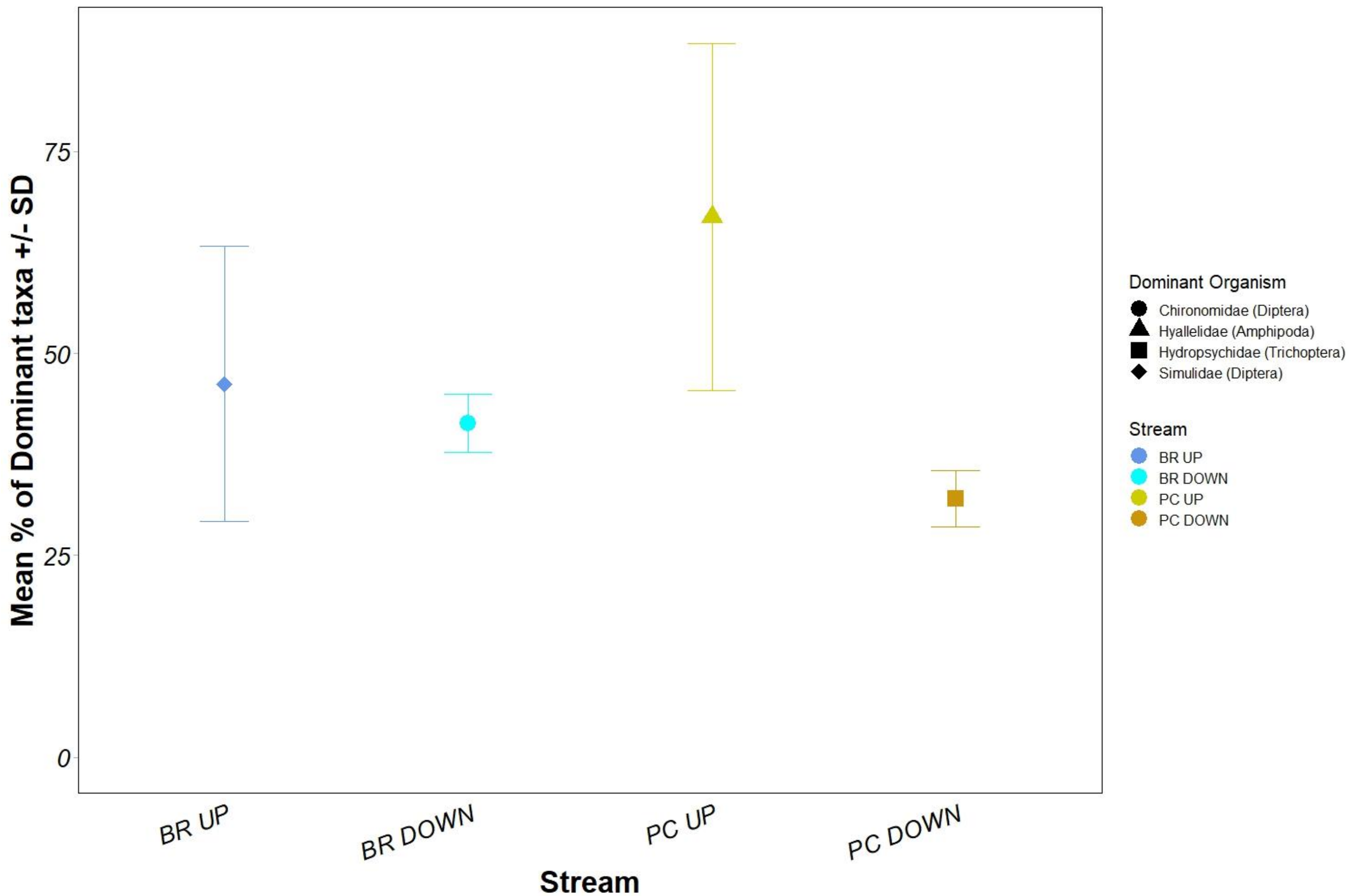


Fig. 3.16. The mean percentage of the dominant taxa in Surber sample macroinvertebrate assemblages (% of total community +/- 1 standard deviation). % dominance was calculated from the maximum abundance of a macroinvertebrate family divided by the total number of taxa identified in each stream at each location. All individual samples for each site in the defined treatment and location combinations were averaged (n=3, aside from BR DOWN, for which n=2). The most dominant family was calculated from the highest abundance of a macroinvertebrate family across a whole stream location and is indicated alongside the Order.

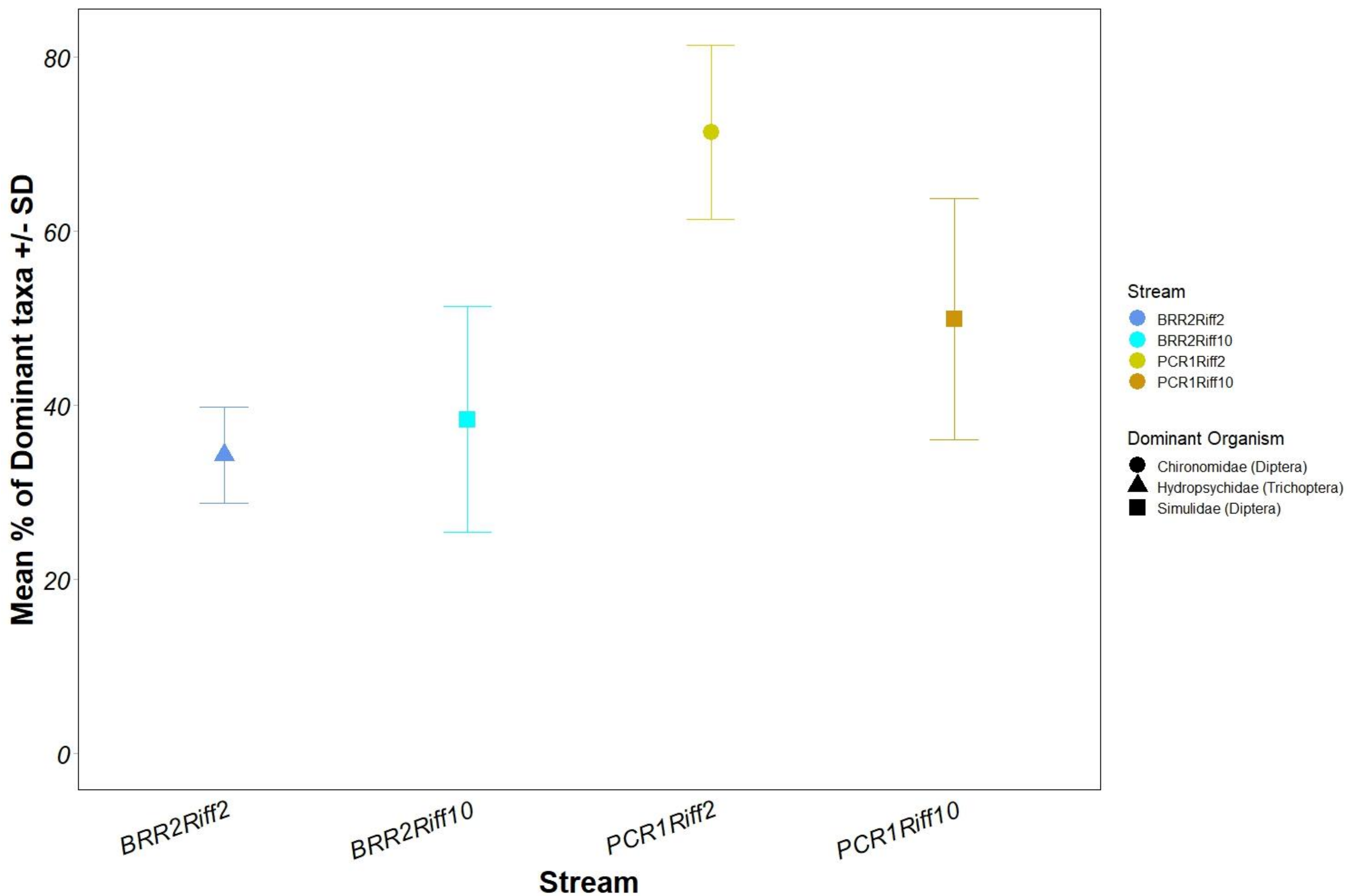
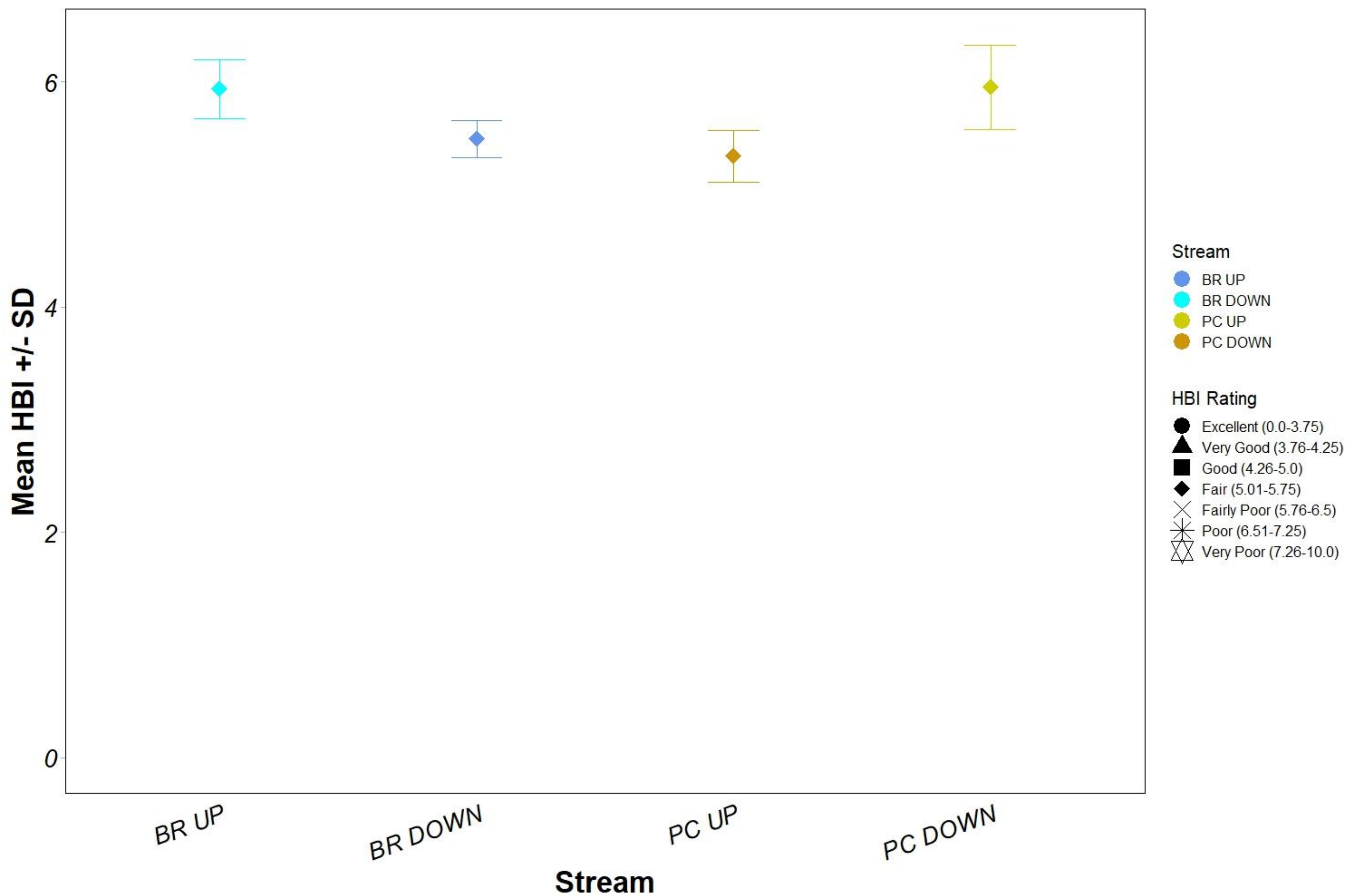


Fig. 3.17. The mean percentage of the dominant taxa in rock basket macroinvertebrate assemblages (% of total community +/- 1 standard deviation). % dominance was calculated from the maximum abundance of a macroinvertebrate family divided by the total number of taxa identified each sample from the representative treatment and control stream at each location. All individual samples for each site in the defined treatment and location combinations were averaged (n=3). The most dominant family was calculated from the highest abundance of a macroinvertebrate family across a whole stream location and is indicated alongside the Order.



3.18. The mean HBI rating of Surber sample macroinvertebrate taxa (+/- 1 standard deviation). The Hilsenhoff Biotic Index (HBI) was calculated using the HBI equation for each stream at each location. All individual samples for each site in the defined treatment and location combinations were averaged (n=3, aside from BR DOWN, for which n=2). The numeric score (between 0 and 10) is also associated with a categorical rating: Excellent (0.0-3.75, Very Good (3.76-4.25), Good (4.26-5.0), Fair (5.01-5.75), Fairly Poor (5.76-6.5), Poor (6.51-7.25), Very Poor (7.26-10.0).

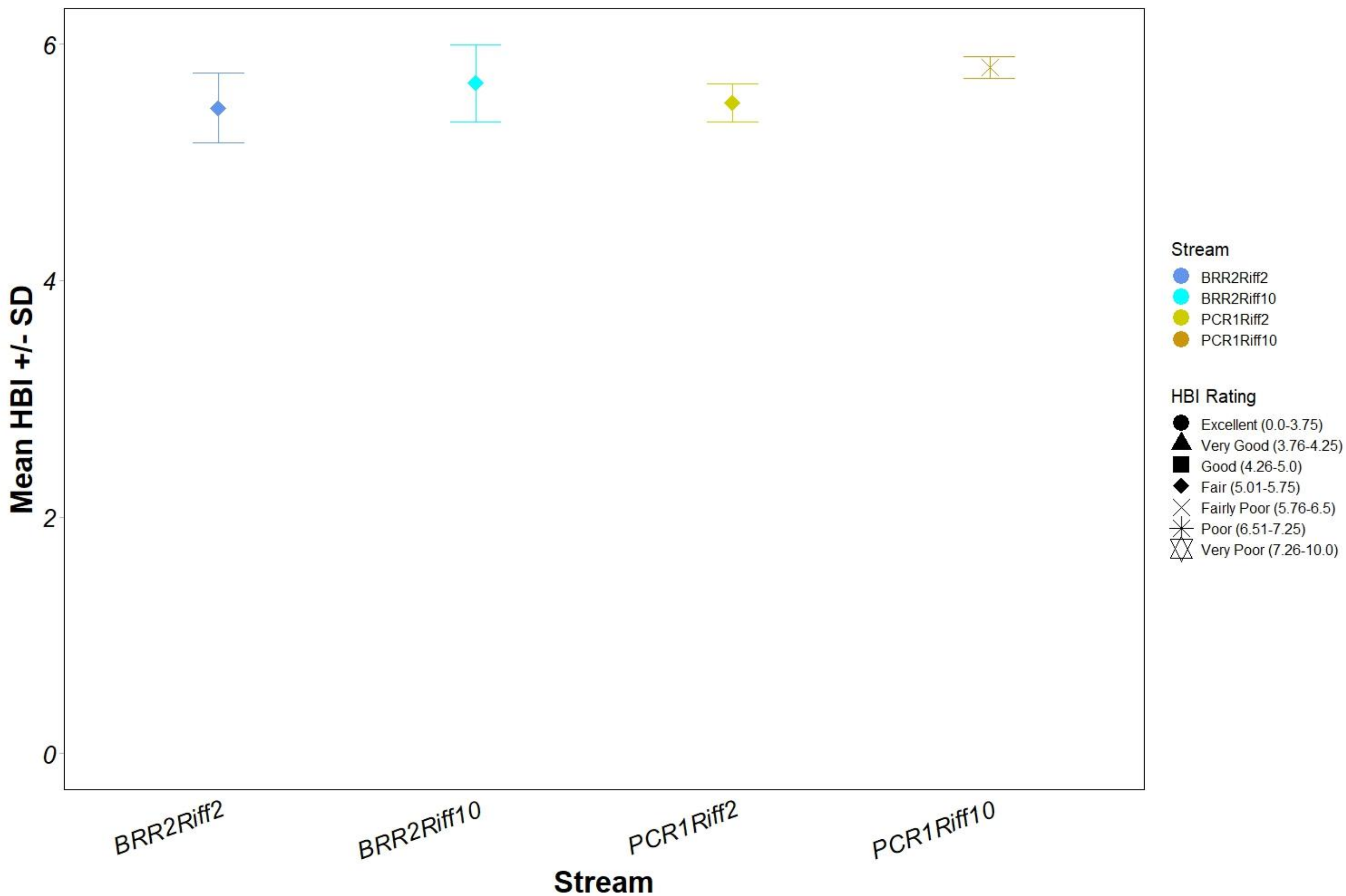


Fig. 3.19. The mean HBI rating of rock basket macroinvertebrate taxa (+/- 1 standard deviation). The Hilsenhoff Biotic Index (HBI) was calculated using the HBI equation for each sample from the representative treatment and control stream at each location. All individual samples for each site in the defined treatment and location combinations were averaged (n=3). The numeric score (between 0 and 10) is also associated with a categorical rating: Excellent (0.0-3.75, Very Good (3.76-4.25), Good (4.26-5.0), Fair (5.01-5.75), Fairly Poor (5.76-6.5), Poor (6.51-7.25), Very Poor (7.26-10.0).

3.3.2 PERMANOVA & SIMPER

Macroinvertebrate community assemblages were found to be significantly different between the Bow River (control) and Pine Creek effluent streams (Table 3.2); however, pairwise comparisons between sampling sites revealed no significant sampling site-specific differences (Table 3.3). Similarly, macroinvertebrate community assemblages in rock baskets differed significantly between the Bow River (control) and Pine Creek effluent streams (Table 3.4), but again pairwise comparisons did not find any pairwise differences between sampling locations (Table 3.5).

Table 3.2. ACWA stream 2020 benthic macroinvertebrate Surber sample (log_{10} transformed) PERMANOVA results. * Indicates significant $p<0.05$.

F-value	df	p-value
2.3731	3	0.014*

Table 3.3. ACWA stream 2020 benthic macroinvertebrate Surber sample pairwise (log_{10} transformed) PERMANOVA results. * Indicates significant $p<0.05$.

Pairwise Comparison	df	p-value
BR UP vs. BR DOWN	1	1.0
BR UP vs. PC UP	1	1.0
BR UP vs. PC DOWN	1	0.6
BR DOWN vs. PC UP	1	1.0
BR DOWN vs. PC DOWN	1	1.0
PC UP vs. PC DOWN	1	0.6

Table 3.4. ACWA stream 2020 benthic macroinvertebrate rock basket sample (log_{10} transformed) PERMANOVA results. * Indicates significant p<0.05.

F-value	**df**	**p-value**
2.6467	3	0.001*

Table 3.5. ACWA stream 2020 benthic macroinvertebrate rock basket sample pairwise (log_{10} transformed) PERMANOVA results. * Indicates significant p<0.05.

Pairwise Comparison	**df**	**p-value**
BRR2Riff2 vs. BRR2Riff10	1	1.0
BRR2Riff2 vs. PCR1Riff2	1	0.6
BRR2Riff2 vs. PCR1Riff10	1	0.6
BRR2Riff10 vs. PCR1Riff2	1	0.6
BRR2Riff10 vs. PCR1Riff10	1	0.6
PCR1Riff2 vs. PCR1Riff10	1	0.6

SIMPER analysis performed on the benthic macroinvertebrate communities from the Surber samples revealed that differences between the Bow River (control) and Pine Creek treatment streams were primarily influenced by only five taxonomic Families: two Diptera families (Simuliidae and Chironomidae), one Trichoptera family (Hydropsychidae), one Coleoptera family (Elmidae), and one Amphipoda family (Hyallelidae) (Table 3.6). Related NMDS ordinations revealed similar separation between stream sites (Fig.A.4). In particular, the family Simuliidae from the order Diptera influenced >5% differences between all but one pair of sites (BR UP and PC UP) and was often the most influential taxa between sites. On average, Simuliidae was generally highly abundant for all sites sampled but tended to spike in single replicates. The family Elmidae from the order Coleoptera was also responsible for differences between all pairs of sites but one (BR UP and PC UP). No taxa identified by the SIMPER analysis was totally absent from any single stream type or location.

A SIMPER analysis for the rock basket samples in the ACWA streams showed that differences between stream treatment and location were influenced by only three families: two Diptera families (Simuliidae and Chironomidae) and one Trichoptera family (Hydropsychidae) (Table 3.7). Related NMDS ordinations generally revealed separation between streams and sampling locations, although the downstream Bow River site was less distinct (Fig.A.5). Most differences were influence by the Diptera families, which were somewhat more abundant in the treatment stream assemblages than the control streams (at upstream and downstream locations). Again, no taxa identified by the SIMPER analysis was totally absent from any single stream type or location.

Table 3.6. SIMPER results for Surber samples taken in each triplicate control and treatment stream for upstream and downstream locations at ACWA in October 2020. The most influential taxa are listed first and % listed is cumulative explanation of difference between sample sites. Only taxa explaining >5% of region difference are included and taxa are colour coded by taxonomic group: red (Ephemeroptera), green (Plecoptera), blue (Trichoptera), yellow (Diptera), pink (Coleoptera), or grey (Other taxonomic group). n=3 for all sites aside from BR DOWN, for which n=2.

Sites	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**
BR UP vs. BR DOWN	Simuliidae (36.7%)	Hydropsychidae (65.8%)	Elmidae (79.8%)	
BR UP vs. PC UP	Hydropsychidae (52.6%)	Chironomidae (78.4%)		
BR UP vs. PC DOWN	Hyallelidae (27.8%)	Elmidae (49.1%)	Hydropsychidae (67.4%)	Simuliidae (80.6%)
BR DOWN vs. PC UP	Simuliidae (46.5%)	Hydropsychidae (65.0%)	Elmidae (82.9%)	
BR DOWN vs. PC DOWN	Simuliidae (32.8%)	Hyallelidae (53.4%)	Elmidae (72.6%)	
PC UP vs. PC DOWN	Hyallelidae (32.3%)	Elmidae (56.3%)	Simuliidae (71.5%)	

Table 3.7. SIMPER results for triplicate rock basket samples taken in one representative control and treatment stream for upstream and downstream locations at ACWA in October 2020. The most influential taxa are listed first and % listed is cumulative explanation of difference between sample sites. Only taxa explaining >5% of region difference are included and taxa are colour coded by taxonomic group: red (Ephemeroptera), green (Plecoptera), blue (Trichoptera), yellow (Diptera), pink (Coleoptera), or grey (Other taxonomic group). n=3 for all sites.

Sites	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**	**Taxa (% cumulative influence)**
BRR2Riff2 vs. BRR2Riff10	Simuliidae (30.0%)	Hydropsychidae (55.5%)	Chironomidae (75.1%)	
BRR2Riff2 vs. PCR1Riff2	Chironomidae (72.7%)			
BRR2Riff2 vs. PCR1Riff10	Simuliidae (38.8%)	Chironomidae (67.7%)	Hydropsychidae (84.7%)	
BRR2Riff10 vs. PCR1Riff2	Chironomidae (75.1%)			
BRR2Riff10 vs. PCR1Riff10	Simuliidae (39.9%)	Chironomidae 71.3%)		
PCR1Riff2 vs. PCR1Riff10	Chironomidae (63.9%)	Simuliidae (87.8%)		

3.4 Discussion

3.4.1 Patterns in benthic macroinvertebrate assemblages and community indices

Patterns of metrics commonly used to assess benthic macroinvertebrate assemblages were inconsistently indicative of effects related to MWWE between different sampling sites. Richness and diversity metrics showed little variation and/or were not patterned to a particular kind of site. Meanwhile, evenness was higher in the control streams for the rock baskets but showed no clear pattern in the Surber samples. However, there was often clear differences between control and Pine Creek effluent streams, although metric effects also tended to align with an upstream vs. downstream effect in the Surber samples versus a stronger control vs. treatment effect for the rock baskets. The overall abundance of taxa followed this pattern, with the higher abundances being observed in downstream sites regardless of treatment (Surbers) and in treatment streams regardless of location (rock baskets). The dominant % of the dominant organisms shifted less clearly in the Suber samples, although on average the dominant organisms were more dominant in treatment stream sites. The rock basket samples were more heavily dominated in the treatment streams.

These results align only partially with other published mesocosm experiments, as there was only limited evidence that cumulative exposure to MWWE drives benthic macroinvertebrate assemblages. Previously, a 5% addition of MWWE in a stream mesocosm study demonstrated correlation with changes in benthic macroinvertebrate community assemblages (Grantham et al. 2012). For *in-situ* systems, benthic macroinvertebrate assemblages downstream of point source MWWE sources tend to have high abundance, lower diversity and richness, and an increase in pollution tolerant taxa (Williams-Subiza et al. 2022, Enns et al. 2023). However, in this case, only some metrics between the two methods demonstrated that these qualities were changing in relation to MWWE exposure, although that a difference existed between the control stream and treatment streams was more consistent.

Benthic macroinvertebrate assemblages observed in the ACWA experimental streams were dominated by Diptera and Trichoptera families, which was especially evident in the dominance of Diptera in artificial rock basket samples from the Pine Creek treatment stream, which aligns with observed nutrient related impacts on benthic macroinvertebrate assemblages (Holeton et al. 2011). However, the percentage of Diptera in Surber samples from in the Pine Creek treatment streams was lower than the control streams, where they were replaced by Coleoptera families, the Trichoptera family Hydropsychidae, and the Amphipoda family Hyallelidae. The consistently strong presence of the Hydropsychidae family from the Order Trichoptera could also be notable due to their role in affecting nutrient enrichment. Hydropsychidae caddisflies were observed, in a stream-based nutrient limitation experiment, to significantly decrease the concentration of chl-α through shaded microhabitats created by their refuge nets (Pan and Lowe 1995). This may perhaps account to some degree of the lack of observed differences between the two treatment streams.

One of the most consistent patterns in the Surber samples was an increase of Coleoptera families in the downstream riffle sites (of both control and treatment streams). There has been sustained argument in the literature for the addition of Coleoptera into EPT-like assessments (Céréghino et al. 2003, Minaya et al. 2013, Masese and Raburu 2017). One study additionally argues that the inclusion of some particularly tolerant EPT, including Hydropsychidae, overinflates EPT assessments of ecosystem health (Masese and Raburu 2017). Removing Hydropsychidae from the % EPT would reduce this metric and adding Coleoptera to % EPT would strengthen the pattern of improved stream health in the downstream sites for the Surber samples. The increase in the relative abundance of Coleoptera in the downstream sites could suggest that there are changes or a gradient in environmental condition further downstream of the immediate input of MWWE in the experimental streams.

However, the HBI for both methods in all streams was similar, ranging from Fair to Fairly Poor and did not appear to be related to any stream treatment or location. HBI, assigning tolerance ratings to all families, should account for the presence of pollution tolerant taxa, such as Hydropsychidae Trichoptera. However, these ratings don't strongly suggest that upstream and downstream differences in the total assemblage indicates better ecological health in the downstream locations, which was suggested somewhat by the increase in Coleoptera for downstream Surber samples. Lastly, although HBI ratings have not been previously assigned to the ACWA streams, the ratings for the streams are somewhat lower than Bow River ratings that have been previously observed in the mainstem at nearby points (Saffran et al. 2009).

In the ACWA streams there are some notable considerations that offer potential explanations for the observed results. Firstly, benthic macroinvertebrate communities in these streams have been colonized naturally through organisms that enter the stream through the nearby Bow River intake or from organisms emerging from nearby waterbodies, including the Bow River. The communities in the streams may have never resembled a Bow River not exposed to MWWE if they were primarily colonized by organisms downstream of significant WWTP outflows in the CoC (Bonnybrook and Fish Creek) and/or there are physical barriers to larval colonization at the intake site. Additionally, emerging insects have complex dispersal mechanisms, meaning that colonization from adult insects in nearby waterbodies is likely inconsistent. Specifically, previous research has suggested that Trichoptera and the Diptera family Chironomidae travel further than Ephemeroptera when adults emerge to lay eggs, with

Chironomidae taxa from a stream being observed up to 17 km away compared to Ephemeroptera taxa at 160 m (Muehlbauer et al. 2014).

Overall, only some community indices provided few clear qualitative differences between control and treatment streams, which from an EEM perspective, does not suggest that there was strong immediate concern. The control and treatment streams did often display different overall profiles from another, although these differences were not always consistent between sampling methods. However, the observed effects commonly related to nutrient effects, such as increased abundance and increased pollution tolerant taxa (such as Diptera) were observed more strongly in the artificial substrate rock baskets. Overall, though, the streams were all typically dominated by pollution tolerant organisms, such as Diptera families, and Hydropsychidae, a particularly tolerant Trichoptera family. There were some notable differences in the upstream and downstream assemblages overall, but these did not appear to translate into indicators of ecosystems health, such as different HBI ratings. While the current condition of the benthic macroinvertebrate assemblages does not suggest that the streams are as healthy as streams unexposed to MWWE, there are some potential confounding reasons related to benthic macroinvertebrate colonization that this may be the case. Generally, these results suggest that the Pine Creek MWWE contributes some degree of cumulative nutrient enrichment, although this may be more apparent in colonization patterns and young communities than in established communities. The MWWE that is added to the ACWA experimental streams, which is added to control streams with previous upstream MWWE exposures, is very high quality and may be a weaker driver than that observed in older, less highly treated effluent studies.

3.4.2 Patterns of difference in benthic macroinvertebrate assemblages

The difference in some trends between method approaches for groups tested in this community assessment provide mixed support for cumulative effects from Calgary MWWE in the benthic macroinvertebrate community composition for the ACWA experimental stream. Overall, the results from the PERMANOVA showed that when grouped into methods and then treatments, control stream replicates were significantly different from the streams with the 5% MWWE exposure, until pairwise comparisons were performed. Additionally, as sites were also compared as upstream or downstream locations, this analysis additionally demonstrated that the upstream and downstream locations were also not significantly different in pairs, regardless of whether they were treatment or control streams. Overall, this suggests that while differences in the stream replicates is likely related to MWWE as a driver of benthic macroinvertebrate assemblages, the differences observed may not be based on consistently, strong overall differences in the assemblages.

The benthic macroinvertebrate communities in the experimental streams at ACWA have not previously had their communities characterized, making this the first study of this type at the facility. Therefore, these assemblage results cannot be compared to previous benthic macroinvertebrate assemblages or results. However, these results differ from expectations of the role of MWWE in shaping benthic macroinvertebrate communities. Typically, studies have shown that MWWE exposures are likely to significantly change the types of organisms present in communities, which may be due to direct changes such as reductions to the dissolved oxygen concentrations or sedimentation processes that reduce habitat quality and quantity (Holeton et al. 2011, Dos Reis Oliveira et al. 2019). These changes may also come about through indirect processes, including shifts to food sources stemming from changes in nutrient and light availability to producers (Piggott et al. 2012). In previous assessments of the ACWA experimental streams, some of these parameters have appeared to be extremely similar between stream treatments, such as dissolved oxygen, which is a key physicochemical measurement often affected by the presence of MWWE (Jackson 2020). This supports the conclusion that while the environmental conditions of the streams are well controlled, additional tertiarily treated Pine Creek MWWE added to streams already exposed to MWWE is likely to be driving the observed differences between streams through direct and indirect impacts that would affect benthic macroinvertebrate community composition.

Other mesocosm experiments outside of ACWA that utilize MWWE exposures have observed changes to benthic macroinvertebrate communities at a 5% MWWE exposure, which is the exposure observed in this experiment. However, not all MWWE mixtures are the same and the constituents and nutrient concentrations may vary significantly depending on a number of factors but particularly the type of treatment used. The Pine Creek MWWE employs tertiary treatment with recent technological advances to produce an extremely high-quality effluent. Particularly, Pine Creek makes use of an effective biological nutrient removal process. Similarly, ESOC removal, although not a formalized process in WWTP treatment, was observed to be the highest at Pine Creek for Calgary's WWTPs (Chen et al. 2015). These effluent qualities may also explain why the presence of Pine Creek effluent in the experimental streams is not as notable as in other mesocosm studies, and similarly, why the impacts from other, larger WWTPs upstream (such as Bonnybrook) have wrought more notable changes in the overall streams than the addition of Pine Creek MWWE would add.

As established, the water used in the control streams is taken from an intake just upstream of facility, meaning that the water is exposed to two WWTP outflows in the nearby upstream. As previous research in the Bow River has demonstrated, nutrient enrichment effects typically last at least 52 kms (in terms of autotroph biomass as a biological endpoint) so the ACWA experimental stream intake would still be within the range of the influence of both Bonnybrook and Fish Creek WWTPs (Hogberg 2004). Additionally, when assessing the distances at which emerging insects can be identified from their larval stream, many insects are likely to be less than a km away. A notable exception is the Diptera family Chironomidae, who are often associated with nutrient enrichment in aquatic systems. The above conditions on colonization processes for the ACWA streams are likely to have had long term effects on the overall types of communities formed and the differences subsequently observed. As supported by the similarity of physicochemical measurements of DO, pH, and nutrients between control and treatment streams, alongside the high quality of the added effluent, each separate mesocosm replicate is too similar to identify strong differences in benthic macroinvertebrate community composition.

Additionally, there did not appear to be strong evidence for a gradient effect in the streams in terms of the overall benthic macroinvertebrate assemblages. While the ACWA streams are not long, comparatively to a natural system, they all contain visible algal and plant (macrophyte) growth engaging in nutrient cycling processes (Newbold et al. 1981). One test of nutrient chemistry performed over the top half of the experimental streams showed that there were significant increases downstream in the concentration of dissolved inorganic nitrogen (DIN), which is inclusive of families of compounds also found in released in MWWE, such as NO_2^- and NO_3^-. Total phosphorus concentration also largely increased from upstream to downstream. Additionally, surveys of macrophyte biomass in the sequential pools of the ACWA streams showed peak biomass in pools 3 and 4, with the lowest biomass observed in pools 9 and 10 (Jackson 2020). As previous work on the ACWA experimental streams has observed consistent shifts in nutrient concentrations between upstream (riffle 1) and midstream (riffle 6) in all streams, it was possible that there would be an observable pattern of uptake that is relevant to the physicochemical conditions shaping the benthic habitat and subsequently, the community assemblage.

However, the previously observed nutrient shifts demonstrated a shift towards more readily biologically available forms of nitrogen (DIN) but a reduction in total nitrogen, which likely is related to ammonification and nitrification processes (Jackson 2020). However, it is possible that these effects are unrelated to the MWWE inputs, not only because of the lack of community distinction between treatment, but also because MWWE tends to add nitrogen that is already undergone one or both of these processes. If the primary source of DIN in the streams was from MWWE, the concentrations would likely shift in the opposite direction. Crucially, one stream mesocosm study investigated nutrient spiralling through reducing nutrient input to a stream, which decreased ambient nutrient levels while levels in harvested producers stayed relatively even. This means that the receiving system has some ability to regulate, which may account for the lack of difference. However, this relationship does suggest that the mesocosm system does provide an opportunity to observe the effects of shifts in nutrients, but the contribution of MWWE to nutrient enrichment in the streams would have to be more notable than the natural processes in the stream to influence these patterns, which may not have been the case.

Previous experiments in the ACWA streams also identified that some ESOCs, in this case a common water additive, fluorine (F), were reduced by exposure to the stream gradient. Alternatively, titanium (Ti), which is used in personal care products and has been demonstrated to affect periphyton growth in mesocosms, was not reduced by exposure to the stream gradient

(Wright et al. 2018, Jackson 2020). While fluorine is unlikely to be of biological relevance, as toxicological studies have demonstrated toxic effects to occur for Trichoptera larvae at concentrations several magnitudes higher than observed concentrations, the suggestion that some ESOCS may be removed through the stream gradient is promising in identifying. Particularly if certain chemicals or groups are shown to be consistently removed while others are not, there is interesting potential to trace ESOC effects through gradient systems. However, with the lack of significant difference between communities, it is unlikely that ESOCs are currently driving strong differences in community composition and concentrations would need to be increased (via the ability to increase MWWE concentration in the streams) to identify what role, if any, they are playing. Additionally, without specific ESOC chemistry performed on the upstream and downstream sites, it is not possible to attribute the patterns in community composition at upstream versus downstream sites to any specific ESOC constituent of MWWE.

3.4.3 Patterns in benthic macroinvertebrate sampling methods

The Surber samplers were intended to capture a "snapshot" type assessment of the benthic macroinvertebrate assemblages at that moment in time, affected by physicochemical and biological drivers up to that point in time. Alternatively, the rock basket samplers, which were deployed over a six-week period and retrieved during the same time period as the Surber samples were taken, were intended to provide a more integrated assessment of physicochemical conditions over the six-week period (Yeardley et al. 2020). A combination of these samplers provides information on both the composition of long-term communities (Surbers) and also how colonization of new substrate from the communities occurs (rock baskets) in the same conditions aside from the additional exposure of MWWE in treatment streams. Additionally, assessment of multiple sampling techniques can provide insight into how different sampling methods can provide different balances of field and processing time in relation to the results provided. Generally, the trends observed from the results of both Surber samples and rock basket samples of benthic macroinvertebrate assemblages in the ACWA experimental streams over this time period were similar, but a handful of key patterns emerged that are relevant to understanding the role of MWWE and the gradients within the streams. However, both methods did not ultimately demonstrate significant differences within the analysis for any type of site.

Traditionally, comparisons of kick methods (which tend to be more similar to the Surber samples), versus artificial substrate methods, have found that artificial substrate samplers show lower abundance, richness, and diversity, but can have higher % EPT. The typically lower abundance is likely related to process of colonizing the artificial substrate (Letovsky et al. 2012, Funnell et al. 2020). In this experiment, diversity was slightly lower in the rock baskets than the Surber samples, but richness was very consistent across all samples. However, the abundance of the rock basket samplers in the treatment stream was substantially higher than the abundance in any of the Surber samples, which differs from previous studies. Furthermore, when identifying the dominant species observed in the rock baskets, the discrepancy in abundance appeared to be particularly related to the prevalence (>50% of assemblage) of a dominating Diptera family (either Simuliidae or Chironomidae) in the treatment streams. Overall, the % Diptera in treatment stream rock baskets was much higher than in the control rock baskets or any Surber samples, typically reaching ~75% of the total assemblage.

Patterns of benthic macroinvertebrate colonization can be utilized to partially explain this result. While a six-week deployment period has been shown to be a reliable time to reach a representative level of colonization, this may also depend on the particular system samplers are deployed in (Waters et al. 2005). In similar previous experiments on colonization of benthic macroinvertebrates into artificial substrate samplers have shown that detritus feeders are often the first taxa observed, for example, Batidae mayflies and Hydropsychidae caddisflies (Hall 1982, McArthur and Barnes 1985). The Diptera families noted here, Simuliidae and Chironomidae, are also detritus feeders, which partially explains their presence as early colonizers. However, this does not explain the increased scale of the abundance for these rock baskets. It additionally does not address the effect of the treatment stream in demonstrating this particular shift has not necessarily been seen in previous comparisons of these method types, although comparisons that include the presence of added MWWE effluent have not previously been performed.

MWWE and other anthropogenic contaminants are associated with an increase in pollution tolerant taxa, such as those from the Diptera family, as well as overall abundance, which could account for the dominance of Diptera in the treatment stream rock baskets (Williams-Subiza et al. 2022). As established, changes to physicochemical conditions, such as nutrient availability and sedimentation, have detrimental effects on sensitive benthic macroinvertebrates (Holeton et al. 2011). However, it is notable that this shift towards Diptera dominance was seen in the rock baskets only and not also in the Surber samples, which had the same dominant Diptera families but only in downstream sites rather than treatment sites and additionally at far lower relative measures (<50% dominant and only making up 20-40% of the overall community in any stream).

Often, Diptera families become dominant in MWWE affected sites due to replacing less tolerant organisms, such as EPT representatives (Enns et al. 2023). However, there were very few representatives of EPT in either Surber or rock basket samples. The dominant representative of the EPT category for both methods in the streams was the caddisfly family Hydropsychidae, which is known to be common in artificial samplers and is considered to often contribute to the inflated EPT scores (Letovsky et al. 2012). Hydropsychidae are a particularly tolerant caddisfly family and their dominance, especially in the absence of Plecoptera families and extremely low abundance of Ephemeroptera and other Trichoptera families, makes it unlikely that MWWE alone accounts for this pattern. Despite their high relative abundance across most samples, it is unlikely that this family are a strong sign of high-quality habitat, with no site having a higher HBI rating than “Fair” (with the lowest sites rating “Fairly Poor”).

Overall, these results suggest that conditions in the treatment streams are providing good conditions for the colonization of pollution tolerant taxa at a much higher rate than other organisms in the mesocosms. While the results from the Surber samples do not suggest that MWWE treatment streams are more dominated by Diptera overall, the rock basket results do suggest that these organisms are able to take advantage of uncolonized substrate more effectively than other organisms. One study of Chironomidae larvae indicated that the best colonizer of clean substrate were organisms that were small, tolerant, and had very short lifecycles (Wotton et al. 1992, Marwein and Gupta 2021). Chironomidae can emerge as adults in a time as short as 10 days, which would support their frequent presence as dominant in the rock baskets, especially when in more eutrophic streams, such as those with the cumulative addition of Pine Creek effluent. Additionally, the artificial substrate rock baskets provide more homogenous habitats than and may display stronger results due to controlling for habitat variation that may have influenced results in the Surber samples.

However, it is necessary also to understand that the experimental design of the two methods differed slightly: Surber samples were replicated through using all three replicate streams for each treatment while triplicate rock baskets were deployed in a single stream riffle for a stream randomly chosen to represent the control and treatment categories. Since there was variation observed between the replicate streams in the Surber samples, it may be that the shift in the pattern of differences seen between the two streams is related to the specific representative streams chosen for the rock basket samples.

Overall, the two methods represented somewhat similar assemblages overall and comparisons are limited, due to the experimental design, but the results indicated some key differences than may be of interest to investigate further. Many indices indicated similar communities, but rock baskets appeared to be somewhat better at establishing differences in what organisms dominate the colonization process in benthic macroinvertebrate assemblages when there is MWWE exposure, while the Surber samples identified a pattern in the way the communities shifted from upstream to downstream within the streams. Additionally, while the potential for improving assessment time in the artificial substrate rock basket was supported by the lower overall benthic macroinvertebrate abundances, the communities characterized by the two methods were not similar enough to confidently replace more traditional methods, such as Surber samples. Identifying the role of different types of samplers in investigating how benthic macroinvertebrate communities are affected by MWWE exposure, especially in controlled stream environments, is key in understanding how the constituents of the complex mixture may affect the biological health of aquatic ecosystems as MWWE exposure increases with population growth.

3.5 Conclusion

The controlled experimental streams at the Pine Creek ACWA facility represent a promising approach to assess if there are differences in benthic macroinvertebrate assemblages exposed to cumulative MWWE loadings. This was assessed through a weight of evidence approach using two benthic macroinvertebrate assemblage sampling methods in control and treatment streams at upstream and downstream sites within a Bow River (control) and 5% Pine Creek MWWE streams. MWWE is a common anthropogenic effluent associated with urban environments but variation in treatment types and community characteristics make it difficult to predict the composition of MWWE and its resulting effect on varying environments (Enns et al. 2023). Artificial stream experiments have been demonstrated to provide key opportunities for assessing point source stressors, including MWWE, and can help disentangle potential effects from complex effluents by limiting variation in the environment (Juvigny-Khenafou et al. 2021). Additionally, the controlled nature of the experimental streams provides a favourable setting in which to examine multiple methods emphasizing different characteristics of benthic macroinvertebrate assemblages. However, the experimental streams indicated strong, if not always convergent, differences existed between control and treatment streams that ultimately may assist in identifying patterns in larger *in-situ* studies of benthic macroinvertebrate assemblages exposed to MWWE.

The first guiding question for this project examined the current status of key endpoints associated with benthic macroinvertebrate assemblages at upstream and downstream sites of control and treatment streams. Overall, key endpoints based on the assemblages observed from multiple samplers for benthic macroinvertebrates indicated MWWE was a driver in the controlled mesocosm streams, although more strongly for the young, colonizing assemblages in the controlled habitat of the artificial substrate rock baskets than the established assemblage snapshots shown in the Surbers, which may have been taken from more varied habitats. The downstream sites did appear to be slightly higher quality, based largely on EPT and Diptera % metrics, in the Surber samples, while the rock basket samples heavily indicate that the colonization patterns in the treatment streams could indicate some favouring of particularly tolerant taxa over others.

The second guiding question for this project was to assess differences in benthic macroinvertebrate assemblages in streams of Bow River water (with no additional MWWE exposure) and streams exposed to Pine Creek MWWE. Significance testing using PERMANOVA showed, ultimately in pairwise testing, no significant differences between upstream and downstream locations within or between control and treatment streams. Ideally, this would also be done in relation to physicochemical measurements to assess what changes occur over the short stream gradient that may be responsible for this pattern. Related to this, future studies should make use of the ability to increase the in-stream concentration of MWWE, possibly making use of a fuller gradient design that utilizes multiple riffles along the experimental stream in order to better determine differences potentially related to changes over gradient.

The final question that guided the sampling performed at ACWA in 2020 was to assess how the answers to these questions shift when different types of sampling methods are utilized. Primarily, it was observed that passive artificial substrate rock basket samplers indicated that patterns of colonization favoured pollution tolerant Diptera families in streams with an additional point source MWWE exposure. There were often notable differences between control and treatment streams in the benthic macroinvertebrate assemblages for the Surbers, especially when combining upstream and downstream sites. However, there was also a weak pattern for the Surber samples suggested that downstream sites and upstream sites differed from one another in both control and treatment streams but not in a clear pattern that indicated one was in notably better ecological health. This pattern could be explored further, especially with measurements of ESOCs in the water column, to identify if there is an effect related to the stream gradient. Overall, these results also suggest that there is most notably value in better understanding how samplers may affect these observations, which metrics strongly support early evidence of nutrient enrichment, and suggest that MWWE may affect or amplify benthic macroinvertebrate colonization patterns through further study.

Chapter 4: Summary and Conclusions

4.1 The Current condition of Benthic Macroinvertebrate Assemblages in the Bow River in relation to WWTP exposures

4.1.1 Objectives

Overall, this study focuses on benthic macroinvertebrates as representatives of basal consumers in the aquatic food web, and aims to understand multiple facets of potential responses to MWWE as a point source pollutant and a complex mixture containing nutrients and a multitude of ESOCs. As part of a larger Bow River study on multiple endpoints (the Bow River Ecosystem Health Assessment or BREHA), this study aims to report on the current condition of the basal aquatic food web and provides insights for the development of a biomonitoring toolbox. These results were developed with the potential to be used to inform the eventual development of an EEM framework for MWWE. Chapter 2 provides an assessment of the current conditions and patterns of benthic macroinvertebrates in the Bow River close to WWTPs, and Chapter 3 describes an artificial stream experiment testing the responses of macroinvertebrate assemblages to cumulative inputs of MWWE. These two chapters were designed to address the following guiding questions:

- Describe and quantify the current status of and differences in key ecological endpoints associated with benthic macroinvertebrate assemblages exposed to a gradient of MWWE in the Bow River.
- Develop predictive relationships exist that link system drivers (MWWE related nutrients or ESOCs) to benthic assemblage responses.

- Discuss the advantages and disadvantages of various experimental designs and benthic macroinvertebrate assemblage sampling methods in understanding the responses of benthic macroinvertebrate assemblages to MWWE exposure.

4.1.2 Summary of Results

This biomonitoring project performed on the Bow River around the urban areas of Canmore and Calgary provides strong evidence that there is a traceable impact of MWWE on biological endpoints, as represented by benthic macroinvertebrate assemblages. This was primarily seen through a cluster analysis that provided clear organization of the sampled sites into regions within the studied areas. The clustered regions appeared to be primarily separated by high exposure to MWWE and then by distance apart (particularly between sites in a montane cordillera ecozone to sites in a prairie ecozone). The largest dissimilarity between groups of sites was between sites downstream of at least one of Calgary's WWTPs and all other sites upstream of Calgary's WWTPs, which includes Canmore. Canmore, which is most distant from all other sites and in a different ecozone, was the next group outlier, while Cochrane and North Calgary sites were more similar. The differences between these regions were sometimes supported by a significant result from PERMANOVA. These relationships of dissimilarity are a notable break with null models, such as the River Continuum Concept, which posits that changes to benthic macroinvertebrate assemblages happen gradually from upstream to downstream. This model indicates that that Canmore, being the most distant, would be the most dissimilar region (Vannote et al. 1980). Instead, the Bow River in this area appears to conform to a patch like system of similar assemblages and physicochemical attributes, disrupted by WWTPs (Naiman et al. 1988, Winemiller et al. 2010).

When the differences within the assemblages were assessed through characteristics of the taxa present, the regional dissimilarity was determined to often be attributed to increasing presence of Diptera families and reduced presence of EPT families downstream of WWTPs. The composition of EPT from its three Orders also varied somewhat, suggesting that this metric is sensitive to the surrounding land characteristics and the sampling seasonality. However, the overall state of the river, as assessed through the Hilsenhoff Biotic Index (HBI), was generally healthy and while some metrics were not very responsive to MWWE exposure, the same metrics also were not strongly impacted throughout the study, suggesting they are also not impacted by alternative pressures, such as urbanization or industry (Walsh et al. 2005).

Measured ESOC concentrations, additionally, were low and did not seem to align with literature observed effects on assemblages, such as analgesics causing higher mortality in detritivore and shredder macroinvertebrates (Lencioni et al. 2020). Similarly, examples of treatments specifically targeting ESOCs in WWTP treatments for other rivers have not demonstrated quantifiable changes in benthic macroinvertebrate assemblages (Johnson et al. 2019). Alternatively, while attempts to link changes in community assemblages at various sites to various physicochemical parameters were not conclusive, the concentration of Total Phosphorus and the specific conductivity were strong drivers of dissimilarity between sites in different regions.

The results of a mesocosm experiment at the Advancing Canadian Water Assets (ACWA) experimental streams did not provide as clear of evidence of the role of MWWE in driving benthic macroinvertebrate assemblages. Given that the control streams are sourced from Bow River water previously exposed to MWWE, this experiment was primarily intended to assess the effect of cumulative MWWE exposure from a high-quality effluent such as the Pine

Creek WWTP. Control and treatment stream categories were sampled using Surber samples (3 streams per treatment, 1 replicate) and artificial substrate rock baskets (1 stream per treatment, 3 replicates). While differences were often seen between control and treatment streams, the patterns of difference, for example whether Diptera were dominant in treatment streams, were not always convergent between methods. While there was evidence of nutrient enrichment for some metrics in the artificial substrate rock baskets, differences between streams for Surber samples did not always align. Additionally, the use of different methods may indicate some differences in colonization patterns related to MWWE but these did not appear to be supported as long-term differences by other sampling methods. The streams were also tested at upstream and downstream locations to test for potential effects of nutrient or ESOC uptake along the stream. In the Surber samples, there was an inconsistent pattern of similarity between upstream and downstream samples, which was particularly observed through the increased presence of Coleoptera families downstream. Overall, patterns of metrics observed in the streams, similarly to the mainstem project, did not necessarily mark previously expected outcomes of MWWE exposed assemblages, such as reductions to richness and diversity.

Synthesis of the *in-situ* and artificial stream projects reinforced understandings of how MWWE affects benthic macroinvertebrate assemblages, particularly in addressing what aspects of traditional monitoring programs should be integrated into a toolbox for future biomonitoring of MWWE. However, the streams did not strongly contribute to detangling nutrient-ESOC specific effects and potential interactions, except in that observed effects are typically those associated with nutrient enrichment. The artificial stream project does suggest that there are limited cumulative effects from multiple WWTP exposures, based on the addition of Pine Creek MWWE to Bow River water downstream of Bonnybrook and Fish Creek WWTP. Both projects indicate the studied WWTPs in Calgary and Canmore, which all have tertiary treatment implemented, demonstrate extremely high-quality treatment in the released MWWE. Additionally, many WWTPs in these areas serve smaller communities and have resultingly lower effluent releases. This conclusion is supported, firstly, by the relatively good health of the overall ecosystem and, secondly, by the somewhat inconsistent results, particularly in the artificial stream experiments between methods. The Canmore WWTP, serving a particularly small community as well as having tertiary treatment, shows minimal quantifiable effects upstream and downstream compared to Calgary's most upstream and largest WWTP (Bonnybrook). Similarly, Pine Creek WWTP, where the ACWA experimental streams are located, is added to the streams in a consistent volume (5% of total stream flow) but is a very high-quality effluent. The control streams are sourced by water upstream of the Pine Creek WWTP but downstream of the Bonnybrook and Fish Creek WWTP. As the recovery zone in the Bow River for impacts of MWWE has been shown to exceed the distance of these upstream WWTPs to ACWA, it may be assumed that the control streams are already impacted by MWWE and these are representative of cumulative effects from this WWTP (Hogberg 2004). The HBI index of health based on the benthic macroinvertebrate community was lower in the streams than in most of the Bow River, but the streams had similar (although slightly lower) ratings (Fair or Fairly Poor) to Bow River sites adjacent to the ACWA facility and this limitation could related to the options for colonization of insects from the nearby river and effects of upstream WWTPs more than the MWWE exposure in the experiment (Muehlbauer et al. 2014).

4.2 Cumulative Effects Assessment

Cumulative effects refer to the overall degradation in health that comes through multiple and repeated (through point and non-point source) impacts, which is particularly relevant in lotic systems (Squires and Dubé 2013). The Bow River, while established as a relatively pristine system, is still exposed to many anthropogenic stressors by the time it reaches the bottom of the CoC. Primarily, given the repeated exposure of MWWE as in the Bow River from various WWTPs, as well from tributaries with their own anthropogenic footprints, it is valuable to establish the likelihood of observing cumulative effects in the longitudinal gradient of the river, as sampled in this project. Additionally, WWTPs do not represent the only point and non-point source contamination in this section of the Bow River, which is also exposed to urban runoff, stormwater effluent release, dams, and some agricultural and industrial land. The sites sampled in this project were intended to isolate a handful of other anthropogenic impacts, such as the Bearspaw Dam and the areas with the highest concentration of the CoC's stormwater culverts, and assess if substantial changes were quantified in the benthic macroinvertebrate assemblages that could be related to pressures aside from MWWE. While a formal cumulative effects assessment was not performed on the watershed, some results from this project indicate cumulative effects could be relevant to studying the Bow River.

Typically, the chosen endpoints from sites associated with non-MWWE pollutants did not indicate quantifiable changes to the benthic macroinvertebrate assemblages observed in the Bow River, aside from some indication of nutrient enrichment in Cochrane above the Bearspaw Dam. However, their presence may still be related to overall impacts on the river before the southernmost site sampled in the CoC (PMF). The strongest evidence of cumulative effects in the Bow River is that some endpoints for assessing sites are reduced in quality further downstream, despite the most significant input of MWWE occurring at the first of the CoC's three WWTPs (Bonnybrook). Nutrient concentrations and, relatedly, specific conductivity, are consistently higher at the PMF site than they are at GRB, which is directly below Bonnybrook. However, the site specific HBI rating for the benthic macroinvertebrate assemblages (which ranges from Fair to Good for the entire South Calgary region) was slightly better further downstream at PMF than GRB (ranging from Fair to Good for PMF and from Fairly Poor to Good for GRB). These results suggest that while river chemistry may demonstrate cumulatively increasing levels of nutrients, these results are translated more immediately into some biotic endpoints than others.

This aligns with the experimental stream results at ACWA. While nutrient chemistry was not measured at ACWA in 2020, the HBI rating of macroinvertebrate biotic communities also did not indicate lower quality in the treatment streams that have additional Pine Creek MWWE exposures, although all stream HBI ratings are similar to MWWE exposed assemblages in the larger Bow River. However, based on a weight of evidence approach to cumulative impacts, these results do still indicate that cumulative impacts should be monitored in this area, at least for multiple point source WWTP exposures. There is a strong spatial correlation of changes in physicochemical results to the presence of additional WWTPs, which are plausibly linked to MWWE release (Culp et al. 2000). However, this observation would be strengthened by the sampling of additional sites in South Calgary, particularly directly below the Fish Creek WWTP.

While each WWTP in Calgary releases high-quality effluent that is well within Canadian National guidelines for nutrient release and the receiving environment shows only moderate signs of related degradation, it is necessary going forward to assess effects based on understandings that MWWE release is likely to continue to increase over time as populations around the Bow River also increase. Additionally, the condition of the Bow River at the downstream point of the area studied in this project is of particular importance to consider for future biomonitoring of the Bow River that may occur downstream of the City of Calgary (CoC), due to the increased presence of agricultural impacts as these impacts are likely to act cumulatively with the CoC stressors. These interactions are likely to be important to downstream users of the Bow River, including farmers, ranchers, towns, and indigenous communities.

4.3 Recommendations

4.3.1 Future Experimental Directions

This biomonitoring work in the Bow River and ACWA projects is primarily based around single year experiments. While this data is informative, it would be advisable to build on data gathered here to identify stronger patterns on an annual basis. Many programs recommend three years of baseline data to establish ranges of tolerable variation for endpoints, which would strengthen the arguments observed (Hewitt et al. 2003). While the seasonal sampling in the Bow River for this project added additional replication to the patterns observed overall, the abundance and richness data for the river does suggest that late summer to early Fall is the peak for benthic macroinvertebrates in the area and should be the focus going forward. This has the advantage of providing similar data to most benthic macroinvertebrate sampling that occurs in North America, including Canada's CABiN program (Environment and Climate Change Canada 2017). Increased physicochemical data over more years would also improve the ability of models to predict relationships between physicochemical parameters and the benthic macroinvertebrate assemblages observed. This would help to identify firstly, what drivers related to MWWE are most dominant in driving observed differences, and secondly, identify certain parameters may be used as rapid assessments to predict change in ecological endpoints.

Similarly, the addition of measured physicochemical parameters to studies performed at ACWA might improve understandings of why control and treatment streams at upstream and downstream locations don't always show consistent differences in community composition. While the current stream set up provides a way to assess cumulative effects of MWWE exposure, it is less able to disentangle specific mechanisms of MWWE in ecological systems. However, as the streams still indicated some (non-treatment aligned) differences, further studies at the ACWA facility should address drivers of difference between the Pine Creek treatment streams and the Bow River control streams. Additionally, increasing the % of MWWE added to the treatment streams would provide an opportunity to further observe cumulative effects changes in the streams that may be brought on by higher concentration exposure. Tracking the physicochemical changes related to increasing the % of MWWE could provide better insight into both the effects of MWWE and help to disentangle the effects of nutrient pollution from other contaminants, such as ESOCs. It would additionally produce further evidence as to whether the increased MWWE increases the value of physicochemical endpoints, such as nutrient concentrations, without necessarily causing further quantifiable change to the benthic macroinvertebrate community. This would help to identify the potential for cumulative effects of multiple WWTP exposures along a stream gradient.

While there are several endpoints from a community standpoint that were excellent signals for MWWE exposure, the effects of ESOCs on benthic macroinvertebrate assemblages did not clearly appear in either *in-situ* or artificial stream studies. The described effects of ESOCs on benthic macroinvertebrate assemblages from the literature are also not numerous. However, there are far more studies observing physicochemical responses and it is possible understandings of nutrient-ESOC interactions would be improved through the addition of physiological endpoints of benthic macroinvertebrates. For example, in some conditions, caffeine has been associated with reduced biomass of various benthic macroinvertebrates (Marshall et al. 2022). Since caffeine was one of the most notable ESOCs observed in the Bow River, understanding physiological impacts such as this could be important for understanding changes higher in the food web as well.

4.3.2 Impact on Future Biomonitoring Programs

Overall, this project demonstrated mixed success in using common benthic macroinvertebrate endpoints to identify and track the effects of MWWE. There are consistent and clear shifts in several endpoints around WWTPs in the Bow River. These shifts were less clearly observed in the ACWA stream mesocosms, where the MWWE outflow is placed at the top of the smaller stream gradient. However, metrics commonly associated with assessing general ecosystem impairment did not respond to MWWE exposure in the Bow River or in the ACWA streams. Bray-Curtis similarity matrices had great utility in organizing the results from this study into larger parts for future analysis but these exact differences were not always supported by subsequent PERMANOVA. As stated in Chapter 2, matrices that were based on what types of taxa made up the benthic macroinvertebrate assemblages, such as % EPT, were more successful in identifying impacts related to MWWE than more anonymous metrics, such as diversity, although total abundance was a notable exception to this. The results in the ACWA experimental streams were somewhat similar, although potential effects of MWWE in this experiment did not lead to consistent results. However, even in this context, what results could be interpreted were more clearly observed in metrics related to % EPT, % Diptera, and abundance over diversity and richness.

This project is part of the Bow River Ecosystem Health Assessment (BREHA), which is a multi-disciplinary initiative intended to study the health of aquatic ecosystems in the South Saskatchewan River Basin. BREHA was also conceptualized in part to establish what an Environmental Effects Monitoring (EEM) program for MWWE would look like. Traditionally, benthic macroinvertebrate endpoints for previous EEM programs (for example, pulp mill effluents and metal mines) have included density, family level richness, evenness, diversity, and similarity (via the Bray-Curtis index), as well as several taxa specific measurements of density, proportion, and presence/absence (Environment and Climate Change Canada 2010, 2012). This project utilized many of these endpoints, as well as additional endpoints, to determine sensitive and informative metrics for monitoring MWWE.

Given the results of this project, not necessarily all the metrics laid out for previous EEM studies would be likely to provide adequate warning of increasing MWWE impacts in the Bow River. Future studies in this area would be more efficient if they focused on indices related to the contribution of EPT and Diptera than those related to shifts in diversity and richness. While abundance, like the density measurements used in EEM programs, was the measurement that showed the most substantial change in relation to MWWE, without information on what kinds of organisms are implicated in this shift, incorrect conclusions could be drawn about the shifts occurring in this area. However, abundance, in relation to the above metrics could provide helpful context to the types of stressors implicated in changes to benthic macroinvertebrate assemblage endpoints. Additionally, tests of significance provide value in estimating the scale of difference but as appropriate tests, including PERMANOVA, are often conservative; the use of tools such as Bray-Curtis dissimilarities is valuable in identifying where changes of interest in a relatively pristine system, such as the Bow River, are occurring. Overall, the large-scale approach of EEM endpoints based on assemblage metrics is appropriate but the specific metrics of interest may need to be prioritized appropriately for a new framework assessing effects of MWWE.

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Appendix

Table A.1. For each site sampled in May 2021, a list of water sample parameters/physicochemical parameter measurement. Parameters are listed based on whether they were collected internally, sourced externally, or averaged from internal and external values to better estimate the range of conditions around the time of sampling. Dates for all internal and external samples are provided, as well as the laboratory used for processing internal water samples and the source of all external parameter measurements. Parameters include Total Phosphorus (TP), Total Kjeldahl Nitrogen (TKN), Specific Conductivity (Conductivity), Total Organic Carbon (TOC), Turbidity, Total Suspended Solids (TSS), and substrate axis length (Substrate).

Site	Internal sample date	Internal only physicochemical parameter measurements	Laboratory for submission of internal water quality samples	External sample date	External only physicochemical parameter measurements	Physicochemical parameter measurements averaged from internal and external measurements	Sources of external measurements
CUS	May 5, 2021	Substrate	ACWA	May 25, 2021	TKN, TOC, Turbidity, TSS,	TP, Conductivity	EPCOR
CDS	May 5, 2021	Substrate	ACWA	May 25, 2021	TKN, TOC, Turbidity, TSS	TP, Conductivity	EPCOR
BWP	May 7, 2021	Substrate	ACWA	May 12, 2021	TKN, TOC, Turbidity, TSS	TP, Conductivity	City of Calgary
NCA	May 7, 2021	Substrate	ACWA	May 12, 2021	TKN, TOC, Turbidity, TSS	TP, Conductivity	City of Calgary
GRB	May 6, 2021	Substrate	ACWA	May 12, 2021	TKN, TOC, Turbidity, TSS	TP, Conductivity	City of Calgary
PMF	May 6, 2021	Substrate	ACWA	May 11, 2021	TKN, TOC, Turbidity, TSS	TP, Conductivity	City of Calgary

Table A.2. For each site sampled in September 2021, a list of water sample parameters/physicochemical parameter measurement. Parameters are listed based on whether they were collected internally, sourced externally, or averaged from internal and external values to better estimate the range of conditions around the time of sampling. Dates for all internal and external samples are provided, as well as the laboratory used for processing internal water samples and the source of all external parameter measurements. Parameters include Total Phosphorus (TP), Total Kjeldahl Nitrogen (TKN), Dissolved Oxygen (DO), Specific Conductivity (Conductivity), Total Organic Carbon (TOC), Turbidity, Total Suspended Solids (TSS), and substrate axis length (Substrate).

Site	Internal sample date	Internal only physicochemical parameter measurements	Laboratory submission of internal water quality samples	External sample date	External only physicochemical parameter measurements	Physicochemical parameter measurements averaged from internal and external measurements	Sources of external measurements
CUS	Sept. 22, 2021	DO, Substrate	Bureau Veritas Calgary	Aug. 31, 2021 & Sept. 23, 2021	TOC, TSS	TP, TKN, Conductivity, Turbidity	EPCOR
CDS	Sept. 22, 2021	DO, Substrate	Bureau Veritas Calgary	Aug. 31, 2021 & Sept. 23, 2021	TOC, TSS	TP, TKN, Conductivity, Turbidity	EPCOR
COCH	Sept. 22, 2021	Substrate	n/a	Aug. 9, 2021 & Sept. 13, 2021 & Sept. 15, 2021	TP, TKN, TOC, TSS	Conductivity, DO, Turbidity	City of Calgary
BWP	Sept. 23, 2021	Substrate	Bureau Veritas Calgary	Aug. 11, 2021 & Sept. 15, 2021	TOC, TSS	TP, TKN, Conductivity, DO, Turbidity	City of Calgary
SGI	Sept. 23, 2021	Substrate	Bureau Veritas Calgary	Aug. 11, 2021 & Sept. 15, 2021	TOC, TSS	TP, TKN, Conductivity, DO, Turbidity	City of Calgary
CUB	Sept. 23, 2021	Substrate	Bureau Veritas Calgary	Aug. 10, 2021 & Sept. 14, 2021	TOC, TSS	TP, TKN, Conductivity, DO, Turbidity	City of Calgary
GRB	Sept. 24, 2021	Substrate	Bureau Veritas Calgary	Aug. 11, 2021 & Sept. 15, 2021	TOC, TSS	TP, TKN, Conductivity, DO, Turbidity	City of Calgary
PMF	Sept. 24, 2021	Substrate	Bureau Veritas Calgary	Aug. 10, 2021 & Sept. 14, 2021	TOC, TSS	TP, TKN, Conductivity, DO, Turbidity	City of Calgary

Table A.3. For each site sampled in November 2021, a list of water sample parameters/physicochemical parameter measurement. Parameters are listed based on whether they were collected internally, sourced externally, or averaged from internal and external values to better estimate the range of conditions around the time of sampling. Dates for all internal and external samples are provided, as well as the laboratory used for processing internal water samples and the source of all external parameter measurements. Parameters include Total Phosphorus (TP), Total Kjeldahl Nitrogen (TKN), Dissolved Oxygen (DO), Specific Conductivity (Conductivity), Total Organic Carbon (TOC), Turbidity, Total Suspended Solids (TSS), and substrate axis length (Substrate).

Site	Internal sample date	Internal only physicochemical parameter measurements	Laboratory for submission of internal water quality samples	External sample date	External only physicochemical parameter measurements	Physicochemical parameter measurements averaged from internal and external measurements	Sources of external measurements
CUS	Nov. 3, 2021	DO, Substrate	Bureau Veritas Calgary	Sept. 23, 2021 & Oct. 26, 2021	TOC	TP, TKN, Turbidity	EPCOR
CDS	Nov. 3, 2021	DO, Substrate	Bureau Veritas Calgary	Sept. 23, 2021 & Oct. 26, 2021	TOC	TP, TKN, Turbidity	EPCOR
BWP	Nov. 1, 2021	Substrate	Bureau Veritas Calgary	Oct. 14, 2021 & Nov. 9, 2021	TOC	TP, TKN, DO, Turbidity	City of Calgary
PMF	Nov. 4, 2021	Substrate	Bureau Veritas Calgary	Oct. 13, 2021 & Nov. 9, 2021	TOC	TP, TKN, DO, Turbidity	City of Calgary

Table A.4. Dates and sites for invertebrate and periphyton sample collection

Site	Date	Invertebrates
CDS	May 5, 2021	Kicknet
BWP	May 7, 2021	Kicknet
NCA	May 7, 2021	Kicknet
GRB	May 6, 2021	Kicknet
PMF	May 6, 2021	Kicknet
CUS	September 22, 2021	Kicknet
CDS	September 22, 2021	Kicknet
COCH	September 22, 2021	Kicknet
BWP	September 23, 2021	Kicknet
SGI	September 23, 2021	Kicknet
CUB	September 23, 2021	Kicknet
GRB	September 24, 2021	Kicknet
PMF	September 24, 2021	Kicknet
CUS	November 3, 2021	Kicknet
CDS	November 3, 2021	Kicknet
BWP	November 1, 2021	Kicknet
PMF	November 4, 2021	Kicknet

Table A.5. Locations and a summary of sampling set up (regions, sites, and replication) within the established longitudinal gradient of MWWE.

Location	Region	Sites and abbreviations		Replicates	Relationship to Longitudinal Gradient of MWWE
Canmore	Canmore Upstream	Canmore upstream	CUS	3x kicknets within site reach	Upstream of Canmore WWTP. Distant from other upstream MWWE exposure.
	Canmore Downstream	Canmore downstream	DUS		Downstream of Canmore WWTP
Cochrane	Cochrane	Cochrane	COCH		Upstream of the city of Calgary. Upstream of Calgary WWTP. Distant from other upstream MWWE exposure.
Calgary	North Calgary	Bowness Park	BWP		Upstream of all MWWE and major tributaries in the city of Calgary. Distant from other upstream MWWE exposure.
		St. George's Island	SGI		Upstream of all MWWE and major tributaries in the city of Calgary. Distant from other upstream MWWE exposure.
		Nose Creek Adjacent	NCA		Upstream of all MWWE but downstream of the Elbow River in the city of Calgary. Distant from other upstream MWWE exposure.
		Cushing Bridge	CUB		Upstream of all MWWE but downstream of the Elbow River and Nose Creek in the city of Calgary. Distant from other upstream MWWE exposure.
	South Calgary	Graves Bridge	GRB		Downstream of the Bonnybrook WWTP.
		Policeman's Flats	PMF		Downstream of the Bonnybrook, Fish Creek, and Pine Creek WWTPs.

Table A.6. The individual chemicals included in each category and their non-detect values. All samples were taken in 2021 between May 10th-11th for the May data, September 13th-14th for the September data, and between November 8th-9th for the November data.

Broader Classification	ESOC	Non-detect value (ng/L)
Hormone/Contraceptive	17a-Estradiol	<10.0
Hormone/Contraceptive	17a-Ethynylestradiol	<0.80
Hormone/Contraceptive	17b-Estradiol	<10.0
Industrial Compounds	4-n-Nonylphenol	<10.0
Industrial Compounds	4-t-Octylphenol	<100.0
Artificial Sweetener	Acesulfame	<50.0
Analgesic	Acetaminophen	<5.0
Hormone/Contraceptive	Androstenedione	<2.0
Cardiovascular Drugs	Atenolol	<5.0
Industrial Compounds	Benzyl butyl phthalate	<20.0
Industrial Compounds	Bisphenol A	<50.0
Industrial Compounds	Bisphenol S	<5.0
Stimulant	Caffeine	<5.0
Antiepileptic	Carbamazepine	<1.0
Antidepressant	Citalopram	<20.0
Antibiotic	Clarithromycin	<5.0
Pharmaceutical (Other)	Codeine	<10.0
Industrial Compounds	Cotinine	<1.0
Industrial Compounds	DEET	<5.0
Industrial Compounds	Di(2-ethylhexyl) phthalate	<1500.0
Industrial Compounds	Dibutyl phthalate	<300.0
Analgesic	Diclofenac	<5.0
Industrial Compounds	Diethyl phthalate	<50.0
Industrial Compounds	Dimethyl phthalate	<100.0
Industrial Compounds	Di-n-octyl phthalate	<100.0
Hormone/Contraceptive	Equilenin	<5.0
Hormone/Contraceptive	Equilin	<5.0
Antibiotic	Erythromycin	<2.0
Hormone/Contraceptive	Estriol	<5.0
Hormone/Contraceptive	Estrone	<5.0
Antidepressant	Fluoxetine	<5.0
Cardiovascular Drug	Gemfibrozil	<5.0
Analgesic	Ibuprofen	<10.0
Pharmaceutical (Other)	Metformin	<1.0
Analgesic	Naproxen	<5.0
Cardiovascular Drugs	Nifedipine	<5.0
Hormone/Contraceptive	Norethindrone	<5.0
Antidepressant	Norfluoxetine	<5.0
Hormone/Contraceptive	Norgestimate	<5.0
Antidepressant	O-Desmethyl-venlafaxine	<1.0

Pharmaceutical (Other)	Pantoprazole	<5.0
Industrial Compounds	PFOA	<10.0
Industrial Compounds	PFOS	<20.0
Antiepileptic	Primidone	<1.0
Hormone/Contraception	Progesterone	<2.0
Cardiovascular Drug	Propranolol	<5.0
Pharmaceutical (Other)	Salbutamol	<5.0
Artificial Sweetener	Sucralose	<10.0
Antibiotic	Sulfamethoxazole	<1.0
Industrial Compounds	TCEP	<5.0
Industrial Compounds	TCPP	<50.0
Hormone/Contraception	Testosterone	<2.0
Industrial Compounds	Triclosan	<10.0
Antibiotic	Trimethoprim	<1.0
Antidepressant	Venlafaxine	<1.0
Pharmaceutical (Other)	Zopiclone	<10.0

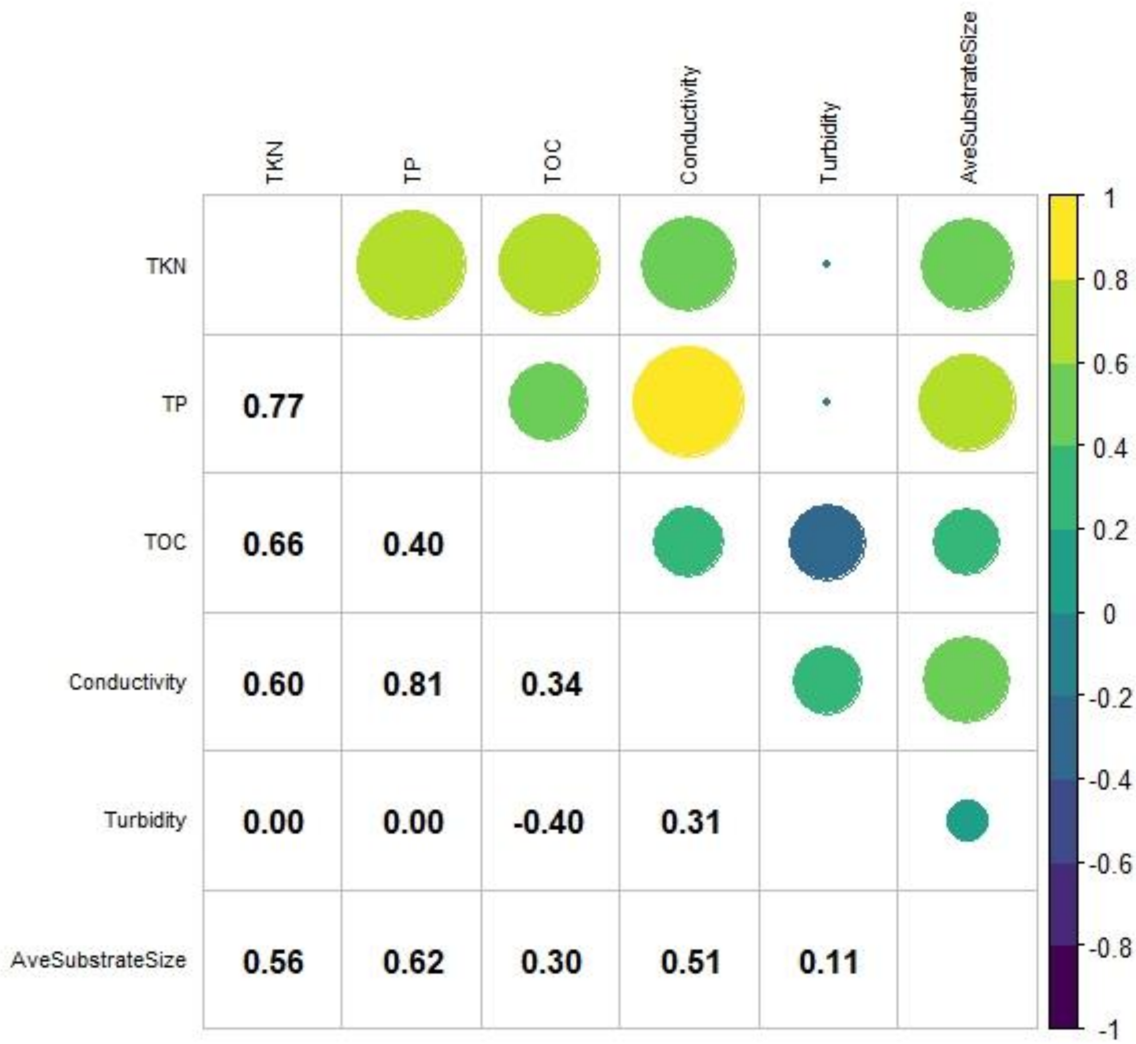

Fig. A.1. Pearson correlation matrix for the physicochemical parameters used in the dbRDA analysis with the Bow River benthic macroinvertebrate assemblages across the open water season (May – November) of 2021.

Table A.7. Sampling sites and dates for all Surber samples and rock basket samples for benthic macroinvertebrate assemblages from the ACWA experimental streams.

Site	Sampler	Date deployed	Date retrieved
BRR1Riff2	Surber	n/a	October 26-27, 2020
BRR1Riff10	Surber	n/a	October 26-27, 2020
BRR2Riff2	Surber	n/a	October 26-27, 2020
BRR2Riff10	Surber	n/a	October 26-27, 2020
BRR3Riff2	Surber	n/a	October 26-27, 2020
BRR3Riff10	Surber	n/a	October 26-27, 2020
PCR1Riff2	Surber	n/a	October 26-27, 2020
PCR1Riff10	Surber	n/a	October 26-27, 2020
PCR2Riff2	Surber	n/a	October 26-27, 2020
PCR2Riff10	Surber	n/a	October 26-27, 2020
PCR3Riff2	Surber	n/a	October 26-27, 2020
PCR3Riff10	Surber	n/a	October 26-27, 2020
BRR2Riff2	Rock Basket	September 2, 2020	October 26-27, 2020
BRR2Riff2	Rock Basket	September 2, 2020	October 26-27, 2020
BRR2Riff2	Rock Basket	September 2, 2020	October 26-27, 2020
BRR2Riff10	Rock Basket	September 2, 2020	October 26-27, 2020
BRR2Riff10	Rock Basket	September 2, 2020	October 26-27, 2020
BRR2Riff10	Rock Basket	September 2, 2020	October 26-27, 2020
PCR1Riff2	Rock Basket	September 2, 2020	October 26-27, 2020
PCR1Riff2	Rock Basket	September 2, 2020	October 26-27, 2020
PCR1Riff2	Rock Basket	September 2, 2020	October 26-27, 2020
PCR1Riff10	Rock Basket	September 2, 2020	October 26-27, 2020
PCR1Riff10	Rock Basket	September 2, 2020	October 26-27, 2020
PCR1Riff10	Rock Basket	September 2, 2020	October 26-27, 2020

Fig. A.2. A rock basket after a six-week deployment partially taken apart to show scour pad sediment traps underneath artifical substrate briquettes (left) and a rock basket deployed in the ACWA streams in September – October 2020 (right).

Fig. A.3. Examples of ACWA stream size, set-up and streamside vegetation in September 2022.

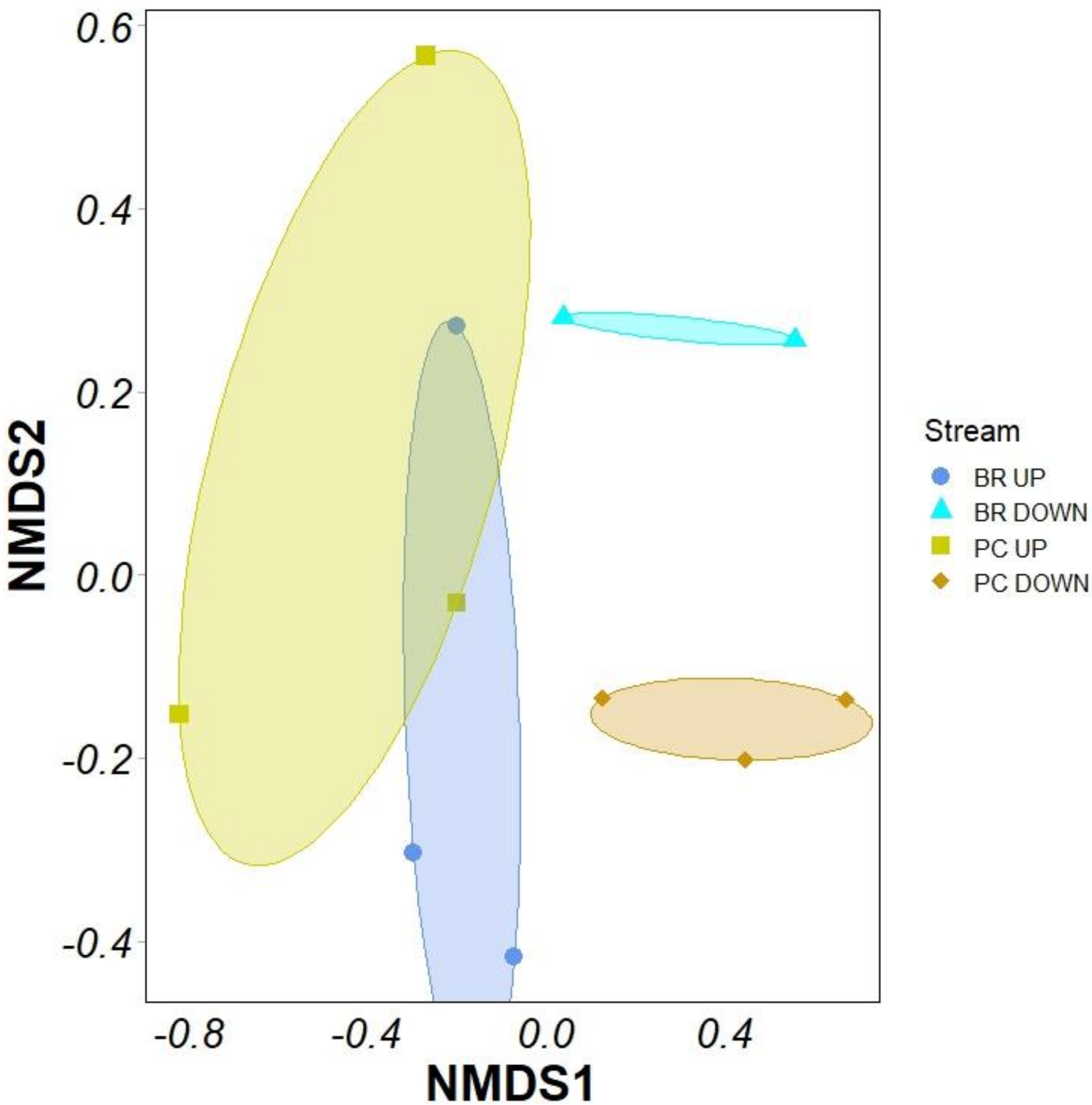

Fig. A. 4. No-metric multidimensional scaling (NMDS) ordination of surber sample replicates of benthic macroinvertebrate communities in the ACWA experimental streams in October 2020.

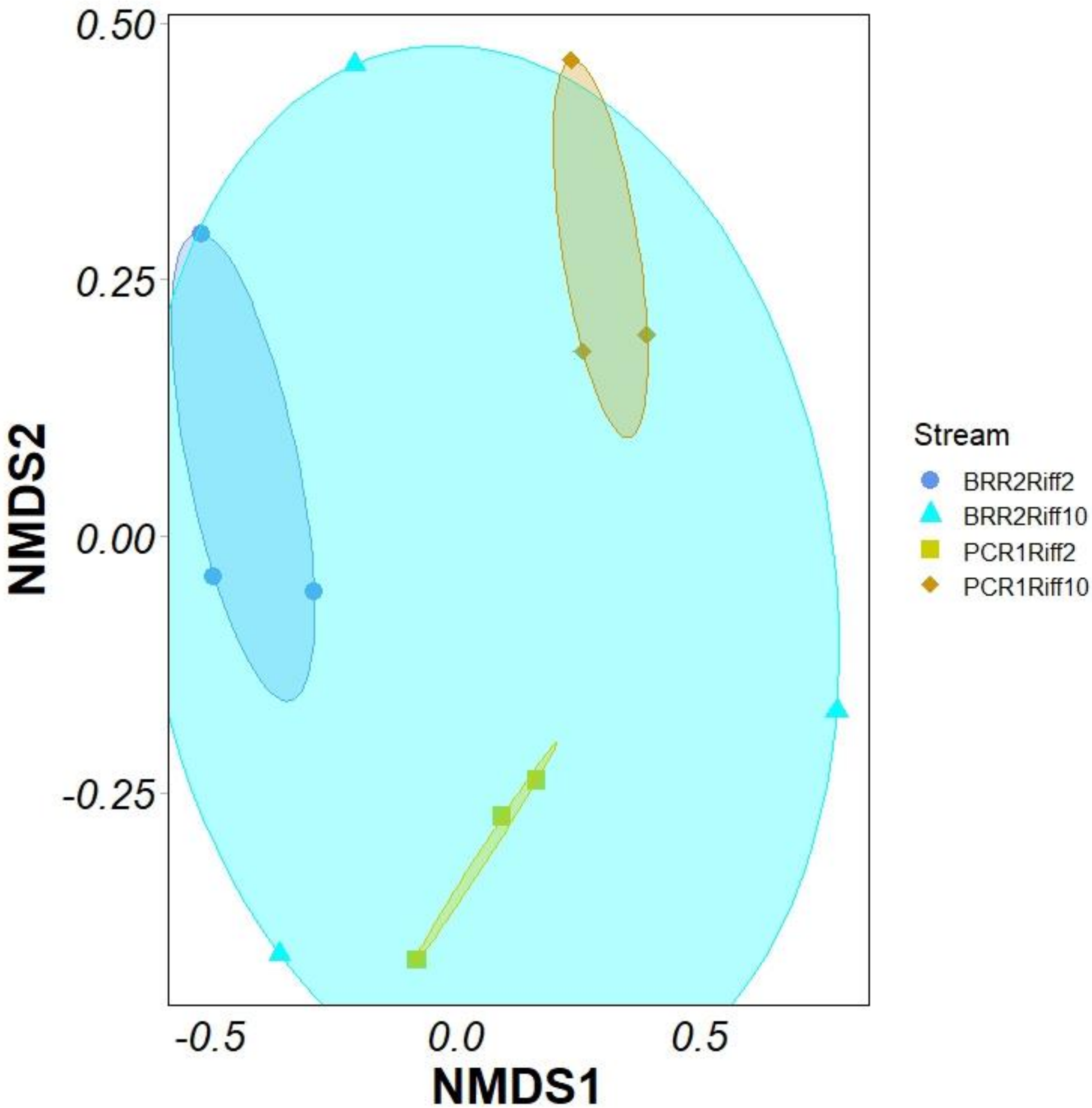

Fig. A. 5. No-metric multidimensional scaling (NMDS) ordination of artificial substrate rock backet sample replicates of benthic macroinvertebrate communities in the ACWA experimental streams in October 2020.