Chitobiase as a surrogate measure of aquatic invertebrate biomass and secondary production in an environmental effects monitoring context

by

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ABSTRACT

The current techniques used to assess aquatic invertebrate community status in the field are typically labour and time intensive, and therefore the development and implementation of new rapid and cost-effective methodologies is warranted. A proposed option is the enzymatic assay to detect and quantify the rate of production of the molting enzyme chitobiase, which can be used for determining impacts on freshwater aquatic systems. Two case studies were performed at: 1) The Prairie Wetland Research Facility at the University of Manitoba, to determine if a relation exists between measures of chitobiase and aquatic invertebrate biomass in a mesocosm setting, as well as to determine if changes in chitobiase activity could detect impacts to aquatic invertebrate communities from sulfamethoxazole and diluted bitumen and; 2) in the Elk River Valley region of British Columbia, to determine if a positive relationship exists between the rate of chitobiase production and benthic invertebrate biomass in lotic freshwater systems. No significant relationship was observed between the chitobiase measures and invertebrate biomass measures, and no effects of the stressors were detected in the first study. A significant positive relationship was observed between the rate of chitobiase production and benthic invertebrate biomass in the second study. It is recommended that additional studies be performed to further assess the potential of chitobiase activity to be used in an environmental monitoring context.

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CONTRIBUTIONS OF AUTHORS

Chapter 3 is based on work conducted in the Elk Valley Region of British Columbia, Canada. Contributing authors include Matthew E. Randell[1], who was involved in conducting the field study, analyzing the chitobiase samples, plotting and interpreting data, and writing the chapter. Michael S. White[2], who was involved in conducting the field study, assisting with the statistical analysis of data collected, assisting in writing, and reviewing the chapter. Shari Weech[2], who was involved in conducting the field study and reviewing the chapter. Patti Orr[2], Carla Fraser[3], and Mark L. Hanson[1], who reviewed the chapter.

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CHAPTER 1. GENERAL INTRODUCTION

The assessment of benthic invertebrate communities is an important consideration when evaluating the impacts of environmental contaminants on aquatic ecosystems, due to their functionally important role in aquatic ecosystems (Wilson 1992; Palmer 1997; Postel and Carpenter 1997; Freckman et al., 2019). The diversity and ecological roles of freshwater benthic species influence the many processes in freshwater ecosystems (Palmer 1997). Generally, species richness and functional significance of benthic invertebrates go largely unnoticed until unexpected or adverse changes occur in ecosystems (Covich et al. 1999). Benthic invertebrates contribute to secondary production, cycling of nutrients, accelerating detrital decomposition, and act as a food source for other invertebrates and fish, and therefore their protection is essential to maintain the structure and function of an aquatic ecological system. They are also widely acknowledged indicator of ecosystem health and are one of the elements that reflect biological quality (e.g., water and sediment quality) and ecological status (assessment of the quality of the structure and functioning of an ecosystem) (Bunzel et al. 2013). This is due to their range of biological traits (e.g., body shape, locomotion, and lifespan), their relative longevity, and well-established responses to different stressors (Reish 1959; Diaz et al. 2004; Pinto et al. 2009). Recent legislative frameworks assessing the status of the aquatic environment such as the Clean Water Act in the United States and in the European Union Water Framework Directive, have emphasized the significance

of the health of benthic communities as they reflect the overall health of the ecosystem (Villnäs and Norkko, 2011).

In Canada it is common practice to use benthic invertebrates to assess and monitor aquatic ecosystem health, particularly through benthic community surveys to evaluate the potential impacts from effluents on fish habitat (Environment Canada, 2010, 2012). This usually takes the form of a benthic community survey as part of an environmental effects monitoring program. This current approach has several drawbacks, including: difficulty accounting for high spatial variance in abundance due to patchy distribution, it requires a considerable amount of analytical time and taxonomic expertise, and it can be costly (Hanson and Lagadic, 2005). These drawbacks support the development of rapid and cost-effective methodologies for assessing effects on invertebrate communities in the field. We propose the implementation of an enzymatic approach to assess the status of invertebrate communities and ecosystem health in lotic and lentic systems. This approach is based on the measurement of free chitobiase, an invertebrate molting enzyme, which is released into the water column (Hanson and Lagadic 2005; Oosterhuis et al 2000; Richards et al 2008). Chitobiase has been found to be predictive of secondary production for marine and freshwater zooplankton (Avila et al 2012, Oosterhuis et al 2000; Sastri and Dower 2009; Sastri and Roff 2000; Vrba and Machacek 1994). Further rationale for the application of the chitobiase assay in a monitoring context is discussed in this thesis.

To investigate this topic, we conducted two studies to determine the suitability of chitobiase in a monitoring context: 1) a mesocosm study at the Prairie Wetland Research Facility (PWRF), at the university of Manitoba, monitoring chitobiase activity in mesocosm tanks exposed to environmentally relevant concentrations of the antibiotic sulfamethoxazole (SMX) and diluted bitumen (dilbit) and; 2) a field study in the Elk River Watershed, British Columbia, an area of British Columbia with coal mining activity, to determine if a positive measurable relationship exists between chitobiase activity and invertebrate biomass in a lotic freshwater system.

1.1 Environmental Effects Monitoring

An essential component in regulating the impacts of industry on the ecosystems is through the implementation of environmental monitoring programs. Since the 1960's it has been used in various countries to monitor the impacts of petroleum extraction and mining (Artiola et al. 2004). Environmental monitoring can be defined as the methodical sampling of air, biota, water, and soil to observe and understand the environment (Artiola et al. 2004). Monitoring is conducted for numerous purposes, including but not limited to the establishment of environmental baselines, trends, and cumulative effects; to inform policy design and decision-making, inform the public about environmental conditions; to assess the effects of anthropogenic influences; and to ensure compliance with environmental regulations (Mitchell 2002). In North America prior to the late 1960's, large amounts of waste by-products from industrial and

agricultural activities were released into the environment without any regulatory consequence (Artiola et al. 2004). Formalized monitoring was introduced in Canada through the Pulp and Paper Effluent Regulations (PPER) under the *Fisheries Act* in 1971 as a government-directed, industry funded program (Curran et al. 2006). Currently, environmental monitoring has become more critical as the human population continues to rise resulting in ever-increasing strains on the environment.

There are three types of environmental monitoring: compliance, surveillance, and effects monitoring (USEPA 2017). Compliance monitoring involves the quantification of industrial impacts on the environment ensuring that applicable permit conditions, orders, laws, regulations, and settlement agreements are obeyed. Surveillance monitoring enforces those compliances set forth on industry through a regulator by performing monitoring and surveillance activities. Finally, environmental effects monitoring (EEM), is an effects-based approach used to identify, quantify, and compare predicted environmental impacts of a proposed industrial project to those observed after the project has been developed (Curran et al. 2006).

EEM was introduced in Canada in 1992 as part of the Pulp and Paper Effluent Regulations (PPER) set forth by the federal government (Borgmann et al. 2010) to act as a condition governing authority to deposit effluent and to evaluate potential effects of effluents on aquatic environments. In 1996 Environment Canada introduced the Metal Mining Effluent Regulations (MMER) to assess the aquatic effects of metal mining (Environment Canada, 2012). Both

the PPER and MMER are in conformance to the Fisheries Act, where the deposition of deleterious substances in water frequented by fish is prohibited through the pollution prevention provision (Section 36) of the Fisheries Act, unless authorized by regulations (Environment Canada 2010). Regulations are currently being developed and expanded for the monitoring of municipal wastewater (CCME 2009). In essence these programs are a series of stages framed into cycle, typically three or four-year sequences of monitoring and interpretation, in which knowledge from previous cycles is incorporated into subsequent cycles of the program (Environment Canada 2010).

1.1.1 Environmental Effects Monitoring Structure

Technical guidance documents created by the Government of Canada provide a detailed outline of how to perform an EEM program for the regulated industries (i.e., pulp and paper and metal mining). The prescribed study design frameworks and sampling methodologies set forth by the guidance documents ensure that a level of consistency for the approach is achieved across Canada. The guidance varies between the MMER and PPER but both contain sections regarding study design; site characterization and general quality assurance and quality control; effects on fish and fisheries resources; effects on fish habitat: benthic invertebrate community survey; effluent characterization and water quality monitoring; sub-lethal toxicity testing; data assessment and interpretation; alternative monitoring methods; public involvement in respective industry effects monitoring; and investigation of cause. MMER contains additional sections on sediment monitoring and report on historical information.

Prior to collection of physical samples in the environment, an EEM program is initiated by creating a study design, characterizing the baseline condition of the site, and making sure that proper general quality assurance and quality control measures are in place. To assess and investigate possible effects and attributing cause from effluent discharges, chemical/toxicological analysis and biological monitoring studies are conducted. Regulatory requirements set forth for the pulp and paper and metal mining industries include conducting biological monitoring studies in aquatic environments that are receiving effluent to determine if it is having an effect on fish, fish habitat or the use of fisheries resources; effluent characterization studies (MMER only); water quality studies; and sub-lethal toxicity testing of effluent. Upon completion of these studies, an interpretive report is prepared to summarize the study results, conduct applicable spatial analysis, specify any "identified" effects, and make recommendations for subsequent EEM programs. Due to the extensiveness of the technical guidance documents and the focus of this study, only the section pertaining to biological monitoring, and specifically benthic community surveys, will be covered in detail below, as these organisms are a focus of the thesis.

1.1.2 Biological Monitoring Studies

As a requirement of EEM, biological monitoring studies must be conducted for the purposes of determining if effluent is having an effect on fish, fish habitat, or the use of fisheries resources (Environment Canada 2010). More specifically, three components comprise the biological monitoring study: 1) studying fish populations to assess effects on fish health; 2) a benthic

invertebrate community study to assess fish habitat and fish food; and 3) a fish tissue study (for contaminant concentrations) looking at the human usability of fishery resources. An effects-based approach allows for the evaluation of a specific set of biological indicators (e.g., fish condition, benthic invertebrate taxon density and richness) through the comparisons of exposure and reference areas or along an effluent concentration gradient. Exposure areas are waters and fish habitat frequented by fish that are subject to effluent deposition meaning significant fish are exposed. Reference areas are waters and fish habitat that are as similar as possible in physical characteristics to that of the exposure area with no exposure to effluent. For the fish population survey and benthic invertebrate community survey, data is collected on specific effect indicators and endpoints. The specific effect indicators for the fish population include growth (energy use), reproduction (energy use), condition (energy storage), and survival. The respective effect endpoints for the fish population survey include size-at-age (body weight relative to age), relative gonad size (gonad size to body weight), condition (body weight to length) along with relative liver size (liver weight to body weight, and age (Environment Canada 2012). The specific effect indicators for the benthic invertebrate community survey include total benthic invertebrate density, evenness index, taxa richness, and similarity index. The respective effect endpoints for the benthic invertebrate community survey include number of animals per unit area, Simpson's evenness, number of taxa, and Bray-Curtis index (Environment Canada 2012). An effect is defined as a "statistical difference between data collected in an exposure area and in a reference area or sampling

areas within an exposure area where there are gradually decreasing effluent concentrations at increasing distances from the effluent discharge" (Environment Canada 2012).

Based on regulatory guidelines, biological monitoring studies are conducted in three or six-year cycles and the requirements for each study are dependent upon the results from the previous cycle. The approach is tiered, such that the results from one phase of the program will determine the study design for biological monitoring during the next phase. The intensiveness of the monitoring prescribed for the next phase is dependent upon the presence of an effect. Furthermore, if effects are present, biological monitoring studies are conducted for the purpose of: 1) describing the magnitude and geographic extent of effects, 2) determining the cause of effects, and 3) identifying possible solutions to eliminate the effects. Hypothetically, if no effects are observed during the biological monitoring study, the individuals conducting the study can continue the monitoring program as initially implemented in accordance with the governing regulatory requirements. Conversely, if a biological monitoring study is conducted and effects are observed, the individuals conducting that study will have to review the data and implement a new study design for the next phase in order to further understand the impacts of the stressor. New implementations to the monitoring program could include determining the concentrations of the stressor present in the ecosystem, extending the range of the study to determine the extents of the impacts/effects of the stressor, amongst other possibilities.

1.2 Benthic Invertebrates and their Use in Environmental Effects Monitoring

1.2.1 Benthic Invertebrates

Freshwater benthic invertebrate species represent a rich fauna, which have evolved from many phyla over millions of years (Covich et al. 1999). New forms are continuously being discovered and many species still remain undescribed, both taxonomically and ecologically (Palmer, 1997). Benthic invertebrates perform a number of major functions in freshwater food webs.. First, benthic invertebrates are essential in aiding the acceleration of detrital decomposition through burrowing, shredding, and feeding. A main energy source for benthic species is dead organic matter and it is estimated that 20 – 73% of riparian leaf-litter is processed by benthic invertebrates (van de Bund et al. 1994; Wallace and Webster 1996; Covich 1988; Hutchinson, 1993). Hieber and Gessner (2002) conducted a field study looking at the contribution of stream detritivores, fungi, and bacteria to leaf breakdown based on biomass estimates in a third-order reach of the Steina, a soft water stream located in the southern Black Forest, Germany. They deployed 140 leaf packs containing alder (*Alnus glutinosa*) and willow (*Salix fragilis*) into a third-order stream for eight weeks; five random leaf packs were removed after 1, 3, 7, 14, 28, and 55 days of submersion. After 28 days <50% of leaf dry mass remained and after 55 days 92% of alder and 74% of willow dry mass was lost. Maximum density of colonized macroinvertebrates occurred within the leaf packs after 28 days with an average of 708 (alder) and 422 (willow) animals per pack, which corresponds to a biomass of 348 (alder) and 209 (willow) mg/pack. Based on literature, Hieber

and Gessner (2002) stated that macroinvertebrates typically consume between 10% and 80% of their body mass per day, which allowed them to determine that invertebrate feeding would account for leaf mass loss of 30% and 24% by day 28, and 64% and 51% by day 55 for alder and willow, respectively.

Second, through feeding activities, excretion, and burrowing into sediments, benthic invertebrates release nutrients, such as nitrogen, into the system. The release and subsequent availability of these nutrients in the water allows for uptake by algae, angiosperms, bacteria and fungi, in turn accelerating microbial and plant growth (Pelegri and Blackburn, 1996; Wallace, Eggert, Meyer, and Webster, 1997). Such activity was observed by Pelegri and Blackburn (1996) who conducted a laboratory study monitoring nitrogen cycling in lake sediments from Slåen Lake in Denmark, a mesotrophic lake undergoing non-anthropogenically mediated anoxic periods, that were bioturbated by *Chironomus plumosus* larvae under differing degrees of oxygenation. C*. plumosus* colonies were incubated in lake water with sediment cores while being saturated with 100% and 39% O_2 and dosed with 40.9 µM $^{15}NO_3^-$. Fluxes of NH_4^+ and NO_3^- plus NO_2^- were measured every second day in order to determine when a steady state was reached. They found that the animal contribution to O_2 and NH_4^+ fluxes were not significantly ($p > 0.1$) affected by the oxygen concentration in the water. An increase of 5.8-fold in NH_4^+ was observed in cultures colonized by *C. plumosus* compared to that of control microcosms. It was also noted that animals enhanced O_2 and NO_3^- uptake by sediment and increased nitrification rates were observed relative to non-inhabited microcosms.

Third, through predation, benthic invertebrates have the ability to control the abundance, location, and size of their prey (Crowl and Covich 1990, 1994). Field and laboratory studies were carried out by Crowl and Covich (1994) monitoring the response of *Atya lanipes*, a freshwater scraper/filterer shrimp, to *Macrobrachium carcinus*, a large predatory shrimp. The field study was conducted in a 1st-order tributary of the Quebrada Sonadora, a 4th-order stream within the Espiritu Santo drainage basin of the Luquillo Experimental Forest, northeastern Puerto Rico. During the field study they found that when *M. carcinus* were added to natural stream pools containing *A. lanipes*, *A. lanipes* emigrated significantly more in pools containing *M, carcinus* than from control pools (F = 30.6, p = 0.0101). On average, control pools increased by 9 *A. lanipes*/m^2 and pools with *M. carcinus* decreased by 13 *A. lanipes*/m^2. The shrimp used in the laboratory study were collected from the same water body as in which the field study was conducted. Similar results were seen in the laboratory study where *M. carcinus* were added to artificial streams containing *A. lanipes*; significantly more *A. lanipes* emigrated from pools stocked with *M. carcinus* than control pools (F = 52.7, p = 0.0001). They also noted that when *A. lanipes* was in the presence of *M. carcinus*, they spent significantly more time in crevices to avoid predation.

Finally, benthic invertebrates act as a significant food source for higher trophic levels such as fishes, reptiles, and birds (Covich et al., 1999). Fish populations within an aquatic system have a structural effect on benthic invertebrate communities (e.g., assemblage structure and density) due to

predation (Dahl and Greenberg 1998; Pierce and Hinrichs 1997; Power 1990). Williams et al. (2003) conducted a study in which they looked at the effects of fish predation on macroinvertebrates in a second-order stream in the Ouachita National Forest, Arkansas, USA. They observed that fish predation had a strong effect on macroinvertebrates, where the presence of fish caused decrease in macroinvertebrate abundance. When fish were removed from individual stream pools it led to a large and significant increase in the density of macroinvertebrates (repeated measures ANOVA, treatment × time; $F = 5.02$, df = 4, $p = 0.01$). They noted that taxa richness was not affected by the presence of fish; fish had an effect on the overall abundance of individual taxa without altering the absolute number of taxa. Additionally, a study was conducted by Culp et al. (1991) monitoring the avoidance response of the larvae of *Paraleptophlebia heteronea* (mayfly species) to *Rhinichthys cataractae* (longnose dace) at night in Jumpingpound Creek, a 4th-order stream located in the foothill's of the Rocky Mountains approximately 30 km west of Calgary, Alberta. During their field observations, they observed two general responses of *P. heteronea* in response to *R. cataractae*: 1) movement into the drift; a 30-fold increase in drift rates was observed in laboratory streams compared to controls without fish. Active entry of larvae into the drift likely acts as a mechanism that decreases the rick of capture to bottom-feeding fish, such as *R. cataractae*. (Beers and Culp, 1990), 2) Retreat into interstitial crevices; approximately 60% of the *P. heteronea* larvae maintained exposed positions in the substrate in the control streams throughout a 24 h period and distributions in the fish and control streams were initially the

same. However, after 15 minutes of foraging by *R. cataractae*, less than 20% of the *P. heteronea* larvae occupied exposed positions in the fish treatment streams.

Abundance and community structure of benthic invertebrates are also shaped by their aquatic habitats, particularly so for riverine areas where riverbed topography and spatial patterns of flow affect the transport and retention of materials such as leaves and sticks (Kobayashi et al. 2013; Wallace et al. 1996). Two major topographic features that make up gravel-bed rivers are riffles and pools, which alternate between shallow areas with fast flow and deep areas with slow flow (Leopold et al. 1964; Kani, 1988). Riffles tend to support more biomass and production of benthic invertebrates than pools due to increased availability of oxygen and food (Logan and Brooker 1983; Grubaugh et al. 1997; Hart and Finelli 1999; Nishimure et al. 2001). However, greater channel velocities are normally associated with increased shear stress that can impede settlement (Brooks, Haeusler, Reinfelds, and Williams, 2005; Morales, Weber, Mynett, and Newton, 2006). Not only are spatial and temporal distributions of benthic invertebrates influenced by current velocity and types of substrata but also by their preferences for particular ranges of temperature and pH (Covich et al., 1999).

Individual diversity of benthic invertebrate species is important; each species is adapted to function under variable conditions, with different species varying in importance to particular ecological processes (Covich et al., 1999). Due to their diversity and ecological roles, benthos influence major processes in

freshwater ecosystems. A shift in the distribution and abundance of a single species within a system can result in unexpected and disproportionate responses by other species in an attempt to compensate for the altered ecosystem services (Frost et al. 1995; Naeem 1998). Liess et al. (2013) carried out a field study where they monitored the impact of land use intensity on benthic invertebrate species richness. They monitored 41 stream sites in Hesse, Germany to encompass effects from land use such as native forests to high-intensity dairy farming, looking at increased nutrient load and increased fine sediment load. They determined that invertebrate taxon richness was negatively affected by land use intensity – land use intensity explained between 13% of the variation in overall invertebrate taxon richness and 20% of the variation in predator taxon richness. They also noted that predator taxon richness increased with grazer taxon richness – the possibility of a causal relationship existing between grazer and predator, where predator numbers increase when prey is more abundant and available. A more diverse predatory community could favor diversified prey strategies and higher prey species richness or vice versa, where a more diverse prey community could favor coexistence of multiple predator species. Loss of species can result in the alteration or degradation of key ecosystems processes due to irreplaceability, although exact consequences of the loss of each species cannot be predicted. Species richness and functional importance of benthic invertebrates often goes unnoticed until unexpected changes occur in an ecosystem (Covich et al., 1999)

1.2.2 Benthic Invertebrate Communities in Environmental Effects Monitoring

The abundance of benthic invertebrates and their wide range of tolerance to different stressors lend them to be a useful tool in identifying environmental impacts that may affect local fish habitat and furthermore fish communities (Brown 2001; Smith et al. 2011). Invertebrates are abundant medium-sized organisms that generally have growth rates and population turnover times that lie midway between those of microorganisms and higher trophic levels, such as plants and animals. They also have effective active and passive dispersal mechanisms that can allow for wide dispersal and rapid recolonization of disturbed habitats (Hodkinson et al. 2002). This allows for benthic invertebrates to serve as effective bioindicators because of their level of responsiveness to changing environmental conditions.

Benthic invertebrates have been utilized as bioindicators for a range of chemical changes occurring within aquatic environments. Common chemical parameters include pH, concentrations of single contaminants such as heavy metal pollutants like cadmium, or excess amounts of nutrients, particularly nitrogen and phosphorous (Broderson and Andersen 2002). A direct relationship can be established between the performance of a bioindicator species and the concentration of a chemical stressor present in the environment. For example, Saraiva et al. (2017) performed a study looking at toxicity of the neonicotinoid pesticide thiamethoxam (TMX) through the use of survival tests (mortality) on *Chironomus riparius*, a standard test species. TMX was observed to be highly toxic to *C. riparius* at field-realistic concentrations (4, 6.5, 10.5, and 18 µg/L),

where field measured concentrations have been detected up to 225 µg/L in freshwater systems close to agricultural development (Anderson et al. 2013). Chronic exposure to low concentrations of TMX (18 µg/L for 10 days) resulted in survival of 35.5% and a significant reduction in *C. riparius* larvae growth ($F_{4,34}$ = 20.02, $p < 0.001$) . At the end of 28 days of exposure, the emergence rate in the control treatment reached 77.5% while the emergence rate in the 10.5 µg/L and 18 µg/L resulted in emergence rates of 12.5 % and 0%, respectively. Therefore, this study shows the direct relationship established between TMX and its toxicity to the standard test species *C. riparius*, demonstrating the usefulness of aquatic insects as bioindicators.

Benthic invertebrates are commonly used in EEM programs to assess possible impacts of effluents on fish habitat (Brown 2001; Hughes et al. 2012; Smith et al. 2011). The objectives of a benthic invertebrate community survey are to determine the magnitude and geographic extent of fish habitat degradation due to organic enrichment or other contamination. The main goal is to determine if there are structural differences (e.g., population sizes, distribution, and abundances) in invertebrate communities in the vicinity of effluent discharge relative to that of reference communities. Biological monitoring studies require a survey if the concentration of effluent in the exposure area is greater than 1% in the area located within 100 meters of an effluent discharge point (Environment Canada, 2010). Collection of benthic invertebrates occurs to determine if there are changes in the effects indicators between exposure and reference areas, or along an effluent concentration gradient. Effect indicators include: total benthic

invertebrate density, evenness index, taxa richness and similarity index. Effect endpoints include: numbers of animals per unit area, Simpson's evenness, number of taxa, and Bray-Curtis index (Environment Canada, 2010, 2012). In order to identify if any 'effects' are present on the indicators, the data are assessed to determine if statistical differences exist between exposure and reference areas. As mentioned previously, biological monitoring studies are repeated in subsequent three to six-year cycles to confirm that observed effects are not a one-time occurrence. If the same effect on the benthic invertebrate community reoccurs in consecutive cycles, the effect is considered confirmed.

Benthic invertebrates are directly related to local fish populations as a food supply, which allows for the assessment of habitat indirectly (Government of Canada 1985). Several studies have been performed that display strong evidence supporting the relationship between macroinvertebrate and fish community compositions. For example, Zimmer et al. (2001) performed a study comparing the size distribution of aquatic invertebrates in two prairie wetlands, 'Rollag' and 'Sagebraten', located on U.S. Fish and Wildlife Service Waterfowl Production Areas in west-central Minnesota, with one supporting a population of fathead minnows and the other being fishless. They observed reduced biomass in all size classes of invertebrates in the wetland containing fish. Diet analysis of the fathead minnows collected during the study (n=240) showed that a variety of aquatic invertebrates were consumed, and that invertebrates accounted for 83% of the diet (by weight) of the fish.

In a similar study performed by Gilliam et al. (1989), they looked at the relationship between fish and benthic macroinvertebrate populations in a warm-water, second-order stream in Albany County, New York. They found that a direct relationship existed between fish and invertebrates abundances, where reductions in total invertebrate volume of 79% to 90% occurred in the presence of juvenile Creek Chub (*Semotilus atromaculatus*) relative to fishless treatments. Their main conclusion was that fish can alter the abundance and size structure of the major invertebrate taxa in the streams. Gilliam et al. (1989) also stated that the recolonization of depleted areas with benthic invertebrates tended to counteract patterns of fish patchiness.

Several things need to be considered when sampling for benthic invertebrates. These include quantitative sampling equipment, mesh size, and the type of habitat in which the organisms reside. The use of quantitative sampling equipment allows for the sampling of a known area or volume of habitat, which allows for comparisons between sampling events or sample locations. Sampling equipment should be non-selective and chosen based on its suitability for habitat class (e.g., depositional or erosional). Samplers that collect benthic communities from the bottom sediments are recommended unless it is not possible due to physical constraints. For PPER and MMER EEM programs, aquatic benthic invertebrates are usually identified to the family-level. This is felt to provide sufficient taxonomic resolution to detect community responses to anthropogenic disturbances (Bowman and Bailey 1997; Warwick 1998a; Warwick 1988b).

1.2.3 Disadvantages of the Benthic Invertebrate Community Survey

There are several weaknesses with the current approach to the benthic invertebrate community survey. First, benthic invertebrates are generally patchy in spatial distribution and therefore require many samples in order to get an accurate estimation of community (Malmqvist, 2002). There is a large temporal variance of many populations resulting in highly variable abundance measurements; the organisms present in spring are likely not to be found in late summer. For example, a field-based investigation was conducted by Reid et al. (1995) to evaluate the temporal variation of benthic invertebrates within three south-central Ontario lakes during ice-off season. They determined that temporal variation was relatively minor among samples collected on the same day (2% temporal variation) but that samples collected over an increased period of time, as little as once per week for a 3-week period, resulted in an increase in variability (6.5% temporal variation). Furthermore, samples collected over a 6-week period showed that temporal variance averaged 26% temporal variation. Additionally, the presence of multiple microhabitats within streams and rivers (e.g., riffles and pools) can influence and support different benthic communities lending to variability in spatial distribution (Hanson and Lagadic, 2005; Malmqvist, 2002). For example, some species are predominantly found in riffle areas of lotic systems on the coarse substratum, which are favourable sites for the growth of algae's and mosses. Meanwhile, other species are found predominantly in pool areas of lotic systems, which tend to have weaker currents,

gentler gradients, and finer substratum particles, allowing for benthos that maintain a burrowing lifestyle (Malmqvist, 2002).

The frequency (3 to 6 years depending on previous monitoring results) in which biological monitoring is carried may be inadequate to capture the majority of potential impacts on benthic invertebrate communities subject to effluent discharge (Environment Canada, 2010; Hanson and Lagadic, 2005). The normal approach results in an impact being detected and reported well after the event has occurred. Due to this, the possible result is observing no strong link between cause and effect, especially if effluents fluctuate in composition. Montz et al. (2010) conducted a study looking to evaluate response and recovery of benthic invertebrate communities exposed to copper at sample sites along the Pelican Brook, the Little Pine River, and the Nokasippi River in Crow Wing County, Minnesota, USA. They reported that for streams exposed to copper (between 0.6 and 0.8 mg/L), major changes in the invertebrate community were seen. Nearly two thirds of pre-treatment taxa were lost; mayflies were eliminated post-treatment, the diversity of caddisflies was substantially reduced, and community composition was overwhelmingly dominated by more tolerant taxa such as Diptera. Recovery of the benthic community was monitored and began to occur within a year after treatment. Within two years post-treatment, taxa numbers were similar to that of pre-treatment numbers. Thus, demonstrating the potential to miss an event that has a significant impact on the benthic invertebrate community due to recovery in a timeframe that is less than the frequency in which mandated biological monitoring is carried out. Ideally, continuous

monitoring would be carried out to capture significant impacts, but this is not realistic. To capture these events with a realistic sampling period, I would recommend that samples be collected pre- and post-effluent release events, if possible. If not possible, I would suggest that sampling be carried out several times from spring melt to fall/winter freeze-up, depending on the geography and climate of the area of interest.

Another drawback to the current benthic community survey is that collection techniques used to collect samples of benthic invertebrates can be destructive to their habitat (e.g., kicknet, hess sampling – both disrupt the bottom of the water body), as well as the sacrifice of organisms for samples. According to the Canadian Aquatic Biomonitoring Network (CABIN), kicknet samples are performed by placing the net downstream of the sampler and while walking, the substrate is kicked and disturbed to a depth of 5 to 10 cm for a period of three minutes. During this process the microhabitats in which benthic invertebrates reside are heavily disturbed or altogether destroyed. All collected benthic invertebrates are then preserved with 95% ethanol, killing the organisms, which are processed at a later date (CABIN).

Finally, a considerable amount of time, taxonomic expertise, and financial resources are required during sample analysis to sort and identify benthic invertebrates to the desired taxonomic level (Chapman, 1999). Benthic invertebrate community surveys involve quantitative sampling using multiple replicates. After the samples have been collected they are sent back to the lab where they are sorted, invertebrates are identified to the required level and

enumerated, and if necessary, the invertebrates need to be processed for weighing to determine biomass. The effort involved requires that a time lag of six months usually exists before biomass numbers are quantified and can prove to be a large expense, which is typically $350.00 per sample at a commercial laboratory (Dr. Michael White and Dr. Shari Weech, personal communication).

1.3 Application of Chitobiase in Environment Effects Monitoring

Chitobiase is one of two chitinolytic enzymes released during invertebrate molting, the other being chitinase (Muzzarelli 1977). During the intermediate stages of invertebrate molting, chitobiase is produced and secreted from the epidermis into the molting fluid that is found between the epidermis and the endocuticle. During the shedding of the invertebrates exoskeleton chitobiase is not re-adsorbed by the organism and released into the surrounding aquatic environment (Spindler 1976). Additional details regarding chitobiase is presented in section 1.4 of this thesis. The potential of chitobiase activity as a metric in EEM is a result of the relationship between benthic invertebrate biomass and liberated chitobiase and the ability to detect liberated chitobiase in the field (Oosterhuis et al. 2000; Sastri and Roff 2000; Sastri and Dower 2009). The production of chitobiase by invertebrates in aquatic ecosystems is a reflection of multiple factors such as number of organisms present, the growth or development rate of the organisms, and the size of the organisms that are actively molting. A simple fluorescence assay can be used to detect released chitobiase in aquatic ecosystems (Oosterhuis et al. 2000; Sastri and Roff 2000). The fluorescence

assay used to detect chitobiase consists of collecting a water sample from the aquatic system of interest and filtering it to remove any bacteria or organic matter. The sample is then incubated in the dark with a substrate which is cleaved by chitobiase. During the incubation, chitobiase cleaves the substrate and the cleaved portion of the substrate is that of which is detected during the fluorescence assay. After incubation, the reaction between chitobiase in the water samples and the substrate is stopped and analyzed using a spectrofluorometer. The amount of substrate that is cleaved is representative of the amount of chitobiase present in the sample, i.e., the more chitobiase in the sample, the more substrate that is cleaved, which results in a higher fluorescence detection and vice-versa. A detailed description of how the chitobiase assay is performed is included later in this thesis in chapter 2 section 2.3.7.

The assay is rapid, where sample collection, preparation, incubation and analysis can all be performed within a day, depending on the sample location and the quantity of samples taken. The assay is also cost effective as it does not require the use of someone with a high level of taxonomic expertise and due to its rapid analysis, it takes significantly less time compared to traditional benthic invertebrate biomass estimate techniques. Therefore, measurement of chitobiase within the water column presents a technique that could be used to assess invertebrate biomass in aquatic ecosystems.

Several studies have been performed demonstrating the positive relationship between measured chitobiase activity and aquatic invertebrate

biomass. For example, Sastri and Roff (2000) performed a laboratory-based study where they looked at the rate of chitobiase degradation as a measure of development rate of the community in three freshwater cladoceran species; *Ceriodaphnia* spp., *Daphnia pulex*, and *Daphnia magna*. Using single-species samples and mixed-species samples under laboratory conditions they were able to show that chitobiase activity is a function of body size ($r^2 = 0.82$, $p < 0.0001$). The relationship between body size and chitobiase activity released into the medium after molting could be described by a single mass-specific regression. They were also able to demonstrate that the rate of decay of chitobiase in the medium can be used as a measure of the average rate of development of the invertebrate community in the laboratory cultures. Similarly, Oosterhuis et al. (2000) looked at the relationship between chitobiase activity and the marine copepod *Temora longicornis*. Under laboratory conditions, biomass was regressed on the daily release of chitobiase and a significant positive relationship was observed, which was very similar to the relationship between increase in body weight and the release of chitobiase from *T. longicornis*. They stated that absolute free chitobiase activity cannot provide a general simple index for secondary production without the additional knowledge of the turnover rate (degradation) of chitobiase. Vrba and Machacek (1994) performed a study that looked at the release of chitobiase during crustacean molting. The experiment utilized *Daphnia pulicaria* under laboratory conditions where *D. pulicaria* were observed under a microscope and those expected to molt were measured (body length) and chitobiase activity was measured immediately after molting. They

found that the individual amount of chitobiase was proportional to the body size resulting in a strong correlation between total apparent chitobiase activity vs. body length ($r^2 = 0.94$, n = 20, $P < 0.001$)

In terms of field studies, Sastri and Dower (2009) carried out a study in the Strait of Georgia, British Columbia, Canada looking at the relationship between the turnover rate of chitobiase and biomass production rate. They found a consistent relationship ($r^2 = 0.949$, $p < 0.001$) between chitobiase and individual dry weight for three morphologically different groups, copepods, decapod larvae and mysids. Similarly, a field study conducted by Avila et al. (2012) looked at the validity of the chitobiase assay to estimate zooplankton secondary production in the Patos Lagoon estuary in Rio Grande, southern Brazil. They reported findings similar to that of Sastri and Dower (2009), where the activity of chitobiase released into the water during the molting process found for the copepod *A. tonsa,* showed similar patterns to that reported for copepoda, mysidacea and decapoda. As well, they noted a similar relationship between chitobiase and copepod body length.

Based on the findings of the previously mentioned studies, it can be concluded that the use of chitobiase to estimate secondary production could be a valid and reliable measure for monitoring. The rate of degradation of chitobiase within an system represents the turnover rate of chitobiase produced by developing arthropods (Sastri and Dower 2006). This assumes a steady-state has been reached, where the amount of chitobiase being produced is equal to the amount being removed or utilized by other factors such as photodegradation,

consumption and absorption via bacteria, dilution into the water column, etc. This rate can be used as a measure of the average rate of development of the arthropod community. Ambient chitobiase activity gives you a direct indication that molting is occurring and that arthropods are present, but it cannot provide a general simple index for secondary production without knowledge of the rate of degradation (Oosterhuis et al. 2000). Therefore, it is necessary to determine the rate of chitobiase degradation within an ecosystem to estimate secondary production. The rate constant of chitobiase degradation can be determined by graphing $\ln(C/C_0)$vs. time, where C is chitobiase activity at time i and C_0 is the chitobiase activity at time 0. The slope of the resultant trend line is the rate constant for degradation of the chitobiase. Assuming that the system from which the sample was taken from is in equilibrium, the chitobiase rate constant for production is equal to the inverse of the rate of degradation.

The implementation of the chitobiase assay in an environmental monitoring context could be a useful additional metric when looking at benthic community status. The assay is straightforward, rapid, and efficient and could prove to be a suitable measure for detecting the impacts of stressors on benthic communities. Please see Chapter 2 Section 2.3.7 for additional details regarding the chitobiase assay. This is true specifically in terms of abundance, as the abundance or biomass of benthic invertebrates may be correlated to chitobiase activity within a system (Hanson and Lagadic, 2005). Community species composition or diversity cannot be estimated by way of this assay, as well, it cannot identify the stressor or impact present in the system. Therefore, the use of chitobiase is more

of a functional measure than a structural one as all invertebrates are incorporated into a single measure. The use of the chitobiase assay to assess benthic arthropod communities in the field would help eliminate some of the previously mentioned deficiencies of traditional benthic invertebrate monitoring. Increased frequency of chitobiase sampling could also occur to develop a historical record of the activity for a specific area where changes in arthropod communities could be monitored, such as in EEM programs for pulp and paper mills and/or metal mining.

1.4 Chitin and Chitinolytic Enzymes

Chitin is fundamentally important for physiological functions in prokaryotes and eukaryotes, including invertebrates, fungi, and diatom algae (Cohen, 2010). It is one of the most abundant extracellular polysaccharides in terms of biomass second only to cellulose and possibly lignin (Cohen, 1993). The annual production of chitin by marine zooplankton, invertebrates, and terrestrial arthropods is estimated to be 10^{10} tons (Gooday, 1990a). Chitin is the main constituent of arthropod exoskeletons and fungal cell walls and contributes to the rigidity and mechanical strength of these structures (Avila et al., 2011; Cohen, 2010). These properties are essential for external support, but restrict growth and development due to limits in shape and size, which is overcome through enzymatic hydrolysis carried out through the use of chitinases and β-*N*-acetyl-glucosaminidases (chitobiases) (Kramer and Koga, 1986).

Chitin hydrolysis (degradation) entails the breakage of β-1,4-glycosidic

bonds via digestive enzymes and the complete degradation results in the generation of GlcNAc monomers (Cohen, 2010). Arthropods grow and develop through a series of successive molting events or ecdysis (Roff, et al. 1994). During ecdysis, two chitinolytic enzymes are produced and secreted from the epidermis into the molting fluid that is found between the epidermis and the endocuticle. The first chitinolytic enzymes are chitinases, family 18 glycosyl hydrolases, which contain a basic structure that includes a catalytic region, a cysteine-rich region and a PEST (proline, glutamic acid, serine and threonine rich peptide sequence)-like region (Kramer and Muthukrishnan, 1997). Chitinases are activated after the release of ecdysterone and begin to hydrolyze the chitin oligomers into dimers and terminal oligomers (Kimura, 1973; Marcu and Locke, 1998). The second chitinolytic enzymes are chitobiases, family 20 glycosyl hydrolases, which are released into the molting fluid prior to the activation of chitinases. Chitobiases are activated after the creation of the smaller chitin dimers and oligomers by chitinases, which are hydrolyzed into monomers (Muzzarelli, 1977). The newly created monomers are then re-adsorbed into the new cuticle and can be used in the formation of new chitin microfibribrils. During ecdysis, chitinases and chitobiases are not reabsorbed by the arthropod and are released into the aquatic environment (Spindler, 1976).

1.4.1 Sources of Chitobiase and Chitinolytic Enzymes in Aquatic Systems

Chitinolytic enzymes are native to many living organisms including bacteria, protists, fungi, invertebrates, fishes, and mammals (Espie and Roff, 1995b; Gutowska, Drazen, and Robison, 2004; Overdijk, Van Steijn, and Odds,

1996; D. C. Smith, Steward, Long, and Azam, 1995). Although many organisms produce chitobiase, not all organisms release chitobiase into the surrounding media and contribute significantly to detectable amounts in aquatic ecosystems.

1.4.2 Invertebrates

During the growth and development of invertebrates, a series of successive molts occur in which the exoskeleton is shed and replaced with a new one (Roff et al., 1994). All invertebrates experience periodic molting of their exoskeleton, which is required for growth and development. Molting initially involves apolysis, the separation of the cuticle from the epidermis, where an increase in several enzymes and hormones occurs and is collectively referred to as the molting fluid (Stevenson, 2019; Skinner 1985). Such enzymes found in the molting fluid include chitinases and chitobiase, along with the molting hormone ecdysone. The initiation of apolysis is triggered by the hormone ecdysone, which results in an increase in concentration of both chitinolytic enzymes (Jeuniaux 1963; Muzzarelli, 1977; Spindler, 1987). During ecdysis, the molting fluid is not reabsorbed by the invertebrate and is liberated into the aquatic environment (Merzendorfer and Zimoch, 2003). Chitobiase activity in aquatic ecosystems due to invertebrates is a reflection of several factors including the number of organisms present, the size of the organisms and the growth or rate of development of the organisms that are actively molting (Hanson and Lagadic, 2005). Therefore, as mentioned before and observed in studies by Vrba and Machacek (1994), Oosterhuis et al. (2000), Sastri and Roff (2000), Sastri and Dower (2009), and Avila et al. (2012), the concentration of chitobiase liberated

into the aquatic environment via aquatic arthropods is relative to the biomass of the organisms releasing it. In lotic systems, benthic invertebrates are likely to be the largest contributor of chitobiase because they are typically the most abundant aquatic macroinvertebrates present (Allan 1995; Wallace and Webster 1996).

1.4.3 Bacteria

Extracellular glycohydrolases, such as chitobiase, have been recognized to fulfill an essential role in the microbial utilization of both dissolved organic carbon (DOC – oligomers, polymers, colloids) and particulate organic carbon (POC) in aquatic environments (Hoppe 1983, 1991; Hoppe et al. 1988; Chrost et al. 1989; Chrost 1989, 1990, 1991; Munster 1991; Munster and De Haan 1998). Because of this essential role, bacteria are a major source of particle-bound chitobiase in freshwater (Vrba et al., 2004). DOC and POC are sources of nutrients for bacteria with their main constituent being chitin (up to 90%) and are broken down via bound chitobiase (Misic and Fabiano 2005; Nedoma et al. 1994). A relationship exists between DOC and chitobiase activity due to increased presence of bacteria utilizing DOC; greater DOC concentrations results in increased chitobiase activity (Findlay et al. 1997). Extracellular chitobiase can be produced and released by bacteria and fungi, which can contribute to the total chitobiase activity that is present in a freshwater ecosystem (Vrba et al. 2004).

1.4.4 Fish

Chitinolytic enzymes, including chitobiase, are endogenous to many living organisms such as fish. As with other organisms that produce chitobiase, it is

used to degrade polymers of chitin and its activity varies greatly between fish species (Fange et al. 1979; Lindsay et al. 1984). Chitinous arthropods are a primary food source for fish in freshwater ecosystems and therefore chitin degradation is critical in the digestive processes (Muzzarelli, 1977). Chitinolytic enzymes are primarily associated with the stomach but are also present in the digestive tract (Lindsay et al. 1984). Not only do chitinolytic enzymes act as digestive enzymes to disrupt chitinous exoskeletons allowing for access to nutrient-rich inner tissues, they also have the added potential to result in additional energy gain from a meal through the breakdown of chitin into single units of *N*-acetyl-glucosamine (NAG) (Fange et al. 1979; Jeuniaux 1993). In a study performed by (Gutowska et al. (2004), chitobiase activity was found in the stomach tissue, stomach contents, intestinal tissue and intestinal contents of 13 species of marine fishes. Chitobiase activity was at its greatest in the stomach tissue, followed by stomach contents, intestinal tissue and finally intestinal contents. Due to the fact that chitobiase activity was greater in tissue samples and progressively decreased during the digestive process, it is logical to assume that an insignificant amount of chitobiase would be released into the aquatic environment by fish, particularly compared to that of the amount released by arthropods.

1.4.5 Diatoms, Fungi and Protists

The cell walls of fungi are comprised of 22 to 44% chitin (Muzzarelli 1977; Muzzarelli et al. 1994). Chitinolytic enzymes produced by fungi perform different functions such as cell division, sporulation, spore germination, branching and

parasitism (Adams 2004; De Marco et al. 2000; Karlssandon Stenlid 2008). The degradation of chitin in filamentous fungi and yeasts is crucial to maintaining standard hyphal growth and is necessary for hyphal branching (Cohen, 2010). In yeast cells, the hydrolysis of chitin facilitates spore germination and release, as well as budding and septum formation (Gooday 1990b). Fungal chitinases also play a major role in ecosystems via the degradation and cycling of carbon and nitrogen from chitin (Kellner and Vandenbol 2010). Vrba et al. (2004) performed a study looking at the relationship between extracellular glycolytic enzymes, such as chitobiase, and bacteria. During their study they observed alternate organisms such as diatoms that contribute to extracellular glycolytic enzymes and that diatom biomass was tightly correlated with glycolytic enzymes. Additionally, Vrba et al. (1996) observed correlation between protistan biomass and chitobiase activity.

1.5 Factors Influencing Chitobiase in the Environment

The chemical and physical properties of an aquatic ecosystem can affect the stability and presence of released chitobiase in the water column. Therefore, the measurement and detection of chitobiase activity within an aquatic system can be influenced by these properties. When looking to utilize and compare chitobiase activity between aquatic systems, extensive background knowledge on the chemical and physical properties need to be established. It should be noted that it is standard practice as part of the EEM framework to measure the chemical and physical properties of the systems being monitored. These

properties must be similar between sites in order to be able to compare chitobiase activity.

1.5.1 Temperature

The effect of temperature on chitobiase activity varies between organisms and species. Measures of maximum chitobiase activity for different species occurs over a range of temperatures; from 30-35°C for *Acartia tonsa* (marine copepod) to 75°C for the fungi *Talaromyces emersonii* (thermophilic fungus) (Avila et al. 2011; O'Connel et al. 2008). Differences in temperatures for maximum chitobiase activity is likely to be explained by the environmental temperature conditions in which the organism resides (Avila, et al. 2011). The temperatures found in natural systems are not likely to directly affect chitobiase after its release, however, these temperatures influence growth rates and emergence of insects (Butler, 1984). Generally, increased growth rates among invertebrates are observed at warmer temperatures and decreased growth rates at lower temperatures. The effect temperature has on invertebrate growth rate is directly linked to the amount of chitobiase being released into the aquatic environment, where increased growth rate results in greater chitobiase activity and vice versa (growth is the product of development = molt). In a laboratory-based study performed by Espie and Roff (1995), they noted that the relationship between chitobiase activity and duration of the molt cycle, as modulated by temperature, was significant ($r^2 = 0.77$) but nonlinear when observing cultures of *D. magna* grown between 6-25°C; as water temperature decreased, a decrease in chitobiase production occurred.

1.5.2 pH

The pH range in which chitobiase maintains enzymatic activity is between 3.2 to 11 (LeCleiret al. 2007; Zielkowski and Spindler 1978). Optimal pH for chitobiase activity is varied between species, Avila et al. (2011) reported an optimal pH range between 4.0 – 7.7 for the chitobiase activity of the copepod *Acartia tonsa*. Meanwhile, Espie and Roff (1995) reported an optimal pH range for chitobiase activity in *Daphnia magna* to be between 5.0 – 5.5. As with most enzymes, when pH values are outside their optimum range the reaction slows down. If the pH is too low or too high it can damage the active sites, essentially changing the shape and substrates will no longer fit thus halting the reaction and not allow for the monitoring/detection of chitobiase.

1.5.3 Discharge and Current

Even mixing of chitobiase within a lotic system should occur fairly rapidly after release due to the dynamics of advection and dispersion, particularly in regards to narrower and shallower systems. The full distribution of chitobiase within the vertical profile of a stream or river should occur after a distance of approximately 12 times the channel depth (Fischer et al. 1979; Hanson and Lagadic 2005). However, due to variability in stream and river discharge, inconsistent discharge can contribute to a difference in chitobiase activity in the same volume of water. In order to avoid inconsistencies of chitobiase activity, standardization of chitobiase activity needs to be performed based on discharge, by multiplying the measured chitobiase activity by stream discharge (Mackenzie,

2016). Standardization needs to be performed prior to making comparisons between sites.

1.5.4 Chemicals

Numerous chemicals have inhibitory effects on molting enzymes including chitobiase activity. Polybrominated diphenyl ethers (PBDEs) are a class of chemical compounds included in plastics, electrical equipment, and textiles due to their flame retardant properties (Alaee, Arias, Sjödin, and Bergman, 2003). PDBEs are persistent in the environment and have the potential to bioaccumulate in aquatic biota such as fish, bivalves and amphipods (La Guardia et al. 2006; de Wit et al. 2010; Tomy et al. 2004; Gustafsson et al. 1999; Tlili et al. 2012) and have been banned in Europe since 2004 (Coquery et al. 2005). Gismondi and Thomé (2014) performed a study in which they found that chitobiase activity was inhibited in female gammarids exposed to the PBDE 2,2',4,4',5-penta-bromodiphenyl ether (BDE-99) for 48 h at any concentration and that chitobiase activity was inhibited in male gammarids exposed for 48 h at 0.1 µg/L.

Other chemicals such as fungicides and insecticides also have inhibitory effects on chitobiase. For example, TNG-chitotriomycin, an anti-fungal and insecticidal agent is responsible for inhibiting chitobiase activity in both fungi and insects but not in plants or animals (Yang et al. 2009). Another pesticide, bromacetic acid, is used in soils in the growing of tomatoes and strawberries as a fungicide, insecticide, and herbicide. (Xie et al. 2007) found that at a concentration of 17.05 ± 0.65 mM, bromacetic acid inhibits 50% of chitobiase

activity. Other chemicals such as hydrogen peroxide, formaldehyde, and dioxane also have inhibitory effects on the enzyme chitobiase and have been well studied (Xie et al. 2004; Xie et al. 2006).

1.6 Mesocosm Studies

Freshwater aquatic mesocosm ponds are artificial ecosystems constructed to study the effects and ecological fates of contaminants and pollutants. Mesocosms are designed to mimic the natural environment but are not a perfect surrogate due to lacking aquatic ecosystem components such as fish communities, certain macrophyte communities, and differences in limnological zones (Williams et al. 2003). They are considered to act as a compromise or an intermediate between laboratory-based testing and full-scale field assessments (Caquet et al. 1996). The use of mesocosms, such as those used in our study, allow for more realistic testing due to interactions that occur within a complex environment compared to that of a laboratory-based study (Solomon, 1996; Van den Brink et al. 2005). Studies that emulate field studies may also provide more realistic exposure scenarios than studies performed in the lab, where compounds will behave as they would in the environment (Liber et al. 1993). Additionally, multiple mesocosm units within a study allow replication and the application of a concentration gradient (Graney et al. 1995).

There are several benefits to using a mesocosm study for the investigation of chitobiase activity and production in relationship to aquatic invertebrate biomass. Multiple species of aquatic invertebrates can live and survive in a mesocosm system compared to the selectivity that needs to be followed in a

laboratory setting. Culturing all of the invertebrates in a laboratory that can be present in a mesocosm setting would be time consuming and could potentially be very difficult, due to the presence of numerous species. The variation in invertebrates present within the semi-field setting can account for the variation in released chitobiase, where different species release different amounts of chitobiase at different rates during their molting process (Espie and Roff 1995a; Sastri and Roff 2000; Avila et al. 2011). Another advantage is that chitobiase activity and production can be monitored over time, which is more difficult and time consuming to do in the field due to preparation time, accessibility, etc.. This can allow for the compilation of data to develop baseline estimates and historical records.

1.7 Compounds of Interest

1.7.1 Sulfamethoxazole

Sulfonamide antibiotics are one of the oldest classes of antimicrobial agents among administered antibiotic classes (Gmurek et al. 2015). A derivative of the sulfonamide antibiotic class is sulfamethoxazole (SMX), chemical name 4-amino-*N*-(5-methylisoxazol-3-yl)-benzenesulfonamide, a synthetic bacteriostatic antimicrobial/antibiotic used in medicine to prevent and treat infections and is also used in feed additives to promote growth of livestock (Gong and Chu 2016; Thiele-Bruhn 2003). SMX is designed to target specific metabolic pathways, more specifically to inhibit bacterial synthesis of dihydrofolic acid by competing with para-aminobenzoic acid (PABA) for binding to dihydropteroate synthetase

(dihydrofolate synthetase) (García-Galán et al. 2008; Schauss et al. 2009). The inhibition of dihydrofolic acid synthesis results in the decrease in synthesis of bacterial nucleotides and DNA, thus acting bacteriostatic in nature. By inhibiting the biosynthetic pathway of folate (a molecule essential to all living organisms), SMX will not only affect bacteria but can also have unknown adverse effects on non-target organisms such as algae, plants, invertebrates, and fish (García-Galán et al. 2008; Kim et al. 2007; Schauss et al. 2009). Numerous studies have demonstrated poor and slow biodegradation of SMX resulting in low removal efficiencies during conventional biological processes (García-Galán et al. 2008; Ingerslev and Halling-Sorensen 2000; Larcher and Yargeau 2011; Perez et al. 2005). Additionally, the presence of SMX in the environment could result in the development, transfer, and promotion of environmental bacterial antibiotic resistance (Gullberg et al. 2011).

SMX is one of the most widely prescribed sulfonamide drugs and has been frequently detected in surface and drinking waters as well as in effluents from wastewater treatment plants (Bahnmüller et al. 2014; Białk-Bielińska et al. 2011; García-Galán et al. 2008; Gao et al. 2014; Hu et al. 2007; Kim et al. 2007). Recent studies have shown that SMX is omnipresent is the environment with a frequency of detection of up to 73% (Mompelat et al. 2009; Nödler et al. 2011; Rodayan et al. 2010; Watkinson et al. 2008). Approximately 15–25% of ingested SMX is not metabolized by humans and animals and is subsequently excreted through urine into the receiving environment (Akhtar et al. 2011; Clara et al. 2005; Paxeus 2004; Rodayan et al. 2010) In a study performed by (Kolpin et al.

(2004) looking at the presence of pharmaceuticals in wastewater samples collected from 139 stream sites across the United States found the maximum detected concentration of SMX to be 1.9 µg/L. Additionally, in a study performed by Watkinson et al. (2009) looking at the occurrence of antibiotics in the watersheds of South-East Queensland, Australia, found maximum SMX environmental concentrations to be 2.0 µg/L.

In a study performed by Ferrari et al. (2003), an acute toxicity test of SMX was performed using *Daphnia magna* and *Ceriodaphnia dubia* looking at the median effective concentration (EC50) resulting in mortality. For both organisms, the reported EC50 was >100,000 µg/L. A chronic toxicity test was also carried out during the same study looking at the no observed effect concentration (NOEC) of SMX on the reproduction of *C. dubia*. Ferrari et al. (2003) observed the NOEC of SMX on *C. dubia* reproduction to be 250 µg/L. Both SMX concentrations for the EC50 and the NOEC for daphnia species are well above the detected maximal environmental concentrations of SMX in the aforementioned studies, which tend to be around 2.0 µg/L. Based on the available literature, environmentally relevant concentrations of SMX should not have a negative effect on aquatic zooplankton or invertebrate species, therefore directly effecting the production of chitobiase. Although little to no effect of SMX on aquatic invertebrates, including the production of chitobiase, is expected..

1.7.2 Diluted Bitumen

Bitumen is a highly-weathered oil that is extracted from naturally occurring oil sands deposits (Meyer et al. 2007). Diluted bitumen (dilbit), also referred to as

crude oil, is derived from bitumen, which is a highly viscous form of petroleum produced via extraction from surficial mining of oil sands. Dilbit is primarily composed of hydrocarbons, including aromatics, resins, saturates, and asphaltenes (King, Mason, Thamer, and Wohlgeschaffen, 2017). Dilbit is also made up of low molecular weight (LMW) saturates and mono- and di-aromatics that are derived from oil-gas condensates (Alsaadi et al. 2018). Aromatics present in dilbit include polycyclic aromatic compounds (PACs) and polycyclic aromatic hydrocarbons (PAHs) (Adams et al. 2014). Typically, diluent (i.e., natural gas condensate, synthetic crude, or a mixture of both) is added to bitumen to achieve a ratio of 30 to 70% diluent to bitumen, resulting in a decrease in viscosity to allow for easier transport via pipeline (Alderman et al. 2016; National Academies Press 2016).

The Oil Sands region of Alberta, Canada is one of the largest oil reserves in the world and is estimated to contain up to 50 billion cubic meters of bitumen (Dupuis and Ucan-Marin 2015; Environment Canada, Fisheries and Oceans Canada, Natural Resources Canada 2013; National Energy Board 2006). The extraction of bitumen has grown exponentially to over 300 million litres per day and most of the product is transported as dilbit via an extensive pipeline network across North America (Alderman et al. 2016; Dupuis and Ucan-Marin 2015). With the increased production and transportation of dilbit via pipeline across North America comes an associated increase in risk of spills or leaks into aquatic environments. Several incidents in North America regarding the accidental release of oil products into the environment have occurred, including the 2010

spill of 3.2 million litres of dilbit into the Talmadge Creek and the Kalamazoo River near Michigan, USA, via pipeline rupture (Crosby et al. 2013). In Canada, a more recent spill released approximately 225,000 L of dilbit into the North Saskatchewan River via pipeline rupture (Government of Saskatchewan 2016). Although spills are infrequent, leaks or spills of dilbit into the aquatic environment can be a major source of concern.

The effects of conventional oils on aquatic ecosystems and species is well studied, including the major constituents of dilbit, but little information is available on the effects of dilbit spills on freshwater ecosystems prior to 2015 (National Research Council 2005; Dupuis and Ucan-Marin 2015; Lee et al. 2015; Madison et al. 2015). Dilbit contains a greater amount of carbon than hydrogen, lower amounts of LMW compounds, and increased amounts of resins and asphaltenes compared to conventional oils resulting in increased adhesiveness and generates more persistent residues (Lee et al. 2015; National Academies of Sciences, Engineering, and Medicine 2016). Therefore, the fate and behavior of spilled dilbit differs than that of conventional oils when it is released into an aquatic environment (Tsaprailis and Zhou 2014). Since dilbit has a lower density than water, it will float and has the potential to contaminate shorelines and aquatic birds and mammals. The rapid evaporation of diluents present in dilbit can result in increased density facilitating its movement into the water column as droplets, the result of which is that the droplets will subsequently adsorb to particulates and thus presents the potential to contaminate sediments (Engineering and Medicine National Academies of Sciences 2015). The

constituents of oil commonly associated with oil toxicity are LMW aliphatics, aromatics, and larger three- to six-ringed PAHs (Adams et al. 2014).

To date, the majority of studies performed on oil toxicity address effects to fish and fish embryos (Robidoux et al. 2018). Robidoux et al. 2018 performed two different laboratory-based studies investigating the toxicological effects of dilbit on aquatic freshwater organisms. One study looked at the acute and chronic effects of unweathered, weathered, and dispersed weathered Access Western Blend (AWB) dilbit on fathead minnows (*Pimephales promelas*). The second study looked at the acute and chronic effects of weathered and unweathered Cold Lake Blend (CLB) dilbit on Daphnia (*Daphnia magna*), Ceriodaphnia (*Ceriodaphnia dubia*), and rainbow trout (*Oncorhyncus mykiss*). The results of the first study showed that acute (96 h) and chronic (7 days) tests using Fathead minnows exposed to weathered AWB (LC_{50}=2.06g/L and 1.31 g/L, respectively) is less toxic compared to the unweathered AWB (LC_{50}=0.628g/L and 0.593 g/L, respectively). The use of dispersant SPC_{1000} (1:10) with the weathered AWB significantly increased mortality (LC_{50}=0.023 g/L and 0.020 g/L, respectively) compared to the unweathered and weathered AWB. The dispersant SPC_{1000} showed 100% lethality at its concentration use (100 mg/L). The results of the second study showed that unweathered CLB demonstrated significant toxicity to rainbow trout (LC_{50}=5.66 g/L). Rainbow trout exposed to 0.32-10 g/L of CLB displayed signs of distress after 10 minutes of exposure and generally died after 24 hours. No mortality was observed in rainbow trout exposed to weathered CLB, even when the initial load concentration was increased to 18 g/L. Daphnia

exposed to 10g/L of CLB showed no mortality while Daphnia exposed to 32 g/L of CLB showed toxicity (LC_{50}>32g/L and mortality of 27%). No mortality was observed in Ceriodaphnia exposed to weathered CLB however, lethal toxicity (LC_{50}=6.43 g/L) was observed when exposed to CLB.

1.8 Hypothesis

It is hypothesized that chitobiase activity and rate of production will have a positive relationship with aquatic invertebrate biomass when evaluated in a mesocosm-based study. Additionally, it is hypothesized that chitobiase activity and production will have a positive relationship with aquatic benthic invertebrate biomass in a field-based study looking at freshwater lotic systems. If these hypotheses are correct, it will support the use of chitobiase as a metric in an environmental monitoring context.

1.9 Research Objectives

Current benthic invertebrate sampling practices employed in North American EEM programs requires a great deal of time and considerable taxonomic expertise and may miss significant impacts on these communities. To address, in part, these concerns, we propose to examine the utility of chitobiase to serve as a metric to complement current EEM programs and help address some of the above noted issues. No studies have been performed looking at the relationship between chitobiase activity and production in regards to aquatic

invertebrate biomass in freshwater lotic systems. Four research objectives were developed with these considerations in mind:

1) Investigate and evaluate the relationship, if any, between the rate of production of chitobiase and zooplankton and emergent insect biomass under aquatic mesocosm conditions during the summer months at the Prairie Wetland Research Facility at the University of Manitoba.
2) Investigate and evaluate the impacts of SMX, if any, on ambient chitobiase, the rate of chitobiase production, and emergent insect and zooplankton biomass under aquatic mesocosm conditions during the summer months at the Prairie Wetland Research Facility at the University of Manitoba.
3) Investigate and evaluate the impacts of dilbit, if any, on ambient chitobiase, the rate of chitobiase production, and emergent insect and zooplankton biomass under aquatic mesocosm conditions during the summer months at the Prairie Wetland Research Facility at the University of Manitoba.
4) Investigate and evaluate the relationship, if any, between the rate of production of chitobiase and benthic arthropod biomass in freshwater lotic systems, specifically during the Fall of 2015 in the Elk Valley Region of British Columbia, Canada.

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CHAPTER 2. THE USE OF CHITOBIASE ACTIVITY AS A MEASURE OF AQAUTIC INVERTEBRATE BIOMASS: A FIELD-BASED MESOCOSM STUDY

2.1 Abstract

Benthic invertebrate communities are an integral component of freshwater ecosystems. The techniques to assess their status in the field are typically labour and time intensive, and therefore the development and implementation of new rapid and cost-effective methodologies is warranted. A proposed option is the enzymatic assay to detect and quantify the rate of production of the molting enzyme chitobiase. In the present study, chitobiase, emergent insect, and zooplankton samples were collected from mesocosms at the Prairie Wetland Research Facility (PWRF) at the University of Manitoba, Canada, exposed to environmentally relevant concentrations of the antibiotic sulfamethoxazole (SMX) and diluted bitumen (dilbit). Sampling of chitobiase was performed for both ambient (standing) chitobiase activity and the rate of chitobiase production. Chitobiase activity was detected and quantified over the course of each study but no significant relationship was observed between the chitobiase measures and invertebrate biomass measures, and no effects of the stressors were detected. It can be concluded that in a mesocosm setting, chitobiase did not prove to be a successful measure of aquatic invertebrate secondary production and is likely due to several factors including the lack of sampling of benthic invertebrates and the relatively low sampling frequency. Additional studies are recommended to further assess the potential of chitobiase activity to be used in an environmental monitoring context.

2.2 Introduction

With an ever-increasing world population, anthropogenic influences induce multiple stressors on aquatic ecosystems and can ultimately result in potential changes in biodiversity and ecosystem functioning. With the social and economic changes of the last few decades, emerging contaminants have been released into the aquatic environment at relevant quantities that can pose a risk to the ecosystem structure and function. The increasing input of contaminants via anthropogenic activities and the enduring changes in the aquatic environment emphasizes the importance of research on methodological approaches to assess aquatic ecosystem health. Currently, benthic community surveys are used in environmental effects monitoring (EEM) programs in Canada to assess aquatic ecosystem health and evaluate the potential impacts of effluents on fish habitat (Environment Canada, 2010, 2012). Due to their abundance and range of tolerance to different stressors, benthic invertebrates lend themselves to being useful tools in identifying environmental impacts in aquatic ecosystems (Brown, 2001; Smith et al. 2011) The current approach has several shortcomings including: requiring a considerable amount of time and taxonomic expertise, difficulty in accounting for variability in abundance due to patchy distribution, requiring many samples to be collected and processed to obtain accurate estimates, and it can be expensive if hired out to a third party (Hanson and Lagadic, 2005). This supports the need for the development of new methodologies that are easier to perform and have faster turnaround times.

An alternative to traditional benthic invertebrate community surveys is the use of the arthropod molting enzyme, chitobiase. Chitin is the primary component of arthropod exoskeletons and is a polymer of β-(1-4) linked N-acetyl-glucosamine (NAG) (Hanson and Lagadic, 2005). Arthropods grow and develop through a series of molting events called ecdysis, where the old exoskeleton is shed and a new one is developed (Avila et al. 2011; Roff et al. 1994). During ecdysis, two chitinolytic enzymes are produced by arthropods and secreted into the molting fluid found between the epidermis and the endocuticle. The first chitinolytic enzyme released is chitinase, which hydrolyzes chitin into oligomers and trimers of NAG. The second chitinolytic enzyme released is chitobiase, which hydrolyzes the oligomers and trimers of NAG to NAG monomers (Muzzarelli, 1977). During ecdysis, chitinase and chitobiase are not re-absorbed by the arthropod but are released into the aquatic environment (Spindler, 1976). It is relatively straightforward to measure chitobiase activity in the water column from arthropod molting using a simple fluorescence assay (Oosterhuis et al. 2000; Sastri and Roff, 2000). Both enzymes can be detected in water samples but the fluorescence assay for chitobiase is easier to perform and is specifically the result of arthropod release (Espie and Roff, 1995b). Chitobiase activity in aquatic ecosystems is a reflection of several factors including: the number of aquatic invertebrates present, the size of the aquatic invertebrates, and the growth or development rate of those invertebrates that are actively molting (Hanson and Lagadic, 2005).

Several studies have been performed supporting the detection and use of chitobiase activity as an estimate of secondary production within aquatic ecosystems. Vrba and Machacek (Vrba and Machacek, 1994) performed a study where they monitored the release of chitobiase during the molting of the crustacean *Daphnia pulicaria*. Under laboratory conditions, *D. pulicaria* were observed using a microscope and the body length of those expected to molt was measured with chitobiase activity being measured immediately after molting. They observed a strong correlation between total apparent chitobiase activity vs. body length ($r^2 = 0.94$, $n = 20$, $P<0.001$). In a similar study, Sastri and Roff (Sastri and Roff, 2000) looked at the rate of chitobiase production as a measure of the development of *Ceriodaphnia* spp., *Daphnia pulex*, and *Daphnia magna*. Under laboratory conditions, they found that the relationship between body size and chitobiase activity could be described by a single mass-specific regression, where chitobiase activity is a function of body size ($r^2 = 0.82$, $p<0.0001$). Additionally, Sastri and Dower (Sastri and Dower, 2009) performed a field study in the Strait of Georgia, British Columbia, Canada, observing the relationship between biomass production rate and the rate of turnover of chitobiase. They reported a consistent relationship between chitobiase and dry weight for three different aquatic invertebrates; copepods, decapod larvae, and mysids.

Chitobiase activity has also been used in studies as an indicator of environmental impacts on aquatic ecosystems. Richards et al. (2008) performed a laboratory-based study looking at the effects of pharmaceuticals atorvastatin, fluoxetine, lovastatin, and sertraline on the chitobiase activity of *Daphnia magna*.

They found that chitobiase activity in the water surrounding *D. magna* increased significantly over a 24-hour period of exposure to 0.1 µg/L of fluoxetine but observed a significant decrease over 72 hours compared to control samples. Additionally, a study was performed by Wittmann and Suominen (Wittmann and Suominen, 2000) observing the effects of pulp and paper mill effluent on chitobiase activity in two separate lakes in Finland. They found that chitobiase activity was severely impaired in samples collected form the lake exposed to effluents compared to that of the control lake. They attributed the cause of the impairment in chitobiase activity as a decrease in zooplankton abundance and diversity due to periods of exposure to pollutants such as chlorine and heavy metals. The above technique maybe be useful in a number of scenarios including assessing effects related to emergent contaminants.

Emerging contaminants are not routinely monitored due to often not being included in current environmental legislation and the environmental fates are not well established or understood (Zenker et al. 2014). Pharmaceuticals are among the emerging contaminants and are one of the most relevant groups of substances in the aquatic environment due to universal use (Zenker et al. 2014). Due to high human population density and an increase in animal feeding operations, aquatic ecosystems are expected to be at high risk for potential contamination of pharmaceuticals(Han et al. 2006). It is estimated that several hundred thousand tons of pharmaceutical substances are being administered each year for human and animal healthcare and livestock farming (Daughton and Ruhoy 2008; Calisto and Esteves, 2009; Kümmerer, 2009). Antibiotics in

particular have been used in large quantities for decades but until recently, the existence of these substances in the environment have received little attention (Kümmerer, 2009).

One of the oldest classes of antimicrobial agents among administered antibiotics are sulfonamide antibiotics (Gmurek et al. 2015). Sulfamethoxazole (SMX) is one of the most commonly prescribed sulfonamide antibiotics used in both human and veterinary medicine (García-Galán et al. 2008; Kim et al. 2007; Santos et al. 2010) and has been frequently detected in surface and drinking waters, as well as in effluents from wastewater treatment plants (Bahnmüller et al. 2014; Białk-Bielińska et al. 2011; García-Galán et al. 2008; Gao et al. 2014; Hu et al. 2007; Kim et al. 2007). SMX is designed to inhibit bacterial synthesis of dihydrofolic acid but can also have unknown adverse effects on non-target organisms such as algae, fish, invertebrates, and plants (García-Galán et al. 2008; Kim et al. 2007; Schauss et al. 2009).

Not all of the antibiotic SMX is metabolized after ingestion, approximately 15-25%, which is subsequently excreted through urine into the receiving environment (Akhtar et al. 2011; Clara et al. 2005; Paxeus 2004; Rodayan et al. 2010). SMX is omnipresent in the environment, with recent studies showing a frequency of detection of up to 73% (Mompelat et al. 2009; Nödler et al. 2011; Rodayan et al. 2010; Watkinson et al. 2008). Kolpin et al. (2004) performed a study on 139 stream sites across the United States looking at the presence of pharmaceuticals in waste water samples and found the maximum detected concentration to be 1.9 µg/L. Additionally, Watkinson et al. (2008) performed a

study in the watersheds of south-east Queensland, Australia looking at the occurrence of antibiotics and observed maximum environmental concentrations of SMX to be 2.0 µg/L. Ferrari et al. (2003) performed an acute toxicity test of SMX on *Daphnia magna* and *Ceriodaphnia dubia* focusing on the median effective concentration (EC50) resulting in mortality. For both *Daphnia* species, the reported EC50 was >100,000 µg/L. Ferrari et al. (2003) also performed a chronic toxicity test looking at the no observed effect concentration (NOEC) of SMX on the reproduction of *C. dubia*. They observed a NOEC of 250 µg/L of SMX on the reproduction of *C. dubia*. Both acute and chronic concentrations of SMX observed to have an effect on the test species were well above the environmentally relevant concentration of SMX, which tends to be about 2.0 µg/L. Based on these results, SMX should not have a negative effect on aquatic invertebrates, and therefore it should not affect invertebrate molting and ultimately chitobiase production.

Another current anthropogenic contaminant of concern is diluted bitumen (dilbit), a derivative of bitumen. Bitumen is a naturally occurring highly weathered and highly viscous oil that is extracted from oil sands deposits (Meyer et al. 2007). Canada contains one of the largest oil reserves in the world, the Oil Sands region of Alberta, and it is estimated to contain up to 50 billion m^3 of bitumen (Dupuis and Ucan-Marin 2015; Environment Canada, Canada Fisheries and Oceans, and Natural Resources Canada 2013; National Energy Board 2006: National Energy Board 2006). Typically, diluents such as natural gas condensate, synthetic crude oil, or a mixture of the two is added to bitumen to

decrease its viscosity to allow for easier transport and resulting in a product known as dilbit (Alderman, et al. 2017; National Academies Press 2016: National Academies of Sciences, Engineering, and Medicine 2016). Dilbit is comprised of hydrocarbons including aromatics, asphaltenes, resins, and saturates (King et al. 2017). The aromatic compounds present in dilbit include polycyclic aromatic compounds (PACs) and polycyclic aromatic hydrocarbons (PAHs) (Adams et al. 2014). Over 300 million litres of bitumen is extracted per day, with most of the product being transported as dilbit via a vast pipeline network across North America (Alderman et al. 2017; Dupuis and Ucan-marin, 2015).

Several spills or leaks of diluted bitumen into aquatic environments in North America have occurred and although infrequent, they can be a major cause of concern. Due to the composition of dilbit, particularly asphaltenes and resins, they possess an increased adhesiveness compared to conventional oils, which has the potential to generate more persistent residues (Lee et al. 2015; National Academies Press 2016). Dilbit has a lower density than water allowing it to float on the surface, which has the potential to contaminate shorelines and aquatic birds and mammals. As well, if rapid evaporation of the diluents in dilbit spills occurs, it can result in an increase in density allowing movement of bitumen into the water column in the form of droplets which can then adhere to particulates presenting the potential to contaminate sediments (National Academies Press 2016).

Prior to 2015, few studies have looked at the effects of dilbit spills on freshwater ecosystems (National Research Council 2005; Dupuis and Ucan-

Marin 2015; Lee et al. 2015; Madison et al. 2015) with the majority of those studies focusing on the effects of oil to fish and fish embryos (Robidoux et al. 2018). Robidoux et al. (2018) performed two studies investigating the potential toxicological effects of dilbit on freshwater organisms. The first study focused on acute and chronic effects of unweathered, weathered, and dispersed weathered Access Western Blend (AWB) dilbit on fathead minnows (*Pimephales promelas*). They found that acute (96 h) and chronic (7 days) tests exposing Fathead minnows to unweathered AWB (LC_{50}=0.628g/L and 0.593 g/L, respectively) is more toxic compared to weathered AWB (LC_{50}=2.06g/L and 1.31 g/L, respectively). In addition of the dispersant SPC_{1000} (1:10) with weathered AWB, mortality was significantly increased (LC_{50}=0.023 g/L and 0.020 g/L, respectively) in the Fathead minnows compared to just unweathered and weathered AWB. 100% lethality of the dispersant SPC_{1000} on Fathead minnows was observed at its concentration use of 100 mg/L.

The second study performed by Robidoux et al. (2018) focused on acute and chronic effects of unweathered and weathered Cold Lake Blend (CLB) dilbit on Daphnia (*Daphnia magna*), Ceriodaphnia (*Ceriodaphnia dubia*), and Rainbow trout (*Oncorhyncus mykiss*). Unweathered CLB displayed significant toxicity to Rainbow trout (LC_{50}=5.66 g/L) with signs of distress being displayed after 10 minutes of exposure to 0.32-10 g/L and death generally occurring after 24 hours. Rainbow trout exposed to weathered CLB displayed no mortality, even with initial load concentrations being increased to 18 g/L. No mortality was displayed in Daphnia exposed to 10 g/L of CLB, with toxicity only being displayed at

concentrations of CLB >32 g/L (LC_{50}>32 g/L and mortality of 27%). The exposure of weathered CLB to Ceriodaphnia resulted in no mortality however, lethal toxicity (LC_{50}=6.43 g/L) was observed when exposed to unweathered CLB. Based on these findings it can be assumed that dilbit could have a negative impact, depending on encountered concentrations, on the production of chitobiase due to negative effects to aquatic invertebrates.

The objectives of this study were to: 1) characterize the ambient chitobiase activity and rate of production in a field-based mesocosm setting, 2) determine if there are any associations between measures of chitobiase and invertebrate community biomass; and 3) determine the impact of the SMX and diluted bitumen, if any, on chitobiase activity and rate of production and the invertebrate community. The overall purpose of this study is to determine if chitobiase activity is a suitable measure for assessing benthic invertebrate community status in an environmental effects monitoring context.

2.3 Methods and Materials

2.3.1 Test facility

The study was conducted at the Prairie Wetland Research Facility (PWRF) at the University of Manitoba (see Cardinal et al., 2014 for more specific details). The facility is comprised of 18 aboveground, circular, flat-bottomed, low-density polyethylene tanks, meant to represent a shallow prairie wetland setting, with two used a reservoir tanks. Each tank measures 2.7 m diameter x 0.72 m height for a 3.49 m^3 total volume. The mesocosms were previously established as model freshwater ecosystems prior to the commencement of the study. In

2011, approximately 0.23 m of soil was added to each tank to act as sediments typical to those of prairie wetlands (Anseeuw Brothers Ltd., Winnipeg, MB) (Cardinal et al., 2014). The sediments are clay-dominated and are comprised of approximately 50.9 % clay, 35.4 % silt, and 13.7 % sand. Organic carbon content was determined to be 2.6 ± 0.1 % while organic matter content was determined to be 4.5 ± 0.2 % (Cardinal et al., 2014).

The biota in the mesocosms were acquired from Oak Hammock Marsh in Stonewall Manitoba (50.152369 latitude, -97.109180 longitude) and consisted of algae, macrophytes, zooplankton, and benthos, as well as natural insect colonization due to the mesocosms being open to the surrounding outdoor environment. The major macrophyte species present included *Lemna* spp. (duckweed), *Myrophyllum sibiricum* (water milfoil), *Potamogeton* spp. (pondweed), *Typha* spp. (cattail), and *Zizania palustris* (wild rice). The mesocosms were supplemented with de-chlorinated water supplied by an adjacent waterline hookup from the City of Winnipeg. The water was run through a carbon filter to remove any residual chlorine. The mesocosms do not have inflowing or outflowing water and are isolated from each other, therefore no water or sediments move between systems.

Each tank was topped up with filtered water as needed, depending on the evaporation rates throughout the summer season to keep water levels constant. Several depth measurements were taken for each tank in seven-day intervals, where average depth was then determined. Water volumes were calculated for each tank using the average depth and tank dimensions.

2.3.2 Sulfamethoxazole ('SMX') treatment and analyses

The sulfamethoxazole study was assigned to six of the 18 available mesocosms. Three mesocosm tanks were treated with SMX (tanks 1, 6, and 8) on July 16, 2018, and three tanks were left untreated as controls (tanks 5, 11, and 16) (Figure 2.1). The SMX (>= 98%, supplied by Sigma Aldrich, Missouri, USA) was added to the treatment tanks to achieve a nominal target concentration of 250 µg/L. Each of the three treatment tanks were dosed with SMX once a week over a period of four weeks to maintain the exposure concentration. The required mass of SMX was dissolved into Milli-Q water and applied via an adjustable hose-end sprayer attached to achieve mixing in the water column. The same volume of Milli-Q water only was applied via the adjustable hose-end sprayer to each of the three control tanks.

Water from the mesocosms was sampled for SMX at one hour pre- and post-treatment and at 1, 4, and 7 days after the start of the study following each of the four weekly treatments, and at 14, 21, and 35 days after the start of the study following the final treatment. Water samples for the analyses of SMX were collected using an integrative water column sampler (Cardinal et al. 2014), resulting in a total combined sample volume of approximately 4 L. Grab samples were then collected from the combined sample and were stored in a 500 mL bottle prior to analysis. Triplicate samples were collected from each of the treated mesocosms on day 1 post-treatment. Rotating triplicate samples were collected on each sample day. Field blanks containing Milli-Q water were open during the

sampling period. SMX concentrations in the water samples were analyzed by LC-MS/MS at the University of Winnipeg.

2.3.3 Diluted bitumen ('dilbit') treatment

Prior to the treatment of the mesocosms with dilbit, two 62 L plastic totes (GSC plastic model 181108), with the bottoms removed were placed in each of the mesocosms. Cylindrical foam was attached to the bottom of each tote to promote buoyancy and the totes were secured in place in each mesocosms with wooden stakes pushed into the sediment. The totes were designed to contain the dilbit during diffusion into the water column and underlying sediments without allowing contact with the inner walls of the mesocosms. The treatment mesocosms were dosed with dilbit on August 18, 2015. There were two treatments, a "low" exposure and "high" exposure, and treatment control (Figure 2.2). The dilbit treatments consisted of Cold Lake Winter Blend dilbit, with the "low" exposure consisting of a 0.5 mL (0.0003 mL/L) addition of dilbit and the "high" exposure consisting of a 5 mL (0.003 mL/L) addition of dilbit. Dilbit was delivered 5 cm under the water surface to the mesocosms via a glass pipette, with each exposure being split between the two containment structures in each mesocosm.

2.3.4 Water quality parameters

Measures of temperature, specific conductivity, pH, and dissolved oxygen (DO) were taken near daily throughout the course of both the SMX and dilbit studies. Water quality monitoring for the SMX study began on May 19, 2015 and

finished on September 16, 2015, resulting in a pre-treatment monitoring period of 58 days and post-treatment monitoring period of 63 days, including day zero when the mesocosms were dosed. Water quality monitoring for the dilbit study began on May 19, 2015 and finished on October 16, 2015, resulting in a pre-treatment monitoring period of 90 days and a post-treatment monitoring period of 60 days, including day zero when the mesocosms were dosed. All measurements were taken near daily, excluding weekends, at consistent water depths using a YSI 650MDS. Photosynthetically active radiation (PAR) was measured in each tank on a weekly basis at approximately 12:00 noon CST throughout the course of the study using the Apogee Quantum Flux MQ-200 PAR meter. Subjective filamentous algae assessments were conducted throughout the course of each study to approximate algal growth. Water depths were measured at six random points in each mesocosm regularly throughout the course of each study when water quality parameters were being measured and were used to calculate water volume in each of the mesocosms.

2.3.5 Emergent insect sampling and analyses

Emergent insects were sampled by the use of emergence traps (Figure 2.3) constructed with a PVC pipe base and frame (approximate base of 0.012 m^2 and an approximate height of 1 m), mesh netting, and a sample bottle attached to the top filled half-way with 70% ethanol. Pieces of cylindrical foam were attached to the base of the traps to promote buoyancy. Each trap was secured in-place in each mesocosms with wooden stakes pushed into the sediment and

covered with a black plastic mesh to protect the traps from deer grazing and bird pecking.

Emergent insect traps were deployed in the morning of the day before sampling to allow for a 24-hour sampling period. Emergent insects were collected over a period of seven weeks starting on July 7, 2015, then every Thursday until August 21, 2015 for the SMX study. Samples were collected over a period of five weeks on every seventh day starting on August 4, 2015 to September 1, 2015 for the dilbit study. Samples were collected by detaching the ethanol jar atop of the trap and pouring the insects into a vial and rinsing out the trap jar with ethanol. The emergent insects collected during sampling were stored in vials with 70% ethanol for future identification and dry mass analyses.

All emergent insects collected in the samples were identified to the order level with the use of a Zeiss Stemi 2000-C dissection microscope and Zeiss Stereo CL 1500 ECO light source (Carl Zeiss AG, Oberkochen, Germany). Emergent insect samples were also analyzed for dry weight. The 70% ethanol used to preserve the insects was disposed of and each sample was rinsed with nanopur (18MΩ-cm) Milli-Q water (Millipore Corporation, Ma, USA). Each sample was then placed into the previously weighed aluminum weigh boat (weighed using a microbalance), which was then placed into a Yamato DKN812 constant temperature oven (Yamato Scientific Co., Ltd., Tokyo, Japan) at 60°C for 24 hours. After 24 hours, the samples were removed from the constant temperature oven and were placed in containers containing desiccant for one hour to remove

any moisture during the cooling off period. The mass of each sample was then measured using a microbalance.

2.3.6 Zooplankton sampling and analyses

Zooplankton in the mesocosms were sampled by the use of activity traps constructed with a 500 mL jar and a funnel attached to the opening of the jar, with the narrow portion of the funnel pointing into the jar, and with s-hooks and elastic bands to hold the funnel in place. Each sampler was placed horizontally on a bracket on the substrate of the mesocosms. Zooplankton traps were deployed the morning of the day before sampling to allow for a 24-hour sampling period as previously described (Lobson et al., 2018). Zooplankton samples were collected once per week over a period of seven weeks starting on July 7, 2015, until August 21, 2015 for the SMX study. Zooplankton samples were collected once per week over a period of five weeks starting on August 4, 2015 to September 1, 2015 for the dilbit study. Samples were collected by removing the traps from the mesocosms, detaching the funnel, and pouring the water and zooplankton in the jar through a sieve made PVC pipe with 53 µm synthetic nylon screen. The zooplankton collected on the nylon screen were then rinsed into sample vials using water from a squirt bottles. After the zooplankton were transferred into the sample vials, 5 mL of soda water was added. The samples were then transported to the lab where 5 mL of 10% sugar formalin solution was added to each sample for preservation.

The dry mass of the zooplankton samples were analyzed at Fisheries and Oceans Canada, Winnipeg, Manitoba, Canada. Prior to weighing the

zooplankton samples, several steps were taken in order separate the zooplankton in the sample from other organics and debris. Samples were poured through an 850 µm sieve attached to 63 µm sieve, to remove any larger objects in the sample, which were then rinsed with nanopur water to remove any formalin still present in the sample. The sample contents that remained on the 63 µm sieve were rinsed into a Petri dish with nanopur water. The sample contents in the Petri dish were then observed under a dissection microscope, where objects such as sand and organic debris were removed from the sample, leaving only zooplankton. The remaining zooplankton sample was then poured into a 20 mL scintillation vial. The zooplankton samples were then filtered onto pre-weighed paper filters using vacuum suction. The resulting filter papers containing the zooplankton were then placed in a Fisher Scientific forced air-drying oven model 6921 (Fisher Scientific International, Inc.) at 21°C and 35% humidity for 24 hours. After 24 hours, the samples were removed from the constant temperature oven and placed in containers containing desiccant for one hour to remove any excess moisture uptake during drying. After the samples had cooled, they were weighed using a Mettler Toledo model XP2U ultra-microbalance (Mettler Toledo, Inc). In order to ensure consistent weights, zooplankton samples were re-dried in the forced-air drying oven at 21°C and 35% humidity for an additional 24 hours and re-weighed using the ultra-micro balance. Any resultant weights for the same sample with a difference of greater than 2% we re-dried until a percent error of 2% or less was achieved.

2.3.7 Chitobiase water sampling and analyses

Surface water samples were used to determine ambient chitobiase activities, as well as, chitobiase production (degradation rate). Water samples were collected from approximately 15 cm below the surface of the mesocosms using a clean 500 mL glass jar. Sample water from the 500 mL glass jar was then sieved through a 53 µm synthetic nylon screen into a clean 250 mL amber glass jar to remove any aquatic organisms and debris. For the collection of a water sample to be analyzed for ambient chitobiase activity, 20 ml of the sieved sample water was drawn into a new 25 mL syringe and filtered through an attached 0.20 µm Filtropur syringe filter (Sarstedt, Inc.) into a pre-labeled 20 mL scintillation vial to remove any bacteria and other small debris. The 20 mL filtered sample was then transferred to a cooler where it was stored at 4°C in the dark until being transferred into a refrigerator for storage at 4°C. The remainder of the sieved water sample in the 250 mL amber glass jar was capped and placed into a cooler to reduce warming and transported to the lab. Samples for determining the rate of chitobiase degradation were collected as 20 mL subsamples from the 250 mL amber jar at times 1, 2, 3, and 6 hours, with a new 25 mL syringe and an aliquot filtered through a 0.20 µm syringe filter into a 20 mL glass scintillation vial in the lab. The subsamples were labeled and placed in a refrigerator in the dark at 4°C. Triplicate samples were collected at random from the mesocosms throughout both studies. Field blanks of nanopur (18MΩ-cm) Milli-Q water (Millipore Corporation, Ma, USA) were opened during water sampling at the PWRF and submitted for chitobiase analyses.

All water samples collected for the analyses of chitobiase were analyzed within 10 hours of their collection. Chitobiase measurements were performed according to the Sastri and Roff (2000) method, as modified by MacKenzie (2016). The assay uses 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (MUF-NAG) (>98% Purity (TLC) from Sigma-Aldrich) as the substrate, which is cleaved by chitobiase into N-acetyl-glucosamine (NAG) and fluorescent 4-methylubelliferone (MUF). A 100 µL aliquot of the 0.2 µm filtered sample was incubated for one hour at 25°C in a 96 well polystyrene microplate with 100 µL of 0.3 mM MUF-NAG in 0.15 M pH 5.5 citrate phosphate buffer. The MUF-NAG stock solution was created by dissolving MUF-NAG in methyl cellusolve (2-methoxyethanol for HPLC ≥ 99.9% from Sigma-Aldrich) to a concentration of 5 mM. The reaction was stopped with the addition of 50 µL 0.25 mM NaOH and fluorescence was measured at 360 nm excitation and 450 nm emission using a SpectraMax M2 spectrofluorometer microplate reader (Molecular Devices, LLC). Fluorescence values were then converted into MUF concentrations using a nine-point MUF calibration curve (0, 2, 4, 8, 16, 32, 64, 128, and 512 nM). Standards were created by serial dilution starting with a 4.12 mM MUF stock. Blanks were prepared using filtered samples in which MUF-NAG and NaOH were simultaneously added. Each sample was measured in quadruplicate and the arithmetic mean of the four measures used in subsequent analyses.

The method detection limit (MDL) and the limit of quantitation (LOQ) for the fluorescence assay were 1 nM MUF and 4 nM MUF, respectively. The MDL and LOQ were determined by using a pH 5.5 citrate phosphate buffer solution as

blank samples and run as if they were field samples. Seven microplate wells of incubated buffer solution were used as recommended for environmental chemistry analysis (Wisconsin Department of Natural Resources, 1996). The MDL was calculated using the standard deviation of the blanks multiplied by the Student's *t*-value for a 99% confidence level (Student's *t*-test value for seven wells is 3.143). The 95% confidence interval for the MDL was obtained by multiplying the MDL value by percentiles of chi-square degrees of freedom (lower confidence limit = 0.64*MDL; upper confidence limit = 2.20*MDL). The LOQ was determined by multiplying the standard deviation of the seven aliquots of the blank samples by 10.

To ensure confidence in the results, quality assurance and quality control (QA/QC) measures were employed. QA/QC measures included: the use of field blanks of nanopure water to identify sources of contamination, where no significant chitobiase activity was found. To increase precision of chitobiase measurements, a minimum of four replicates per sample were run on each assay plate, and three replicates for each standard in the standard curve where the coefficient of variation was required to be less than 5%, or the sample was run again. A 40 nM MUF quality control standard was also included in the standard curve and its detected value was required to be within 4 nM of its expected concentration to ensure accuracy of chitobiase activities. The resultant equation of the line for each standard curve had to have a coefficient of determination (r^2) greater than 0.98, otherwise the microplate was rerun.

Chitobiase production rate was determined by taking the resultant MUF

concentrations at times 0, 1, 2, 3, and 6 hours and graphing the natural log of the concentrations at time i (C_i) divided by the concentration at time 0 (C_0), represented by the formula $\ln(C_i/C_0)$, versus time using SigmaPlot graphing software (Systat Software, Inc. San Jose, California). The slope of the resultant equation is the rate constant for the degradation of chitobiase. Assuming that the system from which the sample was taken is in equilibrium, the chitobiase rate constant for production is deemed equal to the inverse of the rate of degradation (see Sastri and Roff, 2000). Therefore, by taking the inverse of the slope for the degradation curve, the rate of chitobiase production is obtained.

Water samples for the analysis of chitobiase activity were collected once per week over a period of seven weeks starting on July 7, 2015, until August 21, 2015 for the SMX study. Water samples for the analysis of chitobiase activity were collected once per week over a period of five weeks starting on August 4, 2015 to September 1, 2015 for the dilbit study. The sample collection dates correspond to the sampling dates of emergent insects and zooplankton.

2.4 Statistical Analysis

Chitobiase activity was expressed as ambient chitobiase activity, as well as rate of production in the SMX study (n = 6) and the dilbit study (n = 9). Aquatic invertebrate endpoints calculated were: aquatic emergent insect abundance, emergent insect biomass, zooplankton biomass, and total aquatic invertebrate biomass (emergent insect biomass + zooplankton biomass).

Measures of chitobiase activity (ambient chitobiase and chitobiase rate of production (ROP)), abundances of emergent insects, emergent insect and zooplankton biomass, and water quality parameters were assessed using one-way analysis of variance (ANOVA). Two Way Repeated Measures ANOVA was performed on data collected from the mesocosm studies to determine if the treatments (SMX and dilbit) had an effect on chitobiase measures, emergent insect abundance, emergent insect biomass, zooplankton biomass, and total biomass compared to that of the control mesocosms.

Spearman (non-parametric) correlation r-value was used to identify any 'moderate' relationships between the measurements of chitobiase activity and the aquatic invertebrate endpoints. All statistical analyses were conducted using SigmaStat for Windows Version 3.5 (Systat Software Inc.).

2.5 Results

2.5.1 Sulfamethoxazole concentrations

SMX was not detected in any of the pre-treatment samples collected. SMX was added to each of the treatment mesocosms on July 16, July 23, July 30, and August 6, 2015. One-hour post-treatment on July 16, 2015, treated mesocosms had an average SMX concentration of 280.8 (± 12.0) µg/L. One-hour post-treatment of the second treatment of SMX on July 23, 2015, treated mesoscosm had an average SMX concentration of 480.8 (± 28.4) µg/L. One-hour post-treatment of the third treatment of SMX on July 30, 2015, treated mesocosms had an average SMX concentration of 331.6 (± 11.1) µg/L. One-hour post-

treatment of the fourth and final treatment of SMX on August 6, 2015, treated mesocosms had an average SMX concentration of 278.6 (±18.3) µg/L. SMX was not detected in the control mesocosms over the course of the study.

2.5.2 Water quality parameters

Water quality parameters (temperature, specific conductivity, pH, and dissolved oxygen) for the SMX study were not affected by the addition of SMX. Water quality parameters pre- and post-treatment are summarized in Table 2.1. The average water temperature was 19.54 (± 3.23 standard error)°C and ranged from 10.63°C to 25.04°C. The average specific conductivity was 0.496 (± 0.032 standard error) mS/cm and ranged from 0.496 mS/cm to 0.613 mS/cm throughout the study. pH ranged from 8.63 to 9.71 throughout the study. The average dissolved oxygen concentration was 5.66 (±0.21 standard error) mg/L and ranged from 1.98 mg/L to 11.50 mg/L throughout. The average PAR was 894 (±194 standard error) nm over the course of the study. PAR was not affected by the treatment, nor was filamentous algae. The average depth of the water in the mesocosms was 36.0 (±2.6 standard error) cm over the course of the study. The average volume of the mesocosms was 1787 (±129 standard error) L over the course of the study.

Water quality parameters (temperature, specific conductivity, pH, and dissolved oxygen) for the dilbit study were not affected by the addition of dilbit and remained consistent throughout. Water quality parameters pre- and post-treatment are summarized in Table 2.2. The average water temperature was 17.92 (±3.09 standard error)°C and ranged from 11.02°C to 26.63°C. The

average specific conductivity was 0.647 (±0.047 standard error) mS/cm and ranged from 0.583 mS/cm to 0.763 mS/cm over the course of the study. The average pH was 8.90 (±0.39 standard error) and ranged from 8.05 to 9.54 over the course of the study. The average dissolved oxygen concentration was 5.45 (±1.82 standard error) mg/L and ranged from 2.12 mg/L to 10.22 mg/L over the course of the study. The average PAR was 892 (±201 standard error) nm over the course of the study.. The average depth of the water in the mesocosms was 35.9 (±1.7 standard error) cm over the course of the study. The average volume of water in the mesocosms was 1783 (±82 standard error) L over the course of the study.

2.5.3 Aquatic insect emergence enumeration and dry mass

Mean abundances of emergent insects from treatment and control mesocosms during the SMX study can be found in Table 2.3. In total, 2571 emergent insects were collected over the period of the SMX study. Of the total emergent insects collected the majority were chironomids (98%), followed by ephemeropterans (1.6%), and odonates (0.3%). Due to the overwhelming majority of the samples being composed of chironomids, other species were grouped together as “rare insects”. The peak total insect emergence for the control mesocosms occurred on August 21, 2015 (day 36) with 280 insects and the peak total insect emergence for the treatment mesocosms occurred on August 14, 2015 (day 29) with 395 insects. The peak chironomid emergence in the control mesocosms occurred on day 36 of the study, with a mean (n = 3) of 90 chironomids. The peak chironomid emergence in the treated mesocosms

occurred on day 29 of the study, with a mean (n = 3) of 130 chironomids. Peak rare insect emergence in the control mesocosms occurred on day 22, with a mean of 5 rare insects. Peak rare insect emergence in the treatment mesocosms occurred on day 29 of the study, with a mean of 2 rare insects. No significant differences in emergence between the control and treatment mesocosms were observed ($p>0.05$).

Mean abundances of emergent insects from treatment, low dose, and high dose mesocosms during the dilbit study can be found in Table 2.4. In total, 8974 emergent insects were collected over the period of the dilbit study. Of the total emergent insects collected the majority were chironomids (98.4%), followed by ephemeropterans (0.8%), and odonates (0.8%). As with the SMX study, due to the overwhelming majority of the emergent insects consisting of chironomids, other species were grouped together as “rare insects”. The peak total insect emergence for the control mesocosms occurred on September 1, 2015 (day 14), the peak total insect emergence for the low treatment occurred on September 1, 2015 (day14), and the peak total emergence for the high treatment occurred on August 28, 2015 (day 10). The peak chironomid emergence in the control mesocosms occurred on day -7 of the study, with a mean (n=3) of 159 chironomids. The peak chironomid emergence in the low treatment mesocosms occurred on day 14 of the study, with a mean (n=2) of 620 chironomids. The peak chironomids emergence in the high treatment mesocosms occurred on day -7 of the study, with a mean (n=3) of 285 chironomids. Peak rare insect emergence in the control mesocosms occurred on day 10 of the study, with a

mean (n=3) of 10 rare insects. Peak rare insect emergence in the low treatment mesocosms occurred on day 2 of the study, with a mean (n=3) of 5 rare insects. Peak rare insect emergence in the high treatment mesocosms occurred on day 10 of the study, with a mean (n=3) of 6 rare insects. No significant differences in emergence between the control, low, and high treatment mesocosms were observed.

The mean emergent insect biomass for the control and treatment mesocosms during the SMX study can be found in Table 2.5. The results of the analysis of emergent insect biomass for the SMX study are as follows. The peak mean (n=3) emergent insect biomass in the control and treatment mesocosms occurred on August 14, 2015 (day 29), with masses of 28.73 mg and 46.73 mg, respectively. The mean (n=18) emergent insect biomass for the control and treatment mesocosms was 14.07 mg and 14.52 mg, respectively. There was no significant difference in emergent insect biomass between the control and treatment mesocosms.

Mean emergent insects biomass for the control, low dose, and high dose mesocosms during the SMX study can be found in Table 2.6. The results of the analysis of emergent insect biomass for the dilbit study are as follows. The peak mean (n=3) emergent insect biomass for the control and high dose mesocosms occurred on August 28, 2015 (day 10), with masses of 222.8 mg and 459.2 mg, respectively. The peak mean (n=2) emergent insect biomass for the low dose mesocosms occurred on September 1, 2015 (day 14), with a mass of 80.9 mg. The mean emergent insect biomass in the control (n=13), low does (n=14), and

high dose (n=15) mesocosms were 82.7 mg, 43.6 mg, and 142.9 mg, respectively. There was no significant difference in emergent insect biomass between the control, low dose, and high dose mesocosms.

2.5.4 Zooplankton biomass

Mean zooplankton biomass for the control and treatment mesocosms during the SMX study can be found in Table 2.5. The results for the analysis of zooplankton biomass for the SMX study are as follows. The peak zooplankton biomass in the control mesocosms occurred on August 14, 2015 (day 29), with a mass of 31.00 mg. The peak zooplankton biomass in the treatment mesocosms occurred on August 14, 2015 (day 29), with a mass of 38.72 mg. The mean (n=18) zooplankton biomass for the control and treatment mesocosms was 14.45 mg and 13.43 mg, respectively. No significant difference in zooplankton biomass between the treatment and control mesocosms was observed.

Mean zooplankton biomass for the control, low dose, and high dose mesocosms during the SMX study can be found in Table 2.6. The results for the analysis of zooplankton biomass for the dilbit study are as follows. The peak zooplankton biomass in the control mesocosms (n = 3) occurred on September 1, 2015 (day 14), with a mass of 67.8 mg. The peak zooplankton biomass in the low dose mesocosms (n = 3) occurred on September 1, 2015 (day 14), with a mass of 55.0 mg. The peak zooplankton biomass in the high does mesocosms (n = 3) occurred on September 1, 2015 (day 10), with a mass of 41.0 mg. The mean (n=15) zooplankton biomass for the control, low dose, and high does mesocosms was 22.1 mg, 21.1 mg, and 23.6 mg, respectively. No significant differences in

zooplankton biomass between the control, low treatment, and high treatment mesocosms were observed.

2.5.5 Total aquatic invertebrate biomass

Total insect biomass for the control and treatment mesocosms during the SMX study can be found in Table 2.7. Both the emergent insect biomass and zooplankton biomass measures were added to achieve a measure of total biomass. Total biomass in the control mesocosms ranged from 11.8 mg to 69.45 mg with a mean of 28.5 (± 3.6 standard error) mg. Total biomass in the treatment mesocosms ranged from 12.3 mg to 146.0 mg with a mean of 27.9 (± 7.3 standard error) mg.

Total insect biomass for the control, low dose, and high dose mesocosms during the dilbit study can be found in Table 2.8. As in the SMX study, emergent insect and zooplankton biomass measures were added to achieve a measure of total biomass. Total biomass in the control mesocosm ranged from 15.6 mg to 422.1 mg with a mean of 91.4 (±33.2 standard error) mg. Total biomass in the low dose mesocosms ranged from 9.7 mg to 148.4 mg with a mean of 60.3 (± 9.3 standard error) mg. Total biomass in the high dose mesocosms ranged from 28.1 mg to 778.3 mg with a mean of 163.6 (± 62.9 standard error) mg.

2.5.6 Measured chitobiase activity

Mean ambient chitobiase and rate of chitobiase production (ROP) for the control and treatment mesocosms during the SMX study can be found in Table 2.9. During the SMX study, water samples were collected for the analysis of

ambient chitobiase in both control and treatment mesocosms on every seventh day starting on July 7, 2015 and finishing on August 21, 2015. The minimum, maximum, and mean (n = 21) ambient chitobiase concentrations detected in the control mesocosms were 19.38 nM, 186.85 nM, and 68.41 (standard deviation of ± 8.82) nM, respectively. The minimum, maximum, and mean (n = 21) ambient chitobiase concentrations detected in the treatment mesocosms were 18.04 nM, 213.65 nM, and 56.92 (standard deviation of ±9.84) nM, respectively. The minimum, maximum, and mean (n = 42) ambient chitobiase concentrations detected in both the control and treatment mesocosms were 18.04 nM, 213.65 nM, and 62.66 (standard deviation of ±42.69) nM, respectively. Mean ambient chitobiase concentrations for the control and treatment mesocosms can be found in Table 2.9.

Mean ambient chitobiase and chitobiase ROP for the control, low dose, and high dose mesocosms during the dilbit study can be found in Table 2.10. During the dilbit study, water samples were collected for the analysis of ambient chitobiase in control, low, and high treatment mesocosms five times between August 15, 2015 and September 1, 2015. The minimum, maximum, and mean (n = 15) ambient chitobiase concentrations detected in the control mesocosms were 24.07 nM, 156.62 nM, and 71.49 (standard deviation of ±8.85) nM, respectively. The minimum, maximum, and mean (n = 15) ambient chitobiase concentrations detected in the low mesocosms were 62.28 nM, 277.65 nM, and 119.03 (standard deviation of ±14.97) nM, respectively. The minimum, maximum, and mean (n = 15) ambient chitobiase concentrations detected in the high

mesocosms were 26.43 nM, 331.35 nM, and 99.30 (standard deviation of ±21.44) nM, respectively. The minimum, maximum, and mean (n = 45) ambient chitobiase concentrations detected in the control, low dose, and high dose treatment mesocosms were 24.07 nM, 331.35 nM, and 96.61 (standard deviation of ±63.45) nM, respectively. Mean ambient chitobiase concentrations for the control, low does, and high dose mesocosms can be found in Table 2.10.

During the SMX study, water samples were collected for the analysis of the rate of chitobiase production (ROP) in both control and treatment mesocosms on every seventh day starting on July 7, 2015 and finishing on August 21, 2015. The minimum, maximum, and mean (n = 21) ambient chitobiase concentrations detected in the control mesocosms were 0.06 nM/h 0.49 nM/h, and 0.16 (standard deviation of ± 0.02) nM/h^2, respectively. The minimum, maximum, and mean (n = 21) chitobiase ROP detected in the treatment mesocosms were 0.06 nM/h, 0.61 nM/h, and 0.20 (standard deviation of ±0.03) nM/h, respectively. The minimum, maximum, and mean (n = 42) chitobiase ROP detected in both the control and treatment mesocosms were 0.06 nM/h, 0.61 nM/h, and 0.18 (standard deviation of ±0.12) nM/h, respectively. Mean chitobiase ROP for the control and treatment mesocosms can be found in Table 2.9.

During the dilbit study, water samples were collected for the analysis of chitobiase ROP in control, low, and high treatment mesocosms five times between August 15, 2015 and September 1, 2015. The minimum, maximum, and mean (n = 15) ambient chitobiase concentrations detected in the control mesocosms were 0.05 nM/h, 0.23 nM/h, and 0.11 (standard deviation of ±0.01)

nM/h, respectively. The minimum, maximum, and mean (n = 15) chitobiase ROP detected in the low mesocosms were 0.04 nM/h, 0.23 nM/h, and 0.11 (standard deviation of ±0.01) nM/h, respectively. The minimum, maximum, and mean (n = 15) chitobiase ROP detected in the high mesocosms were 0.05 nM/h, 0.23 nM/h, and 0.13 (standard deviation of ±0.01) nM/h, respectively. The minimum, maximum, and mean (n = 45) chitobiase ROP detected in the control, low dose, and high dose treatment mesocosms were 0.038 nM/h, 0.23 nM/h, and 0.11 (standard deviation of ±0.05) nM/h, respectively. Mean chitobiase ROP for the control, low dose, and high dose mesocosms can be found in Table 2.10.

2.5.7 Chitobiase half-life

During the SMX study the minimum, maximum, and mean half-life of chitobiase in both the control and treatment mesocosms were 1.1 hours, 11.9 hours, and 5.3 (±2.8) hours, respectively. During the dilbit study the minimum, maximum, and mean (with standard deviation) half-life of chitobiase in the control, low, and high dose mesocosms were 3.0 hours, 18.4 hours, and 7.4 (±3.3) hours, respectively.

2.5.8 Chitobiase activity and aquatic invertebrate endpoints

No statistically significant relationship was observed between chitobiase activity (both ambient and rate of production) and emergent insect and zooplankton endpoints (enumeration and biomass) (Tables 2.11 and 2.12).

2.5.9 Effects of Sulfamethoxazole and diluted bitumen on chitobiase activity

No statistically significant effect of SMX nor dilbit was observed on chitobiase activity (both ambient and rate of production) over the course of the study.

2.6 Discussion

Benthic invertebrate community studies are a key component of environmental effects monitoring and are used to study the effects of stressors on aquatic ecosystem health, in particular the status of fish habitat. The use of chitobiase activity as a measure of aquatic invertebrate biomass and community status is supported by other studies looking at the relationship between chitobiase activity and aquatic invertebrates in both laboratory and field settings. During the current study, both ambient chitobiase (standing measure) and the rate of production of chitobiase (ROP), were consistently detected and measured in water samples collected from the mesocosms. However, the current study was unable to show a significant statistical relationship between observed measures of chitobiase activity in the mesocosms and observed measures of aquatic invertebrate enumeration and biomass. Additionally, the current study shows that there were no statistically significant effects on chitobiase activity in the mesocosms from exposure to both the antibiotic SMX and dilbit.

The status of the mesocosms remained relatively consistent throughout the duration of the study and functioned well. Measured water quality parameters remained stable in both control and treatment mesocosms in both the SMX and dilbit studies (Tables 2.1 and 2.2) and were consistent with previous mesocosm

studies performed at the PWRF (Cardinal et al. 2014; Lobson et al. 2018; Vanderpont et al. 2018). Insect emergence was observed throughout both studies and results were similar to that of previous years, where emergent insects primarily consisted of chironomids and secondly odonates (Tables 2.3 and 2.4) (Lobson et al. 2018; Vanderpont et al. 2018). Consistent capture of zooplankton in the mesocosms was also observed throughout the duration of both studies, with numbers not varying significantly between control and treatment mesocosms.

The chitobiase assay performed well, where field measures of both ambient chitobiase and chitobiase ROP exhibited very low variability (CV < 5%), implying that the assay is fairly precise. Similarities between both measured ambient chitobiase and chitobiase ROP levels over the course of each study were observed, where a general pattern was exhibited between control and treatment mesocosms (Figures 2.4 and 2.5).

No effect of SMX was observed on any of the biotic endpoints (emergent insect abundance, emergent insect biomass, total aquatic invertebrate biomass) in an aquatic mesocosm setting over the course of the study, where no statistically significant difference ($p<0.05$) was observed. Additionally, SMX did not have an effect on chitobiase activity (ambient chitobiase and rate of chitobiase production) in an aquatic mesocosm setting over the course of the study, where no statistically significant difference ($p<0.05$) was observed. Although SMX was applied to the treatment mesocosms four separate times at a concentration of 250 µg/L, which is well above the published environmentally

relevant concentration of 2.0 µg/L (Kolpin et al. 2004; Watkinson et al. 2008), no effects on biotic endpoints and chitobiase activity were observed. This result was expected and could largely be due to the fact that observed EC50 values of SMX for Daphnid species are >100,000 µg/L (Ferrari et al. 2003). If anything, the assumption could have been made that SMX would increase chitobiase activity detected in the mesocosms due to the possible reduction in bacteria within the mesocosms, where competition for free chitobiase and use of free chitobiase amongst bacteria would have been reduced.

During the course of the dilbit mesocosm study, dilbit had no observed effect on biotic endpoints and chitobiase activity in both the low dose and high dose mesocosms. No statistically significant difference ($p<0.05$) was observed between the control, low dose, and high dose mesocosms when looking at biotic endpoints and chitobiase activity. The most probable cause for no observed effects of dilbit between control and treatment mesocosms is that the amounts of dilbit added to the low dose (0.5 mL of dilbit) and high dose (5 mL of dilbit) mesocosms were not enough to elicit a response by aquatic invertebrates. Robidoux et al. (2018) reported no mortality of Daphnia exposed to 10 g/L of diluted bitumen and that the toxicity of diluted bitumen was only displayed at dilbit dosing concentrations of >32 g/L. Further to this, since diluted bitumen has a lower density than that of water, it is possible that the dilbit added to the treatment mesocosms at the beginning of the study did not disperse into the water column and therefore no opportunity was present for the dilbit and its constituents to come into contact with aquatic invertebrates (Engineering and

Medicine National Academies of Sciences 2015).

No significant relationship ($r > 0.50$, $p < 0.05$) was observed between measures of chitobiase (both ambient chitobiase and chitobiase ROP) and observed measures of aquatic invertebrate biomass (emergent insect biomass, zooplankton biomass, and total aquatic invertebrate biomass)(Tables 2.11 and 2.12). Although general patterns were observed when looking at chitobiase activity or aquatic invertebrate biomass separately, there was no correlation between the two. This observation was unexpected and goes against observations in other studies where significant positive relationships were observed between chitobiase activity and measured aquatic invertebrate biomass (Oosterhuis et al. 2000; Sastri and Dower 2009; Sastri and Roff 2000; Vrba and Machacek 1994). There are several possible factors that could have contributed to this outcome, which include: only emergent aquatic insect and zooplankton samples were collected, leaving out the collection of benthic invertebrates altogether. As previously mentioned, benthic invertebrates are a major component of aquatic ecosystems and the lack of sampling of these organisms leaves a gap in data collected representing the total aquatic invertebrate community. In order to keep the integrity of the mesocosms, benthic invertebrate samples could not be collected due to the destructive nature of the sampling techniques, such as the use of kick nets. Additionally, the frequency or infrequency of sampling could have resulted in missed measures of chitobiase during molting events. Measured half-life times for chitobiase during the SMX and dilbit studies (5.3 ± 2.8 hours and 7.4 ± 3.3 hours, respectively) were

relatively short, which could have resulted in not collecting samples of chitobiase during the most opportune time, which would be shortly after the molting event. This could be especially true when comparing chitobiase activity to measures of emergent insect biomass since collection of chitobiase samples and collection of emergent insects occurs after the molting event, when chitobiase is released into the water column. If chitobiase samples are collected well after the molting event, particularly if past the half life time of chitobiase in the system, detected chitobiase activity could be well below that of what it would have been during or shortly after the actual molting event.

In conclusion, although chitobiase activity was measured consistently and effectively, no significant relationships were observed between measures of aquatic invertebrate biomass and measures of chitobiase activity. Additionally, no statistically significant effects were observed on measured ambient chitobiase or chitobiase ROP when exposed to SMX or dilbit. The absence of an observable relationship between chitobiase activity and aquatic invertebrate biomass in the mesocosms does not support the hypothesis. Due to these negative results, it is recommended that additional mesocosm studies be performed looking at other factors that may influence chitobiase activity such as water quality parameters and the collecting of benthic invertebrates. Additionally, mesocosm studies could be performed looking at difference sampling frequencies to determine if effects exist on the time of when the samples are collected.

2.7 Literature cited

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2.8 Tables and Figures

Table 2.1 Mean (± SD, n = 3) water quality parameters measured in mesocosms pre- and post- treatment with sulfamethoxazole. DO = dissolved oxygen. No significant differences for water quality parameters before (n=) and after (n=) addition of sulfamethoxazole ($p > 0.05$) using repeated measures two-way ANOVA

	Temperature (°C)		Conductivity (mS/cm)		pH		DO (mg/L)	
Treatment	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
Control	17.45 (0.61)	18.82 (0.57)	0.581 (0.006)	0.568 (0.004)	9.55 (0.05)	9.21 (0.03)	8.02 (0.37)	5.33 (0.35)
SMX	17.21 (0.61)	18.85 (0.58)	0.546 (0.005)	0.523 (0.004)	9.60 (0.05)	9.13 (0.04)	7.98 (0.39)	5.80 (0.34)

Table 2.2 Mean (± SE, n = 3) water quality parameters measured in mesocosms pre- and post-treatment with diluted bitumen. DO = dissolved oxygen. No significant differences for water quality parameters before (n=90) and after (n=59) addition of diluted bitumen ($p > 0.05$) using repeated measures two-way ANOVA.

	Temperature (°C)		Conductivity (mS/cm)		pH		DO (mg/L)	
Treatment	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
Control	18.37 (0.47)	14.07 (1.01)	0.616 (0.005)	0.653 (0.007)	9.38 (0.03)	8.72 (0.04)	6.53 (0.35)	6.16 (0.47)
Low	18.42 (0.47)	14.06 (1.01)	0.663 (0.005)	0.695 (0.007)	8.97 (0.05)	8.54 (0.05)	6.22 (0.33)	6.62 (0.43)
High	18.29 (0.48)	14.02 (1.03)	0.645 (0.005)	0.655 (0.007)	9.36 (0.04)	9.06 (0.04)	6.97 (0.31)	7.46 (0.47)

Table 2.3 Mean (± SE, n = 3) abundance of chironomid and rare (Odonates, Ephemeroptera, Trichoptera) insects collected from emergence traps during the sulfamethoxazole study.

	Control		Treatment	
	Mean Chironomid	**Mean Rare**	**Mean Chironomid**	**Mean Rare**
Day -9	4 (3)	1 (1)	5 (2)	0 (0)
Day 1	16 (5)	0 (0)	24 (6)	0 (0)
Day 8	46 (17)	1 (0)	106 (28)	0 (0)
Day 15	42 (11)	0 (0)	52 (13)	0 (0)
Day 22	52 (2)	5 (1)	78 (18)	0 (0)
Day 29	82 (21)	3 (2)	130 (17)	2 (1)
Day 36	90 (10)	3 (2)	108 (42)	0 (0)

Table 2.4 Mean (± SE, n = 3) abundance of chironomid and rare (Odonates, Ephemeroptera, Trichoptera) insects collected from emergence traps during the diluted bitumen study.

	Control		Low		High	
	Mean Chironomid	**Mean Rare**	**Mean Chironomid**	**Mean Rare**	**Mean Chironomid**	**Mean Rare**
Day -17	71 (29)	0 (0)	213 (73)	2 (2)	285 (100)	2 (2)
Day -7	159 (22)	4 (2)	306 (95)	2 (1)	226 (68)	1 (1)
Day 0	119 (16)	4 (1)	145 (39)	5 (3)	154 (48)	1 (1)
Day 10	147 (66)*	13 (4)*	192 (58)	2 (0)	226 (98)	6 (2)
Day 14	211 (109)	7 (7)*	620 (327)*	7 (1)*	197 (66)	2 (2)

*n = 2; loss of sample due to weather conditions

Table 2.5 Mean (± SE, n = 3) biomass of emergent insect and zooplankton biomass pre- and post- treatment for the control and treatment mesocosms during the sulfamethoxazole study. No significant differences in emergent insect biomass and zooplankton biomass between treatment and control mesocosms (p = 0.878 and p = 0.656, respectively) using repeated measures two-way ANOVA.

	Control	Treatment	Control	Treatment
	Emergent Insect Biomass (mg)	**Emergent Insect Biomass (mg)**	**Zooplankton Biomass (mg)**	**Zooplankton Biomass (mg)**
Day -9	11.4 (10.5)	2.3 (0.7)	-	-
Day 1	2.9 (1.4)	2.4 (0.1)	17.28 (2.99)	12.72 (1.77)
Day 8	18.4 (11.7)	9.3 (1.1)	11.83 (1.97)	11.18 (1.51)
Day 15	8.8 (4.4)	7.8 (1.3)	9.16 (0.60)	17.15 (7.69)
Day 22	14.7 (3.6)	10.0 (0.8)	9.27 (2.17)	4.91 (0.17)
Day 29	28.7 (11.0)	46.7 (36.1)	22.56 (4.35)	25.20 (8.41)
Day 36	10.9 (3.9)	10.9 (2.6)	16.62 (1.88)	9.40 (1.28)

*- = no sample collected

Table 2.6 Mean (± SE, n = 3) emergent insect and zooplankton biomass pre- and post- treatment for the control, low dose, and high dose mesocosms during the diluted bitumen study. No significant difference exists between emergent insect biomass and zooplankton biomass for the control, low dose, and high does mesocosms (p = 0.372 and p = 0.959, respectively) using repeated measures two-way ANOVA.

	Control	Low	High	Control	Low	High
	Emergent Insect Biomass (mg)	Emergent Insect Biomass (mg)	Emergent Insect Biomass (mg)	Zooplankton Biomass (mg)	Zooplankton Biomass (mg)	Zooplankton Biomass (mg)
Day -17	18.1 (10.9)	36.0 (11.9)	37.8 (9.5)	12.23 (3.29)	20.63 (10.63)	13.01 (2.11)
Day -7	29.5 (5.9)	44.7 (11.9)	23.1 (0.09)	20.33 (6.94)	14.30 (2.83)	19.49 (8.12)
Day 0	35.2 (12.0)	38.7 (11.8)	26.8 (6.7)	13.19 (1.98)	16.97 (6.17)	19.52 (1.72)
Day 10	222.8 (184.9)*	30.1 (9.7)	459.2 (199.0)	20.79 (4.89)	18.39 (3.85)	24.91 (1.77)
Day 14	190.6 (177.8)*	80.9 (48.4)*	167.6 (154.7)	32.30 (17.79)	27.91 (13.80)	26.35 (7.52)

Table 2.7 Mean (± SE, n=3) total invertebrate biomass for control and treatment mesocosms during the sulfamethoxazole study. No significance difference exists for total invertebrate biomass between the control and treatment mesocosms (p = 0.460) using a repeated measures two-way ANOVA. Not that day -9 was removed as no zooplankton samples were collected on that date.

	Control	Treatment
	Total Biomass (mg)	**Total Biomass (mg)**
Day 1	20.17 (3.01)	15.09 (1.72)
Day 8	30.23 (10.01)	20.44 (2.57)
Day 15	17.92 (3.88)	24.95 (6.67)
Day 22	24.00 (5.74)	14.91 (0.93)
Day 29	51.29 (12.23)	71.94 (37.94)
Day 36	27.52 (2.63)	20.33 (1.36)

Table 2.8 Mean (± SE, n = 3) total invertebrate biomass for control, low dose, and high dose mesocosms during the diluted bitumen study. No significant difference exists for total invertebrate biomass between the control, low dose, and high dose mesocosms (p = 0.387) using a repeated measures two-way ANOVA.

	Control	Low	High
	Total Biomass (mg)	**Total Biomass (mg)**	**Total Biomass (mg)**
Day -17	30.30 (14.19)	56.66 (21.02)	50.78 (7.60)
Day -7	49.83 (12.71)	59.00 (12.62)	42.59 (7.30)
Day 0	48.36 (12.12)	55.67 (17.67)	46.35 (5.20)
Day 10	169.32 (126.59)	48.52 (13.40)	484.15 (199.59)
Day 14	159.33 (113.73)	81.84 (40.12)	193.95 (162.03)

Table 2.9 Mean (± SD, n = 3) ambient chitobiase and rate of chitobiase production (ROP) over the course of the sulfamethoxazole study. No significant differences exist for ambient chitobiase and chitobiase ROP between control and treatment mesocosms (p = 0.525 and p = 0.462, respectively) using a repeated measures two-way ANOVA.

	Control		Treatment	
	Ambient Chitobiase (nM)	**Chitobiase ROP (nM/h)**	**Ambient Chitobiase (nM)**	**Chitobiase ROP (nM/h)**
Day - 9	33.93 (23.17)	0.09 (0.05)	27.93 (9.79)	0.10 (0.06)
Day 1	54.52 (32.29)	0.16 (0.11)	36.07 (3.44)	0.32 (0.06)
Day 8	44.09 (20.88)	0.32 (0.16)	33.23 (22.53)	0.42 (0.21)
Day 15	87.78 (38.25)	0.12 (0.02)	115.29 (85.22)	0.15 (0.03)
Day 22	128.90 (53.90)	0.14 (0.05)	93.23 (39.78)	0.13 (0.07)
Day 29	69.74 (28.32)	0.17 (0.07)	47.16 (19.53)	0.15 (0.08)
Day 36	59.88 (6.45)	0.14 (0.05)	45.54 (17.10)	0.13 (0.01)

Table 2.10 Mean (± SD, n = 3) ambient chitobiase and rate of chitobiase production (ROP) over the course of the diluted bitumen study. No significant differences exist for ambient chitobiase and chitobiase ROP between control, low dose, and high dose mesocosms (p = 0.477 and p = 0.717, respectively) using a repeated measures two-way ANOVA.

	Control		Low		High	
	Ambient Chitiobiase (nM)	**Chitobiase ROP (nM/h)**	**Ambient Chitiobiase (nM)**	**Chitobiase ROP (nM/h)**	**Ambient Chitiobiase (nM)**	**Chitobiase ROP (nM/h)**
Day -17	74.43 (38.29)	0.07 (0.02)	124.54 (53.57)	0.09 (0.04)	116.53 (84.58)	0.09 (0.00)
Day -7	43.04 (17.94)	0.14 (0.08)	84.92 (28.95)	0.14 (0.08)	70.99 (53.75)	0.14 (0.06)
Day 0	65.74 (18.12)	0.10 (0.06)	105.82 (41.91)	0.09 (0.05)	72.63 (29.57)	0.10 (0.04)
Day 10	57.75 (17.12)	0.12 (0.03)	90.91 (36.52)	0.14 (0.08)	60.58 (30.01)	0.16 (0.02)
Day 14	116.51 (34.77)	0.10 (0.02)	188.95 (78.40)	0.08 (0.01)	175.78 (150.74)	0.13 (0.07)

Table 2.11 Results of the Spearman Correlation (non-parametric) of aquatic invertebrate endpoints (enumeration and biomass) with chitobiase activity (ambient chitobiase and chitobiase rate of production (ROP)) collected during the sulfamethoxazole study.

Parameter	Ambient Chitobiase (nM)	Chitobiase ROP (nM/h)	Emergent Insect Abundance	Emergent Insect Biomass (mg)	Zooplankton Biomass (mg)	Total Biomass (mg)
Ambient Chitobiase (nM)	1	**-0.372**	-0.141	-0.187	0.086	-0.084
Chitobiase ROP (nM/h)		1	0.149	-0.132	0.038	-0.125
Emergent Insect Abundance			1	**0.601**	0.013	**0.364**
Emergent Insect Biomass (mg)				1	-0.03	**0.659**
Zooplankton Biomass (mg)					1	**0.644**
Total Biomass (mg)						1

Significant r-values ($r > 0.50$) are highlighted and significant p-values ($p < 0.05$) are given in **bold**

Table 2.12 Results of the Spearman Correlation (non-parametric) of aquatic invertebrate endpoints (enumeration and biomass) with chitobiase activity (ambient chitobiase and chitobiase rate of production (ROP)) collected during the diluted bitumen study.

Parameter	Ambient Chitobiase (nM)	Chitobiase ROP (nM/h)	Emergent Insect Abundance	Emergent Insect Biomass (mg)	Zooplankton Biomass (mg)	Total Biomass (mg)
Ambient Chitobiase (nM)	1	-0.118	0.301	0.111	**0.309**	0.234
Chitobiase ROP (nM/h)		1	**0.406**	0.161	0.225	0.185
Emergent Insect Abundance			1	0.302	0.265	**0.370**
Emergent Insect Biomass (mg)				1	0.249	**0.878**
Zooplankton Biomass (mg)					1	**0.609**
Total Biomass (mg)						1

Significant r-values ($r > 0.50$) are highlighted and significant p-values ($p < 0.05$) are given in **bold**

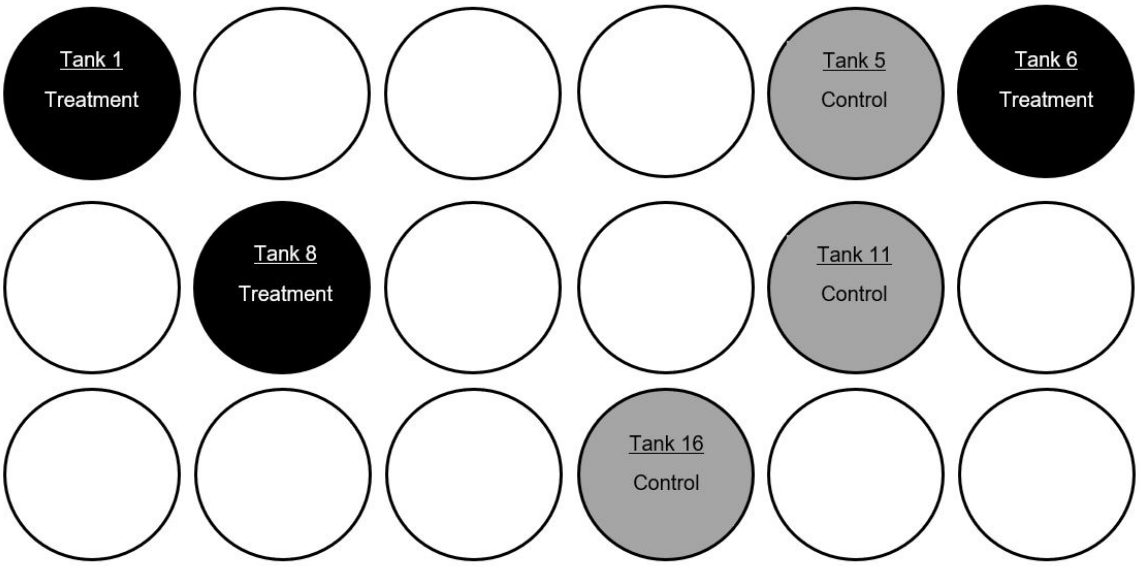

Figure 2.1 Mesocosm layout at the Prairie Wetland Research Facility showing the locations of the control and treatment mesocosms for the sulfamethoxazole study.

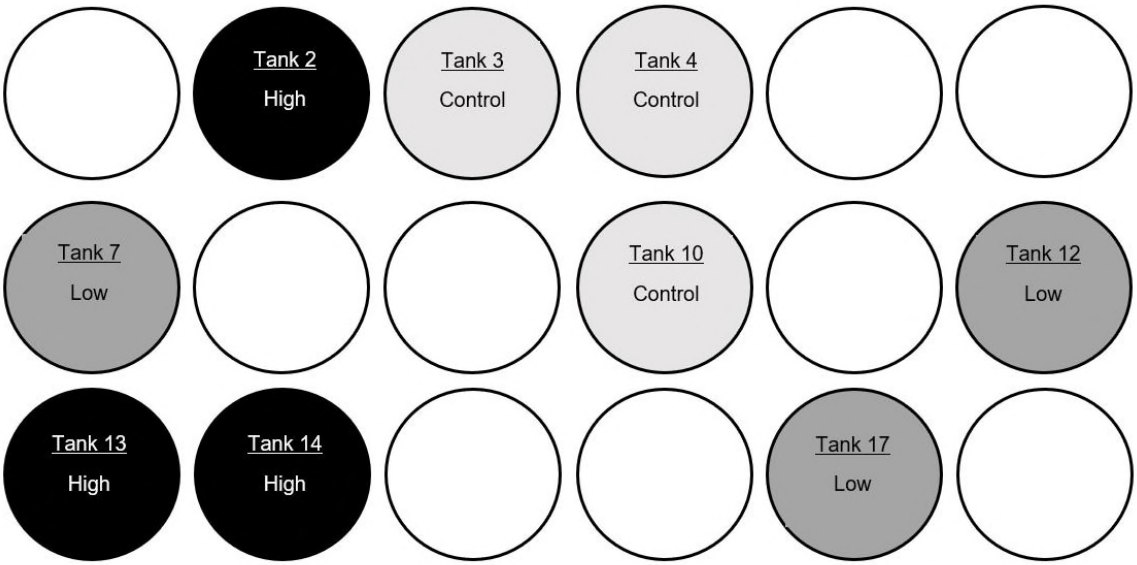

Figure 2.2 Mesocosm layout at the Prairie Wetland Research Facility showing the locations of the control and treatment mesocosms for the sulfamethoxazole study.

Figure 2.3 Photo of aquatic emergence trap set up in a mesocosm at the Prairie Wetland Research Facility at the University of Manitoba, Winnipeg, MB.

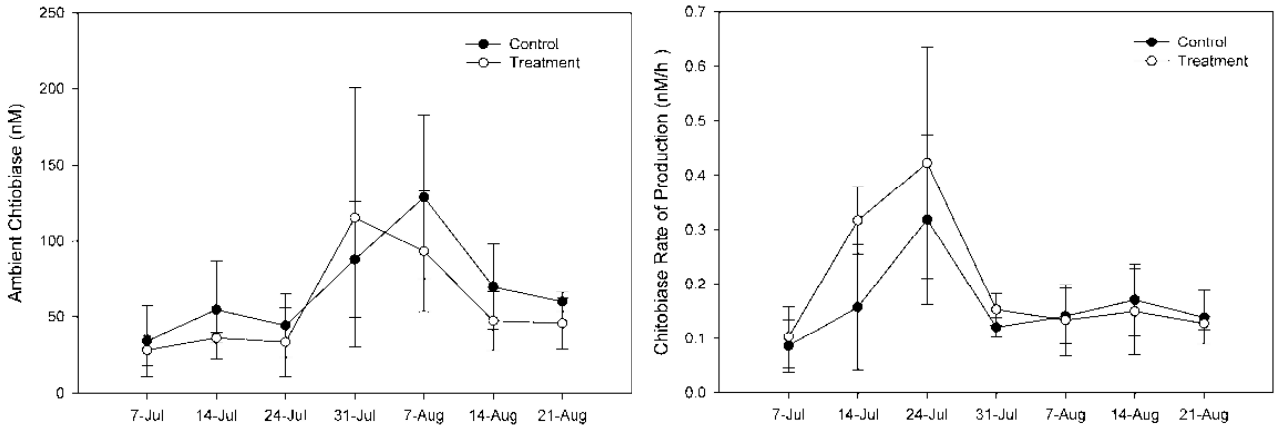

Figure 2.4 Ambient chitobiase and chitobiase rate of production in control and treatment mesocosms during the sulfamethoxazole study.

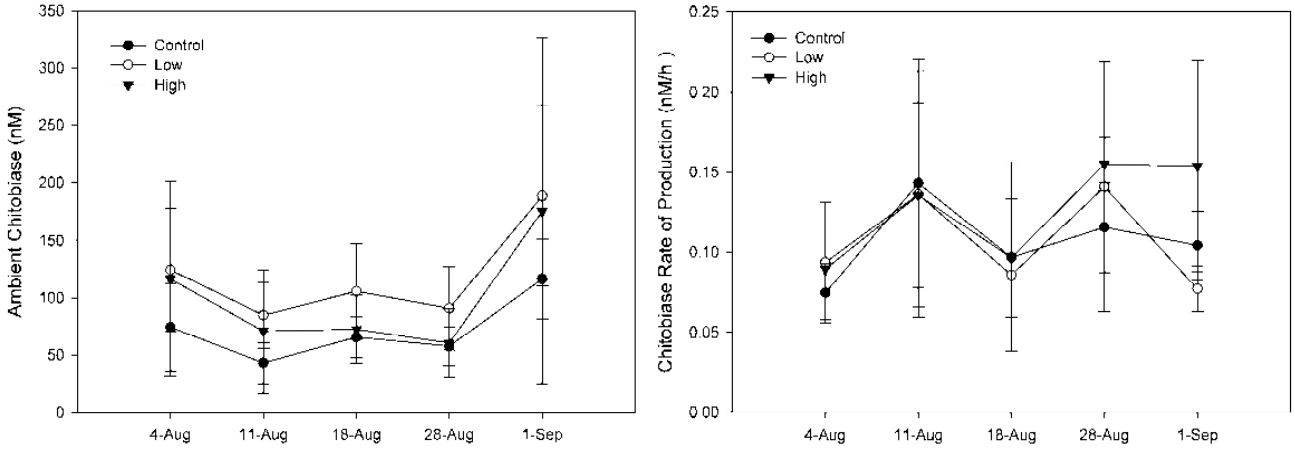

Figure 2.5 Ambient chitobiase and chitobiase rate of production in the control, low dose, and high dose mesocosms during the diluted bitumen study.

CHAPTER 3. RATE OF CHITOBIASE PRODUCTION AS A SURROGATE FOR SECONDARY PRODUCTION IN LOTIC SYSTEMS: A FIELD STUDY IN THE ELK VALLEY REGION OF BRITISH COLUMBIA, CANADA

3.1 Abstract

Chitobiase (*N*-acetyl-β-D-glucosaminidase) is one of two chitinolytic enzymes released by benthic arthropods into the aquatic environment during the molting process. It has been found to be predictive of secondary production for zooplankton in the laboratory and in marine ecosystems. This led to the hypothesis that a positive relationship could exist between the rate of chitobiase production and benthic invertebrate biomass in lotic freshwater systems (i.e., chitobiase measurements could be used as a surrogate of benthic invertebrate productivity in the field). In the present study, benthic invertebrates and chitobiase were sampled from ten freshwater lotic areas located within the Elk River watershed in southeastern British Columbia, Canada. Sampling of chitobiase at these ten areas was performed for both ambient (standing) chitobiase activity and the rate of chitobiase production. The rate of chitobiase production showed a significant positive linear relationship ($p < 0.05$, $r^2 = 0.80$) with benthic invertebrate biomass. Positive relationships were also observed between the rate of chitobiase production and the biomass of Ephemeroptera, Plecoptera, Trichoptera (EPT), and Chironomidae ($r^2 = 0.69$, and 0.77, respectively); taxa groups commonly used as indicators of water quality. These observations support the position that the rate of chitobiase activity could be a useful metric in biomonitoring programs.

3.2 Introduction

The determination of primary and secondary production is essential to understanding energy transfer within aquatic systems. Many freshwater biomonitoring programs routinely sample primary production but not secondary production. This is likely due to the relative ease of sampling chlorophyll-a (chl-a) or ash free dry mass (AFDM) as surrogates for actual primary production. Chl-a is frequently used as an index of phytoplankton and periphyton biomass; however, the relationship between chl-a and biomass (e.g., ash-free dry weight) is complex because the amount of chl-a per unit of biomass varies depending on many factors such as, light intensity, water temperature, community composition, and cell size (Aponasenko et al., 2007; Baulch et al., 2009). Due to this complex relationship, chl-a per biomass unit may vary over two orders of magnitudes (Nicholls and Dillon, 1978). A large proportion of the production in clear and shallow water bodies, typical of the mountain streams in British Columbia, can be attributed to periphytic algae (Dodds et al. 1999). The presence of relatively high algal biomass can indicate eutrophication; while low biomass can be attributed to factors such as nutrient limitations. The relationship between biomass and eutrophication can also be confounded, as high algal biomass can also occur in less productive habitats after long periods of stable flow, and low algal biomass can be due to heavy grazing or a recent storm event (Barbour et al. 1999). These factors can limit the interpretability of periphyton biomass data. Regardless of these limitations, chl-a sampling is a common component of many biomonitoring programs.

Aquatic invertebrates function as dominant secondary producers and primary consumers in most aquatic systems (Wallace et al. 1996). Community secondary production can be determined by enumerating and weighing benthic invertebrate community samples (Cowley and Whitefield 2002; Huryn 1998; Huryn and Wallace 1987). Several studies have used community secondary production as a measure of ecosystem functional response to anthropogenic disturbances (Brooks et al. 2006; Cardinale et al. 2006; Ceballos et al. 2005; Micheli 1999; Mouillot et al. 2006). Estimating secondary production of streams by weighing benthic invertebrate samples is resource intensive and there can be substantial time delays between sample collection and results. Similar to the chl-a relationship with primary production, the molting enzyme chitobiase has been shown to demonstrate a positive relationship with secondary production in marine environments (Sastri and Dower 2009; Oosterhuis et al. 2000; Sastri and Roff 2000; Vrba and Machacek 1994), and we hypothesize it may hold similar utility in freshwater ecosystems,

The chitobiase enzyme is directly related to secondary production as it is synthesized by arthropods to cleave chitin during molting. Specifically, chitin is the main constituent of arthropod exoskeletons (Avila et al. 2011), which grow and develop through a series of successive molting events or ecdysis (Roff et al. 1994). During ecdysis, two chitinolytic enzymes are produced and secreted from the epidermis into the molting fluid found between the epidermis and the endocuticle. The first are chitinases, a family of 18 glycosyl hydrolases, which contain a basic structure that includes a catalytic region, a cysteine-rich region,

and a PEST (proline, glutamic acid, serine and threonine rich peptide sequence)-like region (Kramer and Muthukrishnan 1997). Chitinases are activated after the release of ecdysterone, and begin to hydrolyze chitin oligomers into dimers and terminal oligomers (Kimura 1973; Marcu and Locke 1998). The second are chitobiases (N-acetyl-β-D-glucosaminidase), a family of 20 glycosyl hydrolases, which are released into the molting fluid prior to activation of chitinases. Chitobiases are activated after the creation of smaller chitin dimers and oligomers by chitinases, which are hydrolyzed into monomers (Muzzarelli 1977). Newly created monomers are then re-adsorbed into the cuticle and can be used in the formation of new chitin microfibrils. During ecdysis, chitinases and chitobiases are not re-absorbed but are released into the aquatic environment (Spindler 1976). Both chitinolytic enzymes, chitinase and chitobiase, can be detected and quantified in water samples, but the fluorescence assay for chitobiase is more straightforward, and is specifically the result of invertebrate release (Espie and Roff 1995b). The rate of chitobiase degradation within an aquatic system represents the turnover rate of chitobiase produced by developing arthropods (Sastri and Dower, 2006), and can therefore be used to estimate the average rate of development of the arthropod community. A change in ambient chitobiase activity provides a direct indication that molting is occurring and that arthropods are present, but it cannot provide an absolute estimate of secondary productivity without knowledge of the rate of degradation (Oosterhuis et al. 2000).

Several studies have been performed where chitobiase has been used to

estimate secondary production. For example, in laboratory studies of marine and freshwater zooplankton, a positive relationship has been observed between chitobiase and body size and biomass (Oosterhuis et al. 2000; Sastri and Roff 2000; Vrba and Machacek 1994). A limited number of studies have examined the relationship between chitobiase and secondary production in the field (Espie and Roff 1995a; Vrba et al. 2004). Sastri and Dower (2009) carried out a study in the Strait of Georgia, British Columbia, Canada looking at the relationship between the turnover rate of chitobiase and biomass production rate. They found a significant positive correlation ($r^2 = 0.949$, $p < 0.001$) between the $\log_e$ transformation of both chitobiase activity and individual dry weights of copepods, decapod larvae, and mysids (Sastri and Dower 2009). Another study by Avila et al. (2011) looked at the validity of the chitobiase assay to estimate zooplankton secondary production in the Patos Lagoon estuary in Rio Grande, southern Brazil. They reported findings similar to that of Sastri and Dower (2009), where the activity of chitobiase released into the water during molting demonstrated a positive relationship with the growth of the copepod *Acartia tonsa.* They also noted a relationship between chitobiase and copepod body length. Avila et al. (2011) concluded that the use of chitobiase to estimate secondary production is valid and reliable.

Chitobiase activity has also been used as an endpoint in the evaluation of impacts from stressors, such as in a study performed by Zou and Fingerman (1999) which found that the inhibition of chitobiase activity in the epidermis of the fiddler crab, *Uca pugilator*, was a result of increasing exposure concentrations of

diethyl phthalate; 4-(*tert*)-octylphenol; 2,4,5-trichlorobiphenyl; Aroclor 1242; diethylstilbestrol; endosulfan; and 4-octylphenol as single compounds. Inhibition of chitobiase activity in the epidermis of *U. pugilator* results in the inhibition of molting, preventing the release of chitobiase into the environment. Duchet et al. (2011) found that survival, growth and fecundity of *Daphnia pulex* and *D. magna* were negatively affected by the mosquito larvicides diflubenzuron and spinosad; and that these effects were positively correlated with measurements of chitobiase activity. It should be noted that chitobiase activity only provides evidence that a change has occurred; not the agent causing change. Thus making it a suitable measure for continuous monitoring and comparing sample results for pre- and post-release of a stressor.

Production of chitobiase by arthropods in aquatic ecosystems is a reflection of multiple factors such as number of organisms present, growth or development rate of the organisms, and/or size of actively molting organisms. Therefore, measurement of chitobiase within the water column presents a technique that could be used to quantify active secondary production in aquatic freshwater ecosystems. This study tests the hypothesis that chitobiase is a direct proxy for total benthic invertebrate biomass and that 1) a positive statistical correlation exists between ambient chitobiase activity and benthic invertebrate biomass; and 2) a positive statistical correlation exists between the rate of chitobiase production and benthic invertebrate biomass. Hypotheses were tested in lotic systems within the Elk River watershed of British Columbia, Canada.

3.3 Materials and Methods

3.3.1 Study area and study design

This study was conducted in the Elk River watershed, British Columbia, Canada. It is located in the southeastern corner of the province in the rugged terrain of the Front and Border Ranges of the Rocky Mountains, with peaks up to 3,300 m in the north and 2,200 m in the south.

A total of ten areas were selected for inclusion in the study. Five areas were within the same sub-watershed (labeled as follows: LI24, SLINE, LILC3, LI8, and LIDSL within Line Creek), while the other five (labeled as follows: HENFO, GHCKD, HACKUS, AL4, and MI5) were in separate sub-watersheds (Figure 1). At each area, a single water sample was collected to determine ambient chitobiase activity and an additional sample was collected to determine the chitobiase degradation rate. Concurrently, a total of ten benthic invertebrate samples were taken at each of the five Line Creek areas while three replicates were taken at the other five study areas. All sampling occurred between September 10th and 18th, 2015.

3.3.2 Chitobiase sampling and analysis

Surface water samples were used to determine ambient chitobiase activities, as well as, chitobiase production (degradation rate). Twenty-five mL water samples for ambient chitobiase activity were drawn directly from the surface water of the ten sample areas with a 25 mL syringe and filtered through a 0.2 µm syringe filter (in order to remove bacteria that will degrade chitobiase) into

a 20 mL glass scintillation vial in the field. These samples were then stored in the dark in a cooler at about 4°C until being transferred to a refrigerator for storage at 4°C. Samples for determining the rate of chitobiase degradation were filtered through a 53 µm Nitex mesh into a 250 mL amber jar to ensure exclusion of zooplankton and other pelagic invertebrates (to prevent new release of the enzyme into the vials) and were stored in a cooler to reduce warming. At times 0, 1, 2, 3, 6, and 12 hours, 25 mL subsamples were taken from the 250 mL jar with a new 25 mL syringe and an aliquot filtered through a 0.2 µm syringe filter into a 20 mL glass scintillation vial in the field. Subsamples were labeled and placed in the dark in a cooler at 4°C until storage in a refrigerator at 4°C. Field blanks of nanopure (18MΩ-cm) Milli-Q water (Millipore Corporation, Ma, USA) were opened during water sampling at three (HENFO, HACKUS, and GHCKD) of the ten sample areas and submitted for chitobiase analysis.

All water samples were transported to the University of Manitoba for chitobiase analysis (within 10 days of collection). Chitobiase measurements were performed according to the Sastri and Roff (2000) method, as modified by MacKenzie (2016). The assay uses 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (MUF-NAG) (>98% Purity (TLC) from Sigma-Aldrich) as the substrate, which is cleaved by chitobiase into N-acetyl-glucosamine (NAG) and fluorescent 4-methylubelliferone (MUF). A 100 µL aliquot of the 0.2 µm filtered sample was incubated for one hour at 25°C in a 96 well polystyrene microplate with 100 µL of 0.3 mM MUF-NAG in 0.15 M pH 5.5 citrate phosphate buffer. The MUF-NAG stock solution was created by dissolving MUF-NAG in methyl

cellusolve (2-methoxyethanol for HPLC ≥ 99.9% from Sigma-Aldrich) to a concentration of 5 mM. The reaction was stopped with the addition of 50 µL 0.25 mM NaOH and fluorescence was measured at 360 nm excitation and 450 nm emission using a SpectraMax M2 spectrofluorometer microplate reader. Fluorescence values were then converted into MUF concentrations using a nine-point MUF calibration curve (0, 2, 4, 8, 16, 32, 64, 128, 512 nM). Standards were created by serial dilution starting with a 4.12 mM MUF stock. Blanks were prepared using filtered samples in which MUF-NAG and NaOH were simultaneously added. Each sample was measured in quadruplicate and the arithmetic mean of the four measures used in subsequent analyses.

The method detection limit (MDL) and the limit of quantitation (LOQ) for the fluorescence assay were 1 nM MUF and 4 nM MUF, respectively. The MDL and LOQ were determined by using a pH 5.5 citrate phosphate buffer solution as blank samples and run as if they were field samples. Seven microplate wells of incubated buffer solution were used as recommended for environmental chemistry analysis (Wisconsin Department of Natural Resources 1996). The MDL was calculated using the standard deviation of the blanks multiplied by the Student's *t*-value for a 99% confidence level (Student's *t*-test value for seven wells is 3.143). The 95% confidence interval for the MDL was obtained by multiplying the MDL value by percentiles of chi-square degrees of freedom (lower confidence limit = 0.64*MDL; upper confidence limit = 2.20*MDL). The LOQ was determined by multiplying the standard deviation of the seven aliquots of the blank samples by 10.

To ensure confidence in the results, quality assurance and quality control (QA/QC) measures were employed. QA/QC measures included: the use of field blanks of nanopure water to identify sources of contamination, where no significant chitobiase activity was found. To increase precision of chitobiase measurements, a minimum of four replicates per sample were run on each assay plate, and three replicates for each standard in the standard curve where the coefficient of variation was required to be less than 5%, or the sample was run again. A 40 nM MUF quality control standard was also included in the standard curve and its detected value was required to be within 4 nM of its expected concentration to ensure accuracy of chitobiase activities. The resultant equation of the line for each standard curve had to have a coefficient of determination (r^2) greater than 0.98, otherwise the microplate was rerun.

Chitobiase production rate was determined by taking the resultant degradation MUF concentrations at times 0, 1, 2, 3, 6, and 12 hours and graphing the natural log of the concentrations at time i (C_i) divided by the concentration at time 0 (C_0), represented by the formula $\ln(C_i/C_0)$, versus time using SigmaPlot graphing software (Systat Software, Inc. San Jose, California). The slope of the resultant equation is the rate constant for the degradation of chitobiase. Assuming that the system from which the sample was taken is in equilibrium, where the amount of chitobiase being released into the system by molting invertebrates is equal to the amount being removed by microbes and other degradation processes, the chitobiase rate constant for production is deemed equal to the inverse of the rate of degradation (see Sastri and Roff,

2000). Therefore, by taking the inverse of the slope for the degradation curve, the rate of chitobiase production is obtained.

3.3.3 Benthic invertebrate sampling and biomass analysis

Benthic invertebrate samples were collected at all areas concurrent with water samples for chitobiase analysis. Benthic invertebrates were collected using a Hess sampler with 500 µm mesh. Ten stations were sampled at each of the five Line Creek areas (LI24, SLINE, LILC3, LIDSL, and LI8) and three stations were sampled at the other five sampling areas (AL4, GHCKD, HACKUS, HENFO, and MI5). Stations within areas were located a minimum of 5 m apart. A single sample was collected at each station by carefully inserting the base of the Hess sampler into the substrate to a depth of approximately 5-10 cm. Any gravel or cobble collected with the sample was carefully washed while allowing the current to carry dislodged organisms into the mesh collection net. All organisms collected into the net were rinsed into the collection container at the end of the net, and then into a labeled wide-mouth plastic jar and preserved in a 10% buffered formalin solution.

Benthic invertebrate biomass samples were sent to ZEAS Inc. in Nobleton, Ontario, Canada for sorting, taxonomic identification, and wet mass measurements. All preserved organisms were removed from sample debris (i.e., no sub-sampling) and grouped at the family-level of taxonomy for weighing. Each group was placed onto a fine cloth to remove any excess surface moisture (preservative) before being weighed to the nearest 0.0001 g. Total and family-level biomass were reported for each sample (preserved weight).

3.3.4 Chlorophyll-a and AFDM sampling and laboratory analysis

Periphyton samples were collected at 10 stations at each of the five Line Creek areas (LI24, SLINE, LILC3, LIDSL, and LI8), with stations being located a minimum of 5 m apart, and a single station was sampled at the other five sampling areas (AL4, GHCKD, HACKUS, HENFO, and MI5). Periphyton samples were collected from riffle habitats with a water depth of at least 5 cm, near-bottom water velocity of approximately 0.1-0.4 m/s, and uniform substrate characteristics. When a sampling area with such characteristics was identified, a relatively flat rock of at least 12 cm in length was sampled. If a rock chosen by this method was judged unsuitable for sampling (e.g., too small, highly angular, or uncharacteristic surface texture), an alternative rock in close proximity, having visibly similar periphyton coverage, was sampled instead. This approach was used to try and minimize the variability in chl-a and AFDM that is attributable to variations in natural habitat. At each station, a total of five rocks of similar size were sampled (i.e., large enough to collect separate samples for both chl-a and AFDM analyses) and the periphyton scrapings from the five rocks were combined to form a single composite sample.

After a suitable rock was selected, a thin acetate template with a 4 cm^2 opening was placed on the rock and all periphyton was scraped from the surface of the rock within the opening using a scalpel. This process was repeated with four additional rocks, and all five scrapings were placed on a wetted Whatman® GF/F glass fiber filter (e.g., 90 mm diameter, 0.7 µm pore size) to provide a single, composite sample per station. The filter paper containing the sample was

then folded in half twice and tightly wrapped in aluminum foil. Foil wrapped samples were placed in a labeled Whirl-Pak® bag and stored in a cooler with freezer packs (in the field) until transfer to a freezer later in the day. Samples can be stored frozen for up to 30 days as long as they are not exposed to light (APHA et al. 1998).

The same rocks sampled for chl-a analysis were also used to collect separate scrapings for analysis of AFDM. Each composite sample for AFDM analysis was placed in a small sealed container and kept cool until transfer to a freezer later in the day.

Samples for AFDM and chl-a analysis were shipped frozen to ALS Environmental (Calgary, Alberta, Canada or Burnaby, British Columbia, Canada). Analysis of chl-a was completed using procedures adapted from EPA Method 445.0; involving routine acetone extraction followed by fluorescence detection using a non-acidification procedure (a method that is not subject to interferences from chlorophyll-b). Analysis of AFDM followed procedures modified from American Public Health Association (APHA) Method 10300 C. Total AFDM was calculated as the difference between the dried sample weight and the ash weight, both of which were determined gravimetrically. Dry weight was determined by drying the sample at 105°C, and the ash weight was subsequently determined by ashing the dried sample at 500°C.

3.3.5 Water quality and stream parameters

Temperature, dissolved oxygen, conductivity, and pH were measured *in situ* at all areas when water samples were collected for chitobiase analysis.

Measurements were made using an YSI 650 MDS (Multi-parameter Display System) equipped with an YSI 6820 Sonde. Bankfull width (m), wetted width (m), and bankfull-wetted depth were measured using a tape measure and a wading rod. Channel velocity was measured using a March-McBirney Flo-Mate portable velocity flow meter. Discharge was calculated following Canadian Aquatic Biomonitoring Network (CABIN) Protocol (Environment Canada 2012).

3.4 Statistical Analyses

Chitobiase was expressed as ambient chitobiase activity, as well as rate of production (N = 10). The ambient chitobiase activity was also normalized with discharge (ambient chitobiase times total discharge) to account for dilution of chitobiase, creating an 'absolute' ambient chitobiase activity.

Benthic community endpoints calculated were: total density and total biomass; density, biomass, percent density, and percent biomass of EPT, Diptera, Chironomidae, and Oligochaeta.

Spearman (non-parametric) correlation r-value was used to identify any 'moderate' relationships between the two chitobiase measurements (ambient and production rate) and biotic community endpoints. Any significant ($p < 0.05$) correlations with absolute Spearman correlation r-values greater than (0.50) were further assessed using linear regression. Before linear regression, the data were tested for normality using a Shapiro-Wilks test, if the data failed to meet the normality assumption of the linear regression model, the following transformations were applied: Log_{10}, square root, fourth root, inverse, and logit

(percentage data only). Transformation type was selected based on that which produced the greatest Shapiro-Wilks p-value and inspection of quantile-quantile (Q-Q) plots. Spearman correlation was conducting using SPSS version 12 (SPSS 2003) and all other analysis were conducted using the Real Statistics Resource Pack version 4.3 add-in (Zaiontz 2015) for Microsoft Excel©.

3.5 Results

Inspection of chitobiase degradation curves (found in SI) revealed that two areas failed to demonstrate degradation; areas SLINE and HENFO had determination coefficients of 0.14 and 0.16, respectively. Inspection of the measured degradation curve for HENFO revealed that the '6-hour' measurement was likely invalid, which could have been the result of improper mixing of the 250 mL water sample that was being subsampled, improper filtering, or contamination of the sample with bacteria. Removal of the 6-hour measurement resulted in an acceptable coefficient of determination of 0.97 for the degradation regression and so this site was retained as part of the analysis. Chitobiase was detected in SLINE, but the resulting degradation curve was not statistically significant with no obvious outliers. Therefore, SLINE was excluded from further analysis. This left a total of nine areas with acceptable chitobiase measurements. Detected ambient chitobiase activities for areas ranged between 1.1 to 97.2 nM MUF with an average of 16.8 ± 13.8 (Standard Deviation) nM MUF. The MDL for chitobiase was determined to be 1.58 nM; all chitobiase sample activities used to determine the rate of production were above the MDL. The rate of chitobiase production for the nine areas ranged between 0.020 and 0.222 nM MUF/h (Table

1) with an average of 0.065 ± 0.061 nM MUF/h. Total benthic invertebrate biomass ranged from 4.50 to 69.27 g/m^2 among the ten sample areas (Table 1). Water temperature, pH, specific conductance and dissolved oxygen for the ten areas sampled for chitobiase production ranged between 4.3 – 8.7°C, 7.97 – 8.65, 0.0293 – 1.302 mS/cm, and 10.0 – 12.5 mg/L, respectively (Table 1).

Spearman correlation of chitobiase (ambient and production rate) with biotic endpoints that had an absolute r-value greater than 0.50 and p-values less than 0.05, included the following: eight with production rate, one with ambient chitobiase activity, and one with ambient chitobiase activity divided by discharge (Tables 2 and 3).

Normality tests of the three chitobiase endpoints and the biotic endpoints with which they demonstrated moderate correlation (absolute Spearman r-value greater than 0.50 and p-value less than 0.05) indicated that the data were not normally-distributed (Shapiro-Wilk's p-value > 0.05). A log10 transformation was applied and resulted in all data being normally-distributed. Therefore, linear regression was applied using log10 transformation.

A significant linear relationship was found between rate of chitobiase production and total measured biomass (r^2 = 0.80; Figure 2 and Table 4). Other biotic endpoints (auto-correlated) also demonstrated strong regression relationships; the rate of chitobiase production and Chironomidae biomass (r^2 = 0.77; Fig. 2 and Table 4), Chironomidae density (r^2 = 0.77; Table 3), EPT biomass (r^2= 0.69; Figure 2 and Table 4), Trichoptera biomass (r^2 = 0.75; Figure 2 and Table 4), and Plecoptera biomass (r^2 = 0.59; Table 4). A weak positive

correlation (spearman r = 0.58 and p = 0.10) was found between AFDM and rate of chitobiase production, whereas no statistically significant relationship was found with chl-a (Table 2).

3.6 Discussion

Based on results from this study, the hypothesis that a significant positive relationship exists between the rate of chitobiase production and benthic invertebrate biomass was supported (Figure 2), thus also supporting the hypothesis that chitobiase can be used as a proxy for total benthic invertebrate biomass. While the rate of chitobiase production had the greatest r^2 with total biomass, the relationship is likely driven by EPT biomass within this system (Figure 2) as this group accounted for 43 to 92 % of overall biomass among areas. Four of the five areas sampled within a sub-watershed (Line Creek) and the five areas sampled in other sub-watersheds both demonstrated a linear relationship with the rate of chitobiase production (Figure 2). This suggests that the rate of chitobiase production can be used to compare secondary productivity both within and among streams allowing it to be used for area-specific studies and in a general biomonitoring context. This study supports the utility of chitobiase as a possible surrogate for measures of secondary production as has been shown in other studies of marine systems and within laboratory settings (Oosterhuis et al. 2000; Sastri and Dower 2006, 2009; Sastri and Roff 2000). That said, significant work is required to determine the limitations of the approach in freshwater ecosystems and many technical details remain unexplored.

No significant relationship between ambient chitobiase activity and benthic invertebrate biomass was observed during this study (r^2 = -0.033 and p = 0.932) (Table 2). Further, the relationships that exist between the biomass of a specific taxon group proved to have weaker (lower r-value) linear relationships with ambient chitobiase activity than with rate of production (Table 4). This outcome could be due to the fact that ambient chitobiase activity is not comparable between areas unless they are very similar in nature across a variety of factors simultaneously (volume, depth, discharge, temperature, width, microbial community, hydrology, etc.). A measure of standing chitobiase in a system limits you to only identifying the amount of chitobiase present at that point in time and does not account for factors that may affect its presence and stability. For example, an aquatic system that has a sparsely populated arthropod community (low biomass) and little to no factors effecting the removal of chitobiase, such as degradation by high microbial activity, the ambient activity of chitobiase will be relatively high because it remains *in-situ.* Comparatively, a similar system that has a densely populated arthropod community (high biomass) with numerous factors that contribute to chitobiase degradation or dilution, such as high microbial load, the ambient level of chitobiase may be relatively low although the system supports a large arthropod community. Rate of chitobiase degradation is a more suitable measure when looking at a possible relationship with biomass because confounding factors such as water volume and bacterial degradation are accounted for. The comparison of ambient chitobiase may be more suited to controlled laboratory conditions, where all environmental conditions are equal,

while the rate of chitobiase degradation is more suitable for comparisons in the field. This entails more effort and time commitments for field-level sampling but results in a measure that is fully linked to secondary production. Therefore, the prediction that a positive relationship exists between ambient chitobiase activity and benthic invertebrate biomass can be viewed as tenuous.

The ecology of the communities present in these systems at a given time during the year could have an effect on the ambient levels of chitobiase and production/degradation rates. This study was conducted in September, however area characteristics and the ecology of stream systems are influenced by seasonal characteristics, such as changes in photoperiod and temperature. These changes have a direct impact on aquatic invertebrate communities. Communities are more productive during increased photoperiod and temperature that will result in increased rates of chitobiase production. Espie and Roff (1995a) observed a significant negative trend between molt cycle and temperature (i.e., greater temperature, more rapid molt) for the zooplankter *D. magna*, which in turn was negatively correlated with chitobiase activity (longer molt times, less chitobiase per organism). As many insects enter a diapause stage as water temperature and photoperiod decrease resulting in decreased growth and metabolism (Gillot 1991), this would translate into a decrease in the rate of chitobiase production. Understanding how the rate of chitobiase production varies across seasons in freshwater systems is lacking. Identifying seasonal patterns would provide further information needed to assess its utility in predicting secondary production across seasons.

Among the areas examined in this study, there were distinct differences in primary production with chl-a values ranging from 1.48 mg/m^2 at LIDSL to 180.45 mg/m^2 at LILC3, resulting in an absolute difference of 178.97 mg/m^2. No correlation was present between chl-a and both ambient chitobiase and rate of chitobiase production, however a weak positive correlation was determined between AFDM content and rate of chitobiase production for the nine areas where a rate was determined (Table 2). The lack of a strong relationship between chl-a or AFDM with the rate of chitobiase production suggests that chitobiase provides information concerning ecosystem function that is not provided by these two surrogates of primary production or is strongly influenced by factors other than primary production (e.g., allochthonous inputs).

The use of chitobiase production as a measure of secondary production within a system could complement traditional sampling methods, especially where intensive sampling is not feasible or is cost prohibitive. Estimating secondary production of invertebrates involves quantitative sampling using multiple replicates. The samples are then sorted (invertebrates picked out) and weighed, with the whole process taking up to six months and approximately $350 per sample at a commercial laboratory. Measuring chitobiase is less expensive (about $30 Canadian in supplies and reagents per curve), much faster in the laboratory (hours or days, not months), and requires fewer replicates (as chitobiase released in the water column is more evenly mixed and not ‘patchy’ as benthic communities can be). The reduced cost and analysis time mean secondary production estimates can also be performed more frequently.

Measurement of chitobiase is also non-destructive with no organisms collected or habitat disturbed.

In conclusion, a positive significant correlationship was found between the rate of chitobiase production and total benthic invertebrate biomass in rocky mountain (steep gradient) lotic systems during this study. Although results support our initial hypotheses, extensive further validation of this method, involving a larger dataset that incorporates seasonal and geographical variability, should be considered before recommending wider adoption. This would allow for a more thorough evaluation concerning the potential use of chitobiase as a routine biomonitoring endpoint for secondary production.

3.7 Literature Cited

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3.8 Tables and Figures

Table 3.1 Water quality parameters and measurements of ambient chitobiase (CBA), rate of chitobiase production (CBP), total benthic arthropod biomass and density, chlorophyll-a (Chl-a) and ash free dry mass (AFDM) from sample sites (n=10) in the Elk River Watershed, British Columbia, Canada. The coefficient of determination (r^2) is for the linear regression of chitobiase rate of production (detected chitobiase vs. time).

Site	Date	CBA (nM)	CBP (nM/h)	Total Biomass (g/m^2)	Total Density (#org/m^2)	Chl-a (mg/m^2)	AFDM (g/m^2)	Dissolved Oxygen (mg/L)	Specific Conductivity (µS/cm)	pH	Temperature (°C)	r^2
AL4	09/13/2015	35.32	0.05	18.85	8,116.7	71.0	59.0	10.6	293	8.27	7.3	0.98
GHCKD	09/15/2015	27.46	0.03	4.50	4,696.7	31.4	6.7	10.3	1302	-	8.2	0.99
HACKUS	09/11/2015	11.94	0.06	15.75	8,123.3	150.0	23.7	11.1	717	8.28	4.6	0.80
HENFO[a]	09/16/2015	5.56	0.05	9.24	7,033.3	7.3	3.5	11.0	431	8.20	6.2	0.97
MI5	09/13/2015	27.20	0.03	15.26	11,550.0	1.5	0.6	10.1	386	7.97	8.7	0.84
LI8	09/13/2015	7.13	0.05	15.05	3,523.0	10.1	2.3	10.0	681	8.65	7.6	0.79
LI24	09/10/2015	4.20	0.02	6.10	2,135.0	7.2	16.0	10.2	289	8.27	4.3	0.84
LILC3	09/14/2015	4.62	0.22	69.27	24,862.0	180.5	122.8	12.5	943	8.13	5.2	0.97
LIDSL	09/12/2015	7.08	0.08	26.2	8,072.0	30.9	12.6	10.0	749	8.30	6.5	0.98
SLINE[b]	09/10/2015	3.60	0.01	11.47	4,497.0	19.7	8.3	11.9	336	8.55	3.2	0.14

[a] Six-hour degradation value removed due to suspected error.

[b] Area not included in statistical analysis as degradation coefficient suggests sample contamination.

Table 3.2 Spearman correlation of benthic invertebrate endpoints (biomass) with chitobiase collected by Hess sampler from 9 sampling areas. Bolded cell values indicate absolute r-value > 0.50 and highlighted cells indicate p-values < 0.05.

Endpoint		Statistic	Chitobiase Rate of Production	Ambient Chitobiase	Ambient Chitobiase Divided by Discharge
Biomass (g/m^2) (Absolute Biomass)	Total Biomass	r	**0.783**	-0.033	**0.517**
		p	0.013	0.932	0.154
	EPT	r	**0.817**	-0.067	0.483
		p	0.007	0.865	0.187
	Ephemeroptera	r	0.017	-0.033	0.350
		p	0.966	0.932	0.356
	Plecoptera	r	**0.733**	**-0.683**	-0.167
		p	0.025	0.042	0.668
	Trichoptera	r	**0.817**	-0.033	**0.550**
		p	0.007	0.932	0.125
	Diptera	r	0.433	0.217	0.267
		p	0.244	0.576	0.488
	Chironomidae	r	**0.967**	-0.200	0.200
		p	0.000	0.606	0.606
	Oligochaeta	r	0.483	0.017	0.217
		p	0.187	0.966	0.576
Biomass (%) (Relative Biomass)	EPT	r	0.050	-0.367	-0.050
		p	0.898	0.332	0.898
	Ephemeroptera	r	-0.350	-0.267	-0.050
		p	0.356	0.488	0.898
	Plecoptera	r	0.033	**-0.533**	**-0.833**
		p	0.932	0.139	0.005
	Trichoptera	r	**0.733**	-0.017	0.467
		p	0.025	0.966	0.205
	Diptera	r	0.117	-0.300	0.017
		p	0.765	0.433	0.966
	Chironomidae	r	0.483	0.083	-0.250
		p	0.187	0.831	0.516
	Oligochaeta	r	0.150	0.367	0.183
		p	0.700	0.332	0.637
Primary Productivity	Chlorophyll-a	r	0.367	-0.150	-0.267
		p	0.332	0.700	0.488
	AFDM	r	**0.583**	0.167	-0.117
		p	0.099	0.668	0.765

Table 3.3 Spearman correlation of benthic invertebrate endpoints (density) with chitobiase collected by Hess sampler from 9 sampling areas. Bolded cell values indicate absolute r-value > 0.50 and highlighted cells indicate p-values < 0.05.

Endpoint		Statistic	Chitobiase Rate of Production	Ambient Chitobiase	Ambient Chitobiase Divided by Discharge
# of organisms/m^2 (Absolute Density)	Total Density	r	**0.567**	0.200	0.467
		p	0.112	0.606	0.205
	EPT	r	0.433	0.133	**0.567**
		p	0.244	0.732	0.112
	Ephemeroptera	r	**0.550**	-0.117	0.283
		p	0.125	0.765	0.460
	Plecoptera	r	**0.733**	-0.383	0.000
		p	0.025	0.308	1.000
	Trichoptera	r	0.433	0.283	**0.567**
		p	0.244	0.460	0.112
	Diptera	r	0.433	0.333	-0.033
		p	0.244	0.381	0.932
	Chironomidae	r	**0.767**	0.117	0.100
		p	0.016	0.765	0.798
	Oligochaeta	r	0.000	0.417	0.067
		p	1.000	0.265	0.865
Percentage of Community/m^2 (Relative Density)	EPT	r	-0.267	-0.367	0.000
		p	0.488	0.332	1.000
	Ephemeroptera	r	-0.083	-0.350	-0.050
		p	0.831	0.356	0.898
	Plecoptera	r	-0.033	-0.333	-0.400
		p	0.932	0.381	0.286
	Trichoptera	r	0.017	**0.633**	0.450
		p	0.966	0.067	0.224
	Diptera	r	0.467	0.300	-0.067
		p	0.205	0.433	0.865
	Chironomidae	r	**0.600**	0.217	-0.067
		p	0.088	0.576	0.865
	Oligochaeta	r	-0.300	0.450	-0.100
		p	0.433	0.224	0.798

Table 3.4 Linear regressions of benthic invertebrate endpoints (biomass and density) with chitobiase metrics that had a spearman correlation absolute r-value > 0.50 and a p-value < 0.05. Bolded cell values indicate $r^2 \geq 0.70$.

Endpoint			Chitobiase Metric	Regression	r2
Biomass	Non- Transformed	Total Biomass (g/m^2)	Chitobiase Rate of Production	y = 0.0031x + 0.004	**0.96**
		EPT (g/m^2)	Chitobiase Rate of Production	y = 0.0036x + 0.006	**0.94**
		Plecoptera (g/m^2)	Chitobiase Rate of Production	y = 0.0219x + 0.009	**0.84**
			Ambient Chitobiase	y = -2.3682 + 20.968	0.25
		Trichoptera (g/m^2)	Chitobiase Rate of Production	y = 0.0042x + 0.020	**0.97**
		Chironomidae (g/m^2)	Chitobiase Rate of Production	y = 0.0238x + 0.023	**0.98**
		Plecoptera (%)	Ambient Chitobiase Divided by Discharge	y = -1.4813x + 35.564	0.41
		Trichoptera (%)	Chitobiase Rate of Production	y = 0.0020x - 0.014	0.43
	$\log_{10}$ Transformed	Total Biomass (g/m^2)	Chitobiase Rate of Production	y = 0.7500x - 2.168	**0.80**
		EPT (g/m^2)	Chitobiase Rate of Production	y = 0.5935 - 1.917	0.69
		Plecoptera (g/m^2)	Chitobiase Rate of Production	y = 0.5642x - 1.426	0.59
			Ambient Chitobiase	y = -0.5497x + 1.169	0.25
		Trichoptera (g/m^2)	Chitobiase Rate of Production	y = 0.3959x - 1.572	**0.75**
		Chironomidae (g/m^2)	Chitobiase Rate of Production	y = 0.4101x - 1.267	**0.77**
		Plecoptera (%)	Ambient Chitobiase Divided by Discharge	y = -1.7493x + 2.684	0.65
		Trichoptera (%)	Chitobiase Rate of Production	y = 0.0113x - 1.738	0.60
Density	Non-Transformed	Plecoptera (#org/m^2)	Chitobiase Rate of Production	y = 6E-05x + 0.0068	0.32
		Chironomidae (#org/m^2)	Chitobiase Rate of Production	y = 1E-05x + 0.0303	**0.96**
	$\log_{10}$ Transformed	Plecoptera (#org/m^2)	Chitobiase Rate of Production	y = 0.0004x - 1.6227	0.44
		Chironomidae (#org/m^2)	Chitobiase Rate of Production	y = 0.3707x - 2.4007	**0.77**

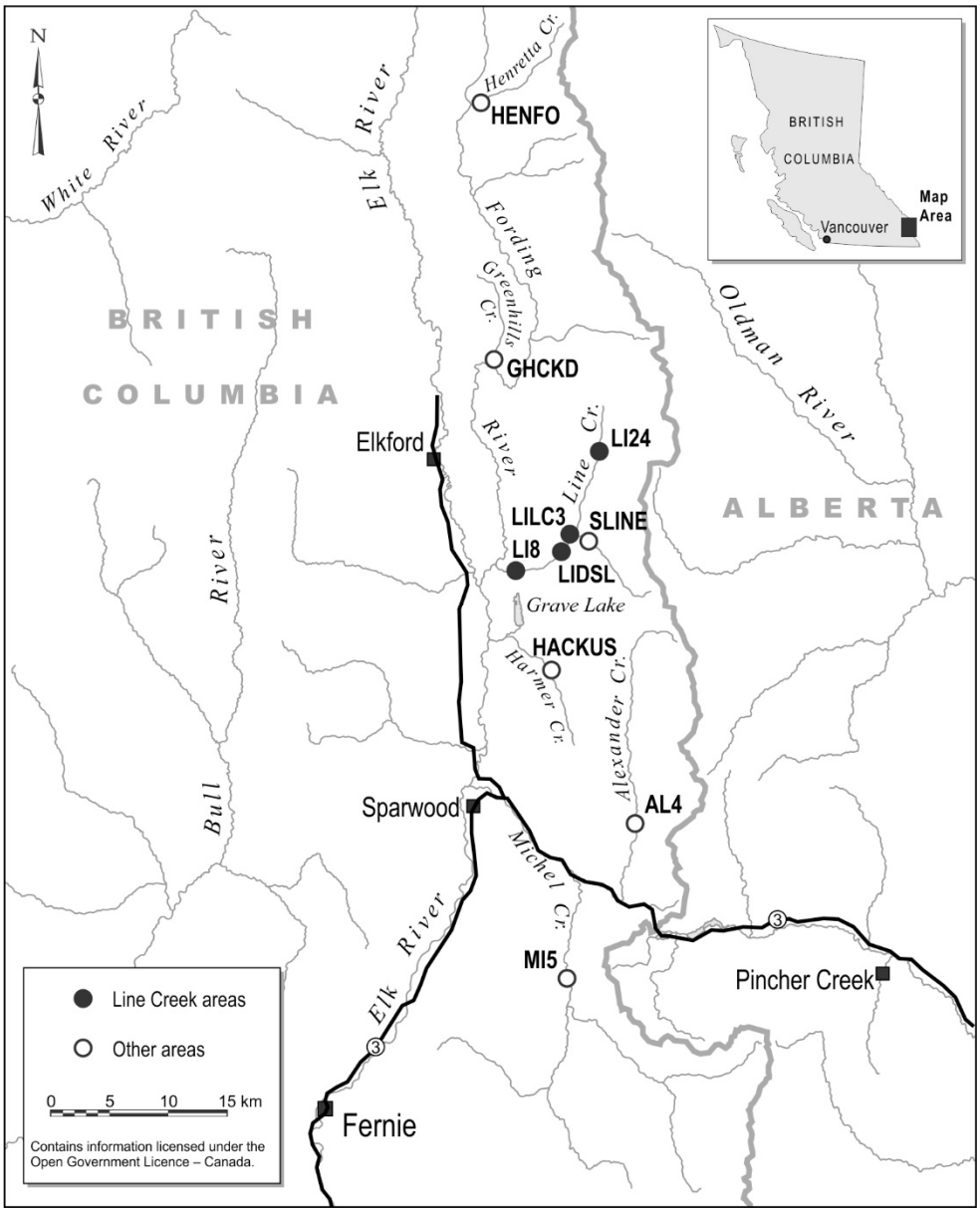

Figure 3.1 Sample locations of study areas located in the Elk River Watershed, British Columbia, Canada.

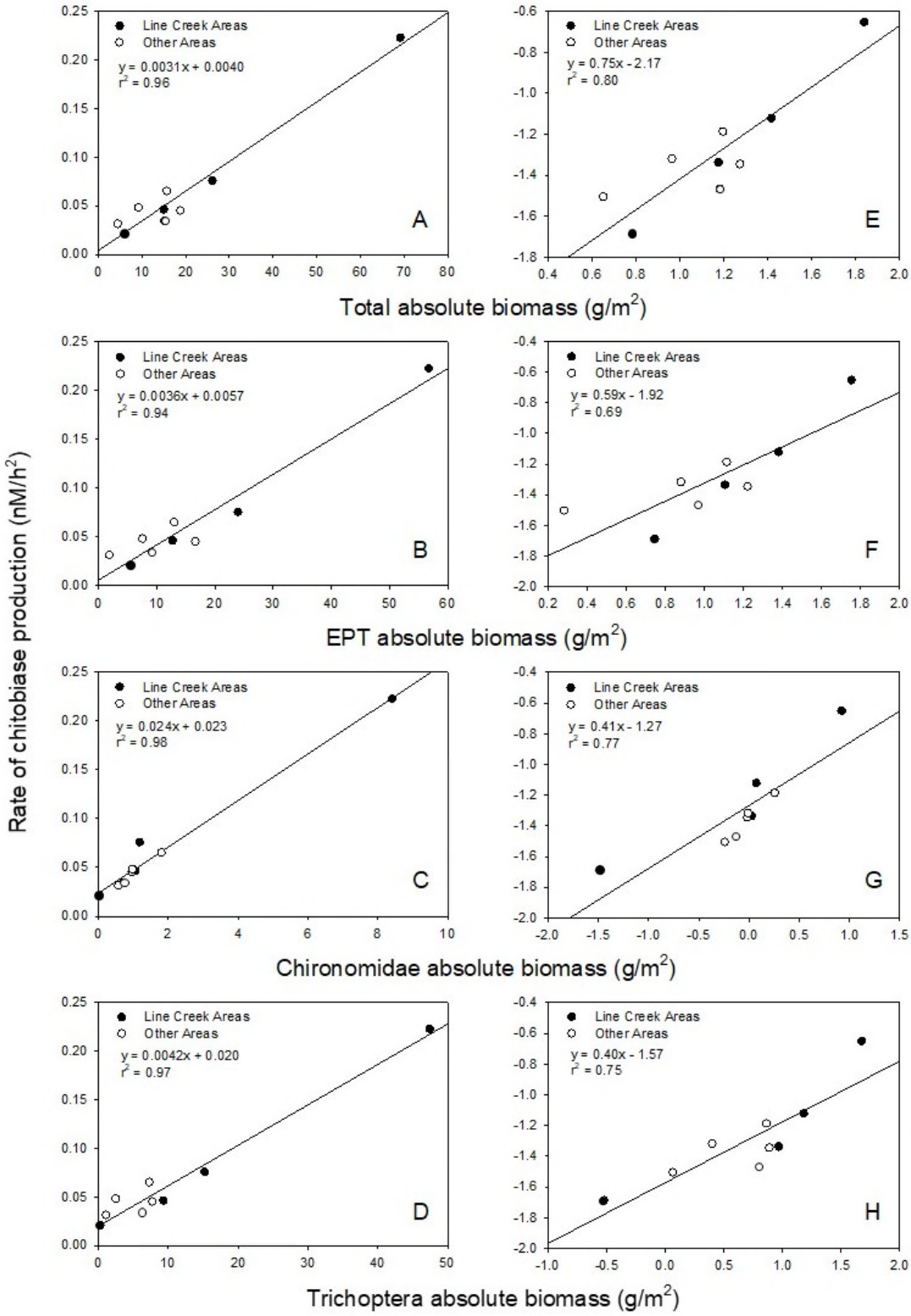

Figure 3.2 Linear regression showing the relationships between measured rate of chitobiase production and total invertebrate absolute biomass; Ephemeroptera, Plecoptera, and Trichoptera (EPT) absolute biomass; Trichoptera absolute biomass; and Chironomidae absolute biomass in the Elk River Watershed from 9 areas sampled between September 10 – 16, 2015. Graphs A-D are non-transformed, graphs E-H are $\log_{10}$ transformed.

SUMMARY

The main objectives of this thesis were to: 1) investigate and evaluate the relationship, if any, between the rate of production of chitobiase and zooplankton and emergent insect biomass using wetland mesocosms (see Chapter 2); 2) investigate and evaluate the impacts of sulfamethoxazole (SMX), if any, on ambient chitobiase, the rate of chitobiase production, and emergent insect and zooplankton biomass using wetland mesocosms (see Chapter 2); 3) investigate and evaluate the impacts of dilbit, if any, on ambient chitobiase, the rate of chitobiase production, and emergent insect and zooplankton biomass using wetland mesocosms (see Chapter 2); and 4) investigate and evaluate the relationship, if any, between the rate of production of chitobiase and benthic arthropod biomass in freshwater lotic systems under full field conditions (see Chapter 3).

Contrary to expectations, no statistically significant relationship was observed between chitobiase activity and aquatic invertebrate biomass, (zooplankton biomass, emergent insect biomass, and total aquatic invertebrate biomass) under the studies performed at the PWRF during the summer of 2015. Previous studies (Oosterhuis et al. 2000; Sastri and Dower 2006, 2009) had observed that chitobiase activity could be used to estimate arthropod biomass in marine systems. Additionally, laboratory-based studies (Sastri and Roff 2000; Vrba and Machacek 1994) also reported a significant and consistent a relationship between measured chitobiase activity and aquatic invertebrate biomass. However, it should be noted that the relationships observed during

these studies were focused on the biomass of individual aquatic species. One possible reason that a relationship was not observed is due to the fact that aquatic benthic invertebrates were not sampled during the studies. As such, the study was missing a major component of the aquatic invertebrate community and biomass of those invertebrates that were not collected were not represented in measures of aquatic invertebrate biomass. Another possible reason is that sampling time and frequency did not capture a representative measure of chitobiase activity within the aquatic mesocosms. The determined average half-life of chitobiase over the course of the SMX and dilbit studies were 5.3 (±2.8) hours and 7.4 (±3.3) hours, respectively. Due to the relatively short half-life of chitobiase that was observed during the study, it is possible that chitobiase samples were not collected during the window of opportunity to obtain a representative measure of chitobiase activity in the mesocosm systems. This is especially true when comparing chitobiase activity to measures of emergent insect biomass, as the molting event during which the aquatic invertebrate forgoes its larval aquatic stage and enters its adult emergent stage has occurred during its capture. Although no half-life times were reported in the studies looking at the relationship between chitobiase and aquatic invertebrate biomass, Oosterhuis et al. (2000) reported that measured chitobiase activity within marine samples decreased by 20% over 24 hours. This would represent a much longer half-life, which could be estimated at approximately 60 hours if the degradation follows a linear pattern. Therefore, it is possible that chitobiase might degrade at an increased rate in freshwater conditions, allowing for a missed opportunity to

get a more accurate measure of chitobiase when it is released during molting. If chitobiase measures are collected well after the emergent insect molting event has occurred when the invertebrate changes from its aquatic larval stage to its emergent adult stage, resultant measured chitobiase activity could be well below that of what it would have been closer to the molting event.

However, over the course of the summer of 2015, chitobiase activity, both ambient chitobiase and chitobiase ROP, were consistently and successfully measured in all of the mesocosms involved in both the SMX and dilbit studies (n = 15). The assay for chitobiase activity exhibited very low variability under field conditions (CV<5%) implying that the assay was fairly precise.

During the SMX study, no statistically significant difference in chitobiase activity, both ambient chitobiase and chitobiase ROP, was observed when comparing control and treatment mesocosms exposed to SMX. Therefore, it was concluded that SMX did not have a noticeable effect on chitobiase activity throughout the duration of the study. The treated mesocosms were spiked with 250 µg/L of SMX four separate times over the course of the study and was successfully measured after each application. Average one-hour post-treatment SMX concentrations ranged between 278.6 µg/L to 480.8 µg/L, which is well above environmentally relevant concentrations, which rarely exceed 2.0 µg/L (Kolpin et al. 2002; Watkinson et al. 2009). The absence of an impact of SMX on chitobiase activity was expected as measured concentrations are well below reported EC50 values of >100,000 µg/L for Daphnia species (Ferrari et al. 2003). Similarly, no statistically significant difference between chitobiase activity in

control, low dose, and high dose mesocosms exposed to dilbit was observed. Therefore, it was concluded that dilbit did not have a noticeable effect on chitobiase activity throughout the duration of the study. The most probable reason for dilbit not having a noticeable effect on chitobiase activity was that the amounts of dilbit added to the low dose (0.0003 mL/L of dilbit) and high dose (0.003 mL/L of dilbit) were not enough to elicit a response by aquatic invertebrates. In a study performed by Robidoux (2018), no mortality was displayed in Daphnia exposed to 10 g/L of diluted bitumen and toxicity was only displayed at dilbit concentrations >32 g/L. Additionally, diluted bitumen has a lower density than water, therefore it is possible that throughout the duration of the study it did not disperse into the water column and therefore did not come in contact with aquatic invertebrates (Engineering and Medicine National Academies of Sciences 2015).

It should be noted that SMX and dilbit were used during these studies due to other studies running concurrently at the PWRF. SMX would not be an ideal stressor to focus on the effects of invertebrates in aquatic monitoring studies due to the fact that environmentally relevant concentrations are quite minimal and that the target organism of this pharmaceutical are bacteria. As well, the amount of dilbit introduced to the mesocosms was quite small and did not appear to impact aquatic invertebrates in any way. It appeared that during the dilbit study, the dilbit and its constituents remained isolated on the surface of the water and did not come in contact with organisms within the water column. In order to address the issue with SMX, I would recommend that more suitable or

environmentally relevant pharmaceuticals or wastewater by-products be used during mesocosms studies when observing effects of stressors on aquatic invertebrate communities. In terms of the dilbit study, I would recommend either adding a higher amount of dilbit to each mesocosm or to facilitate the mixing of the dilbit into the water column, where observable effects might exist.

As it relates to my fourth thesis objective; a significant positive relationship was found between the rate of chitobiase production and total benthic invertebrate biomass in freshwater lotic systems during the study performed in the Elk Valley watershed in southeastern British Columbia, Canada. Benthic invertebrates and chitobiase were sampled from ten freshwater lotic areas located within the watershed. The chitobiase ROP exhibited a significant positive linear relationship ($p < 0.05$, $r^2 = 0.80$) with benthic invertebrate biomass. Additionally, positive relationships were also observed between the chitobiase ROP and the measured biomass of two taxa groups that are commonly used as water quality indicators: Chironomidae and Ephemeroptera, Plecoptera, Trichoptera (EPT) ($r^2 = 0.77$ and 0.69, respectively). No significant relationship was observed between ambient chitobiase activity and benthic invertebrate biomass ($r^2 = -0.033$, $p = 0.932$) during this study. These results suggest that the rate of chitobiase production can be used to compare secondary productivity both within and along streams and supports the utility of chitobiase activity as a possible surrogate for the measure of secondary production, which has been shown in other studies of marine systems and within laboratory settings (Oosterhuis et al. 2000; Sastri and Dower 2006, 2009; Sastri and Roff 2000).

Based on the results observed for both the mesocosm studies and the field study, it can be concluded that the use of chitobiase activity as a measure of aquatic invertebrate biomass for freshwater ecosystems has potential, but there is still significant uncertainty in the approach. Although no significant relationship was observed between chitobiase activity and measures of biomass in the mesocosm studies, it should not be ruled out until further studies are performed. The observation of a positive relationship between the rate of chitobiase production and measured aquatic benthic invertebrate biomass during the Elk River Valley study points towards the possibility of the inclusion of this method in EEM. This method could be used in addition to current benthic invertebrate assessment techniques, where performing it prior to collecting physical invertebrate samples would allow for the comparison of chitobiase measures to measured invertebrate biomass as a confirmation of its validity. Although, I would recommend further studies focusing on the measurement of chitobiase as a surrogate measure for aquatic invertebrate biomass in an EEM context to observe its validity and repeatability.

Recommendations for further studies include 1) the sampling of benthic invertebrates in addition to zooplankton and emergent insects during mesocosm studies to achieve a more total measure of aquatic invertebrate biomass within an aquatic ecosystem, 2) looking at sample collection frequencies for both aquatic invertebrate biomass and measures of chitobiase to determine if effects exist on the time of when samples are collected, and 3) performing studies over

longer periods of time or at similar time points over the course of several seasons to compile a baseline and determine repeatability of this method.

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