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Molecular investigation of stalk formation and growth rates in Didymosphenia geminata

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Molecular investigation of stalk formation and growth rates in *Didymosphenia geminata*

by

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Abstract

Didymosphenia geminata, a freshwater diatom, can produce thick benthic mats of stalk material. The cause of excess stalk formation is proposed to be low soluble reactive phosphorus (SRP) concentrations or a mat-forming genetic variant. Molecular studies on *D. geminata* are few, partly due to the lack of a reliable DNA extraction technique for the diatom, but have the ability to elucidate the drivers of mat formation. Specifically, comparison of gene expression between mat and non-mat-forming sites using reference genomes, may reveal underlying mechanisms behind growth and stalk formation. The objectives of this study were to: 1) identify gene products, expressed only at a mat or non-mat-forming site in Alberta (AB), involved in cell division or stalk formation from the reference genomes of *P. tricornutum*, *T. pseudonana* and *C. crescentus*; 2) compare SRP and growth rate, using frequency of dividing cells (FDC), between a mat-forming and non-mat-forming site; 3) compare FDC between multiple mat-forming sites in AB and British Columbia (BC); and 4) compare the quantity and quality of DNA obtained from mat-forming *D. geminata* using various preservation buffers, storage temperatures and extraction methods, including organic extraction, the Qiagen DNeasy® Plant Mini Kit, and a version of the kit procedure modified for *D. geminata*. Sixty-six putative gene products from the reference genomes were expressed only in the mat-forming sample, while 172 were expressed only in the non-mat-forming sample. FDC and SRP were higher at the non-mat-forming site, with no significant difference between FDC in AB and BC. Collectively, these results support previous findings that *D. geminata* forms excess stalk material under low SRP and increases cell division under higher SRP.

However, an anomalous observation of mat formation at a site with 0.00986 mg/L SRP indicates stalk formation may be influenced by additional factors. In addition, the results of this study indicate that DNA extracted from samples stored in 95% EtOH at -80°C, using the modified kit method, had the best combination of quantity and quality.

Preface

This thesis is original, unpublished, independent work by the author, K. M. Larsen.

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List of Symbols, Abbreviations, and Nomenclature

Symbol	Definition*
CA	Carbonic Anhydrase
COWN	Cowichan River (48.770450, -123.893517)
CTAB	Cetyltrimethyl Ammonium Bromide
DOUG	Douglasdale (50.943091, -114.012255)
EDNA	Edna Lake Outflow (53.079900, -118.032933)
EDWR	Edworthy Park (51.064567, -114.154650)
EPS	Extracellular Polymeric Substances
EPT	Ephemeroptera, Plecoptera, and Trichoptera
FDC	Frequency of Dividing Cells
FLS2	Flavonol Synthase 2
GMD	GDP-mannose 4,6-dehydratase
ITS	Internal Transcribed Spacer
KOKS	Koksilah River (48.642763, -123.738842)
LOUC	Louise Creek (51.418283, -116.204600)
NGS	Next-Generation Sequencing
OE	Organic Extraction
OST	Oligosaccharyl Transferase
PAP	Plastid-lipid Associated Protein
PCR	Polymerase Chain Reaction
Pho	Phosphate
phoD	Alkaline Phosphatase D
PMM	Phosphomannomutase
QUAL	Little Qualicum River (49.313500, -124.557217)
rbcS	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit
SITs	Silicon or Silicic Acid Transporters
SOD	Superoxide Dismutase
SRP	Soluble Reactive Phosphorus
TP	Total Phosphorus

* Where coordinates are given, they are latitude (N) and longitude (W) in decimal degrees.

CHAPTER 1: GENERAL INTRODUCTION

Invasive Species

Climate change and invasive species are two of the greatest threats to biodiversity on earth (Sala et al. 2000, Thuiller 2007). Species invasions are widespread and a noted cause of extinctions (Clavero and Garcia-Berthou 2005, Molnar et al. 2008). Invasive species can lead to the extinction of native species because invasives have the ability to quickly colonize new geographical locations and, in the process, outcompete native species, disrupt local food webs and degrade habitat (Elton 1958, Kolar and Lodge 2000). Climate change may increase the extent of invasions (Thuiller 2007) as environmental change stresses native species and gives invaders a competitive advantage. Due to growth and reproductive characteristics, invasives have broad tolerance to environmental change; further, invasives thrive in disturbed areas created by more frequent natural disasters (Hellmann et al. 2008, Bradley et al. 2010). In conclusion, climate change and invasives are major threats to biodiversity globally, and climate changes will increase the negative effects of invasives.

Freshwater systems frequently experience negative impacts from the introduction of invasive species (Sala et al. 2000). For example, Asian carp, a group of fish including grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molotrix*) and bighead carp (*Hypophthalmichthys nobilis*), are characterized by rapid reproduction following invasions that decimate plankton populations (Kolar et al. 2005). Asian carp have had substantial negative ecological effects on the Mississippi River Basin and

threaten to invade the Great Lakes (Kolar et al. 2005). Another example, Eurasian watermilfoil (*Myriophyllum spicatum*), a non-native invasive aquatic plant, has spread through most of North America over the last 70 years. Eurasian watermilfoil attains high levels of biomass, shades out native aquatic plants, and grows so densely that it interferes with recreational activities and irrigation systems (Aiken et al. 1979, Newroth 1985, Boylen et al. 1999).

"Native invaders" are indigenous species that become detrimental to ecological systems due to anthropogenic change that allows an increase in numbers or biomass (Valery et al. 2008, Simberloff 2011). Anthropogenic changes can include climate change, predator removal, fire suppression, increases or decreases in nutrient concentrations, habitat fragmentation or other influences (Carey et al. 2012). For instance, in the Northeastern USA, where predator numbers have declined, white-tailed deer (*Odocoileus virginianus*) populations have greatly increased. White-tailed deer graze the forest floor removing young trees and understory. The community structure of the forest changes and prevents young trees from reaching a size where they can provide suitable bird habitat (Horsley et al. 2003). Another example is the juniper (*Juniperus spp*.) in western North America. Due to anthropogenic fire suppression, juniper shrubs are now outcompeting other native plants and changing the community structure of their native habitat (Ansley and Rasmussen 2005). Similar to non-native invaders, native invaders also cause ecological harm.

Mitigation of the negative effects of native and non-native invasive species is important as transport of humans and goods globally, as well as the impact of climate change on our environment, continues to increase (Bryan 1996, USBC 2001). Knowledge

of both native and non-native invasive species must be obtained to create effective regulatory policies and minimize damage that invasives may cause to ecosystems (Kolar and Lodge 2000). Examples of important knowledge include the origin and vector of introduced invasive species and mechanisms that cause indigenous and exotic species to become invaders (Morton and Schemerhorn 2013). This study will focus on the freshwater diatom *Didymosphenia geminata*, which is likely both a native and non-native invader (Kilroy and Unwin 2011, Taylor and Bothwell 2014), and the molecular mechanism underlying nuisance stalk formation.

Didymosphenia geminata

Didymosphenia geminata (Lyngbye) M. Schmidt is a raphid pennate, freshwater, single-celled diatom that recently has begun to exhibit invasive behaviour in lotic systems (Kravtsova et al. 2004, Spaulding and Elwell 2007). *D. geminata* cells have a silica outer cell wall called a frustule, which is composed of two distinct halves. Cells are large in size compared to many other diatoms, approximately 60−140 μm long and 25−43 μm wide, and have a distinct 'Coke bottle' shape (Figure 1) (Krammer and Lange-Bertalot 1997, Bhatt et al. 2008).

D. geminata cells primarily reproduce asexually through mitotic division when phosphorus concentrations are sufficient (Bothwell and Kilroy 2011). Cells that divide vegetatively become smaller with each division. Though it has not been documented recently in *D. geminata*, cells have been observed reproducing sexually in the same manner as other pennate diatoms (Meyer 1929). To sexually reproduce, diatoms produce gametes, which come together to create an auxospore (Spaulding and Elwell 2007).

Auxospores are reproductive cells that are needed to restore diatom cell size. In addition sexual reproduction in diatoms contributes to genetic variation (Lewis 1984).

D. geminata cells produce extracellular polymeric substances (EPS), including mucilage, which facilitates movement, and mucopolysaccharide stalk material. Stalks attach to benthic surfaces, increase in length, and elevate the associated cell(s) in the water column under conditions where cell division is limited by SRP (soluble reactive phosphorus) (Spaulding and Elwell 2007, Bothwell & Kilroy 2011). The purpose of stalks is unknown; however, stalks are hypothesized to provide attached cells with support in moving water and increased access to light and growth-limiting SRP (Kilroy and Bothwell 2011) or, possibly organic phosphorus, since some stalks stain positive for alkaline phosphatase (Bray et al. 2016).

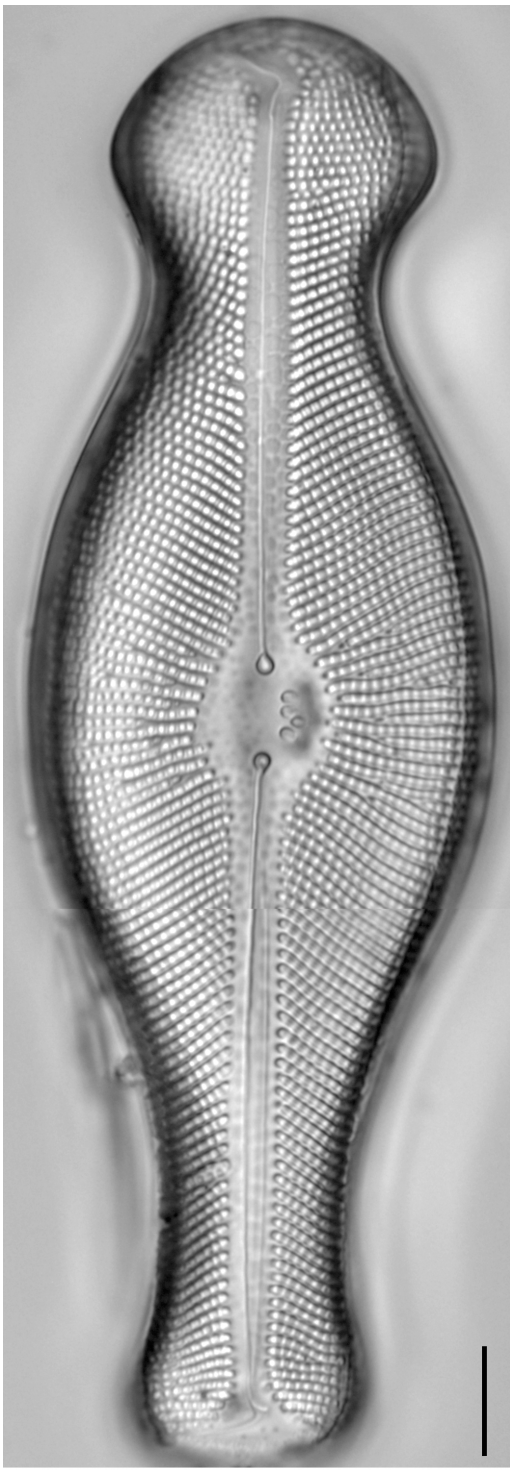

Figure 1. Scanning electron micrograph of a *D. geminata* cell (Spaulding 2010). Scale bar=10 μm.

When a stalked *D. geminata* cell divides vegetatively, the associated stalk also divides. Cells that are attached to the same stalk are genetically identical clones (Kilroy 2004). Cumulatively, branched stalks form mats, whitish-yellow to light brown in colour and slimy in appearance, which can be upwards of 20 cm thick (Kilroy and Bothwell 2011).

Mats composed of excess stalk material form under very low phosphorus concentrations. Mats will not usually form if SRP concentrations are consistently >0.002 mg/L (Kilroy and Bothwell 2012). An increase in algal biomass under low nutrient conditions is unusual as most species have a tendency to gain biomass under higher nutrient conditions (Schindler 1977).

Distribution

D. geminata is considered native to North America, South America, Europe, and Asia (Taylor and Bothwell 2014). Sporadic *D. geminata* mats have been documented in Europe since the 1800s (Stoermer et al. 1993, Sarmaja-Korjonen and Alhonen 1999, Blanco and Ector 2009). Nuisance mats have been documented increasingly and in an expanding range of geographical locations since the 1990s (Kilroy 2004, Bothwell et al. 2009, Whitton et al. 2009, Gillis and Chalifour 2010, Reid et al. 2012). Nuisance *D. geminata* mats now occur globally and have been found in New Zealand, across North America, and in various European, Middle Eastern, Asian and South American countries (Kilroy 2004, Bothwell et al. 2009, Whitton et al. 2009, Gillis and Chalifour 2010, Reid et al. 2012).

Nuisance *D. geminata* mats were first documented in North America in 1989, on Vancouver Island (Bothwell et al. 2009). Thereafter, excess stalk material appeared across North America, primarily in oligotrophic, high light, low temperature lotic systems. However, nuisance *D. geminata* mats have also been documented in an expanding range of conditions (Spaulding and Elwell 2007).

Excess stalk material was first observed in Alberta in the late 1990s in the upper Bow River (Kirkwood et al. 2007). In 2004, a two-year Alberta based study was commenced to investigate environmental variables that may facilitate the growth of excess stalk material. Over the course of the study, *D. geminata* was consistently detected in the Red Deer and Bow Rivers. Flow, water temperature, turbidity, pH, conductivity and total phosphorus (TP) were found to differ significantly between sites where *D. geminata* was present and those where it was absent (Kirkwood et al. 2007). Further research clarified that sites with regulated flow, such as those downstream from dams, were favorable for the growth of *D. geminata* mats (Kirkwood et al. 2009). Additional research in Rocky Mountain streams of Western Canada, where *D. geminata* was found to be present at 88% of sites sampled, also observed stabilized flow and increased water temperature (up to a threshold of 14°C) to be favourable, but was unable to identify a consistent combination of environmental variables that would result in nuisance mat formation where *D. geminata* cells were present (Jackson et al. 2016).

The first documented occurrence of *D. geminata* in the Southern Hemisphere occurred in 2004 on New Zealand's South Island, where *D. geminata* is considered a non-native invasive (Kilroy 2004). *D. geminata* is of great concern in New Zealand as the majority of the South Island's rivers are plagued by thick mats. New Zealand's North

Island is currently free of *D. geminata*. The growth of excess stalk material has since been observed in additional Southern Hemisphere countries including Chile and Argentina (Reid et al. 2012).

The full extent of native and introduced ranges of *D. geminata* is currently unknown as the historical record of freshwater diatoms present is lacking in some locations. However, based on fossil records, *D. geminata* can likely be classified as native in most parts of the world where nuisance mats are found (Taylor and Bothwell 2014), except New Zealand (Kilroy and Unwin 2011).

Ecological Consequences

Excess *D. geminata* stalk material has been shown to negatively impact aquatic ecosystems in multiple ways. The presence of mats greatly increases the biomass of periphyton present in a river (Kilroy et al. 2009), which in turn affects ecosystem habitat and trophic interactions. Mats have been shown to directly alter the community composition of aquatic invertebrates. Abundance of Chironomidae and Gastropoda increase in the presence of *D. geminata* mats; conversely, the Ephemeroptera, Plecoptera and Trichoptera orders (EPT) decrease in abundance (Kilroy et al. 2009, Gillis and Chalifour 2010). EPT orders are used as biological indicators of water quality. Normally, a decrease in EPT abundance would occur due to a decrease in water quality. However, *D. geminata* can thrive in pristine, oligotrophic systems (Sherbot and Bothwell 1993, Bothwell et al. 2009). Changes to macroinvertebrate communities may modify fish diets and, consequently, modify the physical condition of native fishes (Larson 2007, Shearer et al. 2007). In addition, a riverbed altered by extensive thick mats could negatively

impact high quality salmonid spawning habitat (Bickel and Closs 2008). Further, *D. geminata* mats are visually unappealing, clog hydroelectric intakes, reduce the quality of recreational fishing by fouling lines and interfere with enjoyment of other river-related recreational activities (Spaulding and Elwell 2007, Kilroy et al. 2009).

Other than attempting to stop the spread of *D. geminata* by educating river users, there are currently no effective means in place to control excess stalk material (Floder and Kilroy 2009). That being said, there is promise for controlled flood events and phosphorus addition programs (James et al. 2015, Kilroy and Larned 2016) to aid in future *D. geminata* control as it has been demonstrated that mats favour regulated flow and SRP <0.002 mg/L (Kirkwood et al. 2009, Kilroy and Bothwell 2011, Cullis et al. 2012). The effectiveness of various herbicides has been studied. However, a suitable chemical with minimal impacts on non-target species has yet to be found (Clearwater et al. 2011).

Hypotheses for Mat Formation

It is hypothesized that the increase in nuisance blooms globally is the result of a new genetic variant that may have evolved in response to environmental changes and/or was accidentally transported by anglers (Kirkwood et al. 2007, Spaulding and Elwell 2007, Whitton et al. 2009). Due to the belief that *D. geminata* will become a nuisance once introduced, the predominant strategy for managing *D. geminata* is to educate river users about the importance of cleaning their fishing gear. Felt-soled wader boots are banned in New Zealand and parts of the United States (Invasive Species Action Network 2014, Fish and Game New Zealand 2016), as moist felt pores can harbor live cells for

weeks and only a single viable *D. geminata* cell can initiate an invasion (Floder and Kilroy 2009).

Phylogenetic studies provide some evidence for multiple strains of *D. geminata*. Studies performed on *D. geminata*'s nuclear ribosomal internal transcribed spacer (ITS) regions and 18S rDNA, exploring phylogenetic relatedness between *D. geminata* from various locations (Kelly 2009, Cary et al. 2014), suggest that *D. geminata* was introduced to North America from Europe and to New Zealand from North America (Kelly 2009). However, phylogenetic relatedness is not well resolved due to the highly conserved nature of the 18S and ITS regions (Bast et al. 2009).

A further hypothesis for mat formation proposes that *D. geminata* forms stalks in response to very low (<0.002 mg/L) SRP concentrations (Kilroy and Bothwell 2011, Bothwell et al. 2014). For instance, studies conducted in New Zealand demonstrated that *D. geminata* mats will not form in New Zealand rivers when mean SRP is consistently >0.002 mg/L (Kilroy and Bothwell 2012) and that excess stalk material persists in rivers where cell division is limited by 0.001 mg/L SRP concentration (Bothwell and Kilroy 2011). Further, the addition of phosphorus to rivers in South Dakota significantly reduced the biomass of nuisance *D. geminata* mats (James et al. 2015). It is possible that SRP concentrations are declining in some watersheds due to logging, agriculture, commercial fishing and anthropogenically induced climate change (Bothwell et al. 2014). However, low SRP may not be the only explanation for *D. geminata* mats as phosphorus concentrations are rising in freshwater bodies globally, largely due to phosphorus fertilizer entering water bodies (Tilman et al. 2001, Sharpley et al. 2013).

Despite the hypothesis of a mat-forming genetic variant, molecular research on *D. geminata* is lacking, in part, because *D. geminata* does not have a reference genome and is difficult to culture in the lab. Also, cells are found in a complex periphyton matrix that includes *D. geminata* stalks (Pandey et al. 1996, Monteiro et al. 1997, Kuhajek and Wood 2014). Polysaccharide *D. geminata* stalks are problematic for molecular protocols, as they inhibit enzymes involved in PCR and may also interfere with DNA isolation (Pandey et al. 1996, Monteiro et al. 1997).

The reliable extraction of a sufficient amount of high quality DNA is critical to molecular studies and largely dependant on preservation and extraction methods. First, after collection, samples must be preserved to reduce degradation of DNA by enzymes (Yagi et al. 1996, Adams et al. 1999, Prendini et al. 2002). Second, an appropriate extraction method must be used to ensure the desired yield of high-quality DNA. *D. geminata* has been extracted using commercial kits (e.g. Agencourt® Genfind v2 – Blood and serum gDNA Kit, Invitrogen – Chargeswitch® Plant gDNA Kit, Invitrogen – Purelink™ Genomic DNA Kit, Invitrogen – Purelink™ Plant DNA Kit, Qiagen – DNeasy® Plant Mini Kit, Roche – High Pure PCR Template Preparation Kit) and traditional isolation methods such as cetyltrimethyl ammonium bromide (CTAB) and organic extraction (OE) methods. *D. geminata* DNA yields obtained from kits and traditional methods have produced up to 3180 ng of DNA, but are variable and inconsistent (Coyne et al. 2001, Cary et al. 2007, Uyua et al. 2014). The amount of DNA required depends on the downstream application. Common downstream applications include PCR and next-generation sequencing (NGS). PCR reactions usually involve 25-

200 ng of DNA, while Illumina recommends >1 μg of DNA for library preparation and NGS.

Frequency of Dividing Cells

D. geminata usually reproduces asexually. Each half of the frustule develops a new hypotheca and two daughter cells are formed. One of the newly formed cells will be the same size as the original, while the other will be smaller. Asexual reproduction results in a variety of cell sizes and many smaller than the original (Macdonald 1869, Pfitzer 1869) (Figure 2). When cell size needs to be restored and environmental conditions are favorable, most diatoms will sexually reproduce by forming gametes, which join to create a zygote that develops into an auxospore (Mouget et al. 2009). The auxospore divides to restore maximum cell size (Mann 1982, Mann 1984). Therefore, *D. geminata* likely undergoes asexual and sexual reproduction.

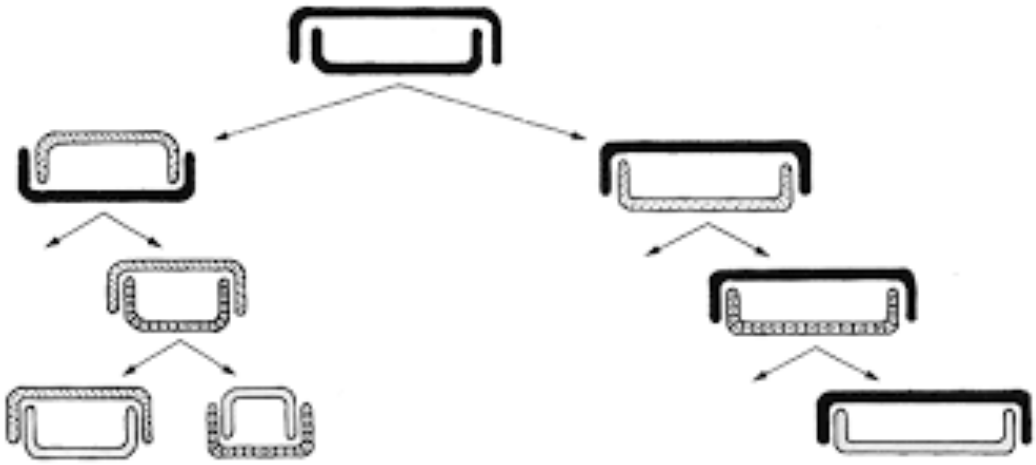

Figure 2. MacDonald Pfitzer hypothesis of asexual diatom reproduction (MacDonald 1869, Pfitzer 1869).

The value of frequency of dividing cells (FDC) provides an estimate of cellular growth rate and is defined as the percentage of total cells that have completed cytokinesis, but still share a cell wall. FDC has been used successfully for many organisms, including *D. geminata* (Swift and Durbin 1972, Lewin and Rao 1975, Smayda 1975, Weiler and Chisholm 1976, Campbell and Carpenter 1986, Tsujimura 2003, Yamamoto and Tsukuda 2009, Bothwell and Kilroy 2011). For example, FDC has been used to investigate nutrient limitation and mat formation in *D. geminata* (Bothwell and Kilroy 2011). Artificial streamside channels in New Zealand's South Island were supplemented with orthophosphate (PO_4^{-3}), in concentrations ranging from 0.0025–0.05 mg/L, resulting in a sustained increase in FDC regardless of accompanying nitrate addition (Bothwell and Kilroy 2011). Artificial streamside channel experiments suggest that *D. geminata* cell division is phosphorus limited. FDC will be adopted in the current study to investigate differences in *D. geminata* growth rate at mat-forming and non-mat-forming sites. Further investigation of the relationship between FDC, SRP and mat formation in *D. geminata* in additional geographical locations could support the low SRP hypothesis for mat formation.

Gene Expression

There are few studies of gene expression in invasives, even though such studies are recognized an important way to identify molecular and physiological reasons for invasions (Bock et al. 2015). Gene expression studies require the analysis of the transcriptome of the subject organism. The transcriptome is made of messenger RNA that

varies depending on an organism's life stage and environment. The transcriptome provides information on which genes are expressed and the corresponding proteins that are produced under specific conditions. Comparing gene expression between organisms in different environments or life stages at a given time provides information as to which gene is responsible for a difference in phenotype or behaviour. Gene expression studies are required to identify the trigger of nuisance stalk formation in *D. geminata* because empirical studies have a limited capacity to demonstrate a direct relationship between mechanism and phenotype.

D. geminata's invasiveness lies in that it behaves differently from other aquatic stalk-forming organisms, producing excessive stalks under very low nutrient concentrations. Related diatoms, from the Cymbellaceae and Gomphonemataceae families, form similar branched sulfated xylogalactan stalks in smaller masses and under higher nutrient conditions (Huntsman and Sloneker 1971, Wang et al. 2000, Gretz 2006 et al., Gretz 2008, Khan-Bureau et al. 2014). Studies suggest that low SRP concentrations cause stalk formation in *D. geminata* but have not proven it as the only trigger (Bothwell et al. 2009, Kilroy and Bothwell 2012, Bothwell et al. 2014, Taylor and Bothwell 2014, James et al. 2015).

Molecular and genetic studies can identify the underlying causes of invasiveness in invasive species. Two genetic approaches may be taken: top-down or bottom-up. The top-down approach is used when a known phenotypic difference exists between invasive and non-invasive organisms of the same species and when candidate genes, which may be responsible for the difference, are identifiable (Bock et al 2015). The top-down approach includes quantitative trait locus mapping and candidate gene analysis. For

example, the DRD4 gene was identified from a list of previously selected candidates as a contributor to invasive behaviours in birds (Mueller et al. 2014). The bottom-up approach is used when the traits contributing to invasiveness are unknown. Differences in genetic sequences between native and invasive organisms, combined with knowledge of the function of differentially expressed genes, can identify the underlying cause of invasiveness (Bock et al. 2015). For example, differential gene expression in response to heat stress has shown that, in warming waters on California's coast, invasive blue mussels are more successful than their native counterparts (Lockwood et al. 2010). Differential gene expression analyses have also revealed that the invasive golden apple snail (*Pomacea canaliculata*) is successful due to its ability to metabolize pesticides (Mu et al. 2015). The genetic approach must be selected based on knowledge of traits that cause an organism to be invasive and the underlying genes involved.

The cause of *D. geminata* nuisance mats may be revealed by molecular studies on the underlying mechanisms of stalk formation. Nuisance stalk formation is the reason for *D. geminata*'s invasiveness (Spaulding and Elwell 2007) and low SRP is a hypothesized trigger of nuisance stalk formation (Bothwell and Kilroy 2011, Kilroy and Bothwell 2011, Kilroy and Bothwell 2012, Bothwell et al. 2014). Therefore, we can predict that candidate genes related to phosphorus metabolism and EPS production may only be expressed during stalk formation. For example, if transcripts coding for alkaline phosphatases were only expressed in mat-forming *D. geminata*, it could indicate that *D. geminata* is forming stalks in reaction to low SRP concentrations. Because low SRP may not be the only trigger of stalk formation, this study will not limit its scope to candidate genes. This study will identify all genes that are expressed exclusively in a mat-forming

or non-mat-forming sample in an attempt to reveal additional causes of nuisance stalk formation.

To investigate the function of genes the are exclusively expressed in mat-forming or non-mat-forming *D. geminata*, a reference genome is required. A reference genome is an electronic nucleic acid sequence assembly that is considered representative of a specific species. *D. geminata* does not currently have a reference genome. Therefore, reference genomes of closely related organisms are required to compare to *D. geminata* sequences. Candidate reference genomes for *D. geminata* include marine diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* (Armbrust et al. 2004, Bowler et al. 2008). *D. geminata* and *P. tricornutum* are both pennate diatoms, therefore, likely have more genes in common than *D. geminata* and *T. pseudonana,* as *T. pseudonana* is a centric diatom (Bowler et al. 2008).

An additional candidate reference genome belongs to the bacterium *Caulobacter crescentus* (Nierman et al. 2001). Many diatom genes originated in bacteria (Armbrust et al. 2004, Bowler et al. 2008, Allen et al. 2011); thus it is possible that the genes responsible for stalk production in *D. geminata* originated in stalk-forming bacteria such as *C. crescentus*. Like *D. geminata*, *C. crescentus* also forms stalks in phosphorus-limited environments (Gonin et al. 2000). Genes that are exclusively expressed in mat-forming *D. geminata* may exhibit alignment with genes of interest responsible for nutrient processing and stalk formation in *C. crescentus*.

In addition to the lack of a reference genome, *D. geminata* is a challenging organism for gene expression studies due to it being part of a periphyton matrix. It is difficult to isolate *D. geminata* from periphyton, which contains many organisms

including bacteria. Metatranscriptomics is the study of all messenger RNA present in a sample containing multiple organisms. Metatranscriptomics is useful when it is difficult to physically separate organisms, a survey of genes expressed in a community is needed, or when making a comparison between communities under different conditions. Metatranscriptomics is commonly used for microscopic organisms including plankton and bacteria (Marchetti et al. 2012, Puente-Sanchez et al. 2015). Metatranscriptomics will be used in this study on *D. geminata* gene expression.

Study Objectives

The proposed causes of nuisance stalk formation in *D. geminata* include a mat-forming genetic variant (Kirkwood et al. 2007, Spaulding and Elwell 2007, Whitton et al. 2009) and low (<0.002 mg/L) SRP concentrations in rivers where nuisance *D. geminata* is present (Bothwell and Kilroy 2011, Kilroy and Bothwell 2011, Kilroy and Bothwell 2012, Bothwell et al. 2014). Molecular studies are needed to reveal the underlying mechanism behind *D. geminata*'s nuisance stalk formation, as empirical studies have a limited ability to do so. Specifically, gene expression studies are required to identify genes that are exclusively expressed in mat-forming and non-mat-forming *D. geminata.* To further investigate molecular differences between mat-forming and non-mat-forming *D. geminata*, a protocol for the extraction of high quality *D. geminata* DNA of sufficient yield is needed.

If the results of this study support the hypothesis that *D. geminata* is forming stalks in reaction to low SRP concentrations, a phosphorus addition program (James et al. 2015) may be more effective than a management strategy aimed at stopping the spread of

cells. If the results of this study do not support the low SRP hypothesis, then current management practices aimed at stopping the spread of cells, such as felt soled wader bans, should remain in place until the underlying mechanism of nuisance stalk formation is confirmed.

The overall goals of my thesis were to investigate triggers of cell division and stalk formation in *D. geminata* using FDC and gene expression, and perform a comparative DNA extraction experiment. The specific goals of my research were to 1) compare FDC and SRP between a mat-forming and non-mat-forming site on the Bow River; 2) compare FDC between mat-forming sites in Alberta and British Columbia; 3) sequence RNA from a mat-forming and non-mat-forming site on the Bow River; 4) identify gene products from reference sequences that are exclusively expressed at either the mat-forming or non-mat-forming site and may be involved in stalk formation or cell division; 5) test various DNA storage and isolation methods from periphyton containing *D. geminata* cells.

I expected SRP and FDC to be significantly higher at the non-mat-forming site, as compared to the mat-forming site. FDC from Alberta and British Columbia mat-forming samples should be statistically similar if *D. geminata* is behaving the same physiologically regardless of geographical location. If mat-forming *D. geminata* from BC and AB have significantly different FDC, it may indicate that SRP concentrations are not the only trigger of cell division and/or mat formation.

I hypothesized that the physiological processes of *D. geminata* cells that are forming stalks differ from those that are not forming stalks. *D. geminata* cells associated with thick mats have a lower frequency of division compared to non-mat-forming cells

(Kilroy and Bothwell 2012). As a result, different genes are expressed, creating the proteins necessary to accomplish stalk growth and cell division. If the low SRP hypothesis is correct, genes identified as expressed in mat-forming cells may include those coding for phosphorus processing products such as alkaline phosphatases. Cells that are not forming stalks are more likely to be dividing and may be expressing genes related to silica processing and mitosis. I expected that gene products of *P. tricornutum* and *T. pseudonana* that are expressed in *D. geminata* may include those involved in nutrient processing and cell division. There could be similarities between the physiological functioning of *C. crescentus* and *D. geminata* during stalk formation, as *C. crescentus* forms stalks under low phosphate conditions (Gonin et al. 2000). Accordingly, there may also be similarities in proteins that are being produced at the time of stalk formation.

For the periphyton storage and DNA isolation comparative experiment, I hypothesized that samples stored in RNA*later* at -80°C should yield the most DNA. The modified kit method should yield higher amounts of DNA than the unmodified kit method. The modified kit method has been developed with longer incubation times for organisms such as *D. geminata* that pose a challenge for DNA extraction. The organic extraction method should obtain approximately 150−300 ng of DNA from *D. geminata* (Uyua et al. 2014).

This study will be novel in that it addresses FDC and SRP in rivers where *D. geminata* is likely a native invader. It also is the first study to investigate gene expression and identify possible candidate genes involved in stalk formation and cell division in *D. geminata*.

This thesis was written in manuscript style including a general introduction (Chapter 1), research chapters (Chapters 2 and 3) and a general conclusion (Chapter 4), which summarizes important findings. Chapter 2 examined differences in gene expression between mat-forming and non-mat-forming *D. geminata*, compared FDC and SRP between a mat-forming and non-mat-forming site and FDC between British Columbia and Alberta sites. Chapter 3 focused on a DNA extraction comparative experiment.

CHAPTER 2: FREQUENCY OF DIVIDING CELLS, SOLUBLE REACTIVE PHOSPHORUS CONCENTRATIONS AND GENE EXPRESSION

Introduction

Invasive species have ecological and evolutionary implications that have been studied to a greater extent in recent years (Mooney and Cleland 2001). However, implications are difficult to understand as many interactions between invasives and their environments are poorly understood (Levine et al. 2003). Furthermore, invasive species vary in their response to environmental change and ecosystems differ in their vulnerability to invasion (Vila et al. 2011).

Didymosphenia geminata is described as a non-native invasive species in New Zealand (Kilroy 2004), while acting as a native invader in areas where it existed before becoming a nuisance (Taylor and Bothwell 2014). A native invader is an indigenous species that becomes harmful ecologically because of an anthropogenically induced change such as predator removal, climate change, fire suppression, increases or decreases in nutrient concentrations, and habitat fragmentation (Valery et al. 2008, Simberloff 2011, Carey et al. 2012).

Although *D. geminata* is native to the Northern Hemisphere, prior to its discovery on Vancouver Island, excess *D. geminata* stalk material had only been documented in certain rivers and streams in northern Europe (Skulberg 1984, Lindstrom and Skulberg 2008). The first excess stalk material appeared in cold rivers exposed to sunlight with very low nutrients (Blanco and Ector 2009). Mats of excess stalk material are now present in rivers across North America, Europe, Asia, New Zealand, Chile and Argentina, with more diverse nutrient ranges than rivers where *D. geminata* mats were originally

found (Kilroy 2004, Reid et al. 2012). Due to poor diatom records, the full native and non-native ranges of *D. geminata* are unclear.

Regardless of geographical location, *D. geminata*'s invasiveness is due to problematic polysaccharide stalks. Mats are formed from many branched stalks and can be upwards of 30 cm thick, blanketing entire riverbeds. Yet, an understanding of the mechanisms that contribute to this growth remain unknown.

It is hypothesized that *D. geminata* mats form due to very low SRP (soluble reactive phosphorus) concentrations, as mats can form when SRP concentrations <0.002 mg/L (Kilroy and Bothwell 2012) and phosphorus enrichment has shown promise to reduce the growth of mats (James et al. 2015, Kilroy and Larned 2016). *D. geminata* contradicts conventional knowledge, as algae are generally known to increase in biomass under high nutrient conditions (Schindler 1977).

Frequency of dividing cells (FDC), which has been shown to be highly correlated with cellular growth in *D. geminata*, is lower when SRP is low. A percentage value for FDC can be obtained by counting the number of daughter cells that still share a cell wall out of a total number of cells. FDC is a proxy for growth rate and is used in research involving various organisms (Swift and Durbin 1972, Lewin and Rao 1975, Smayda 1975, Weiler and Chisholm 1976, Campbell and Carpenter 1986, Tsujimura 2003, Yamamoto and Tsukuda 2009) that primarily divide asexually, including *D. geminata* (Bothwell and Kilroy 2011).

Another method to assess the mechanisms associated with stalk formation is the characterization of gene expression. Gene expression is often considered a molecular phenotype that can provide information on an organism's physiological response by

revealing genes that may be responsible for differences in phenotype or behavior under specific conditions (Dalziel et al. 2009). Gene expression varies based on genotype, life stage, and environmental conditions. Individuals with identical genotypes can differ in gene expression under different environmental conditions (Ranz and Machado 2006, Pavey et al. 2010).

Gene expression studies of mat-forming and non-mat-forming *D. geminata* may reveal genes responsible for nuisance stalk formation. Low SRP is a hypothesized trigger of nuisance stalk formation (Bothwell and Kilroy 2011, Kilroy and Bothwell 2011, Kilroy and Bothwell 2012, Bothwell et al. 2014). Therefore, we can predict that candidate genes related to phosphorus metabolism and EPS production may only be expressed during stalk formation. For example, if transcripts coding for alkaline phosphatases were only expressed in mat-forming *D. geminata*, it could indicate that *D. geminata* is forming stalks in reaction to low SRP concentrations.

Low SRP has not been proven as the only trigger of stalk formation; therefore, this study will not limit itself to a top-down, candidate gene approach. This study will identify all genes that are expressed exclusively in a mat-forming or non-mat-forming sample in an attempt to reveal additional causes of nuisance stalk formation.

As the genome of *D. geminata* has not been fully sequenced and annotated, identifying genes that may be involved in stalk production or cell division requires reference genomes. *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* are the only diatoms with fully sequenced and annotated genomes (Armbrust et al. 2004 and Bowler et al. 2008). Therefore, *T. pseudonana* and *P. tricornutum* are logical candidates for reference sequences. *T. pseudonana* is a centric diatom while *P. tricornutum* is

pennate. The pennate and centric diatoms diverged 90 million years ago and share 57% of their genes (Bowler et al. 2008). As a result, *D. geminata,* a pennate diatom, is likely more genetically similar to *P. tricornutum*.

Although there are only two reference diatoms, bacterial genomes may also provide a suitable reference for *D. geminata*, as many diatom genes originated in bacteria (Armbrust et al. 2004, Bowler et al. 2008, Allen et al. 2011). *Caulobacter crescentus* is a bacterium that, similar to *D. geminata,* forms stalks under low inorganic phosphorus conditions (Schmidt 1968, Gonin et al. 2000). The genome of *C. crescentus* has been fully sequenced and annotated allowing for a comparison to *D. geminata* (Nierman et al. 2001). It is possible that *D. geminata* has genes similar to *C. crescentus*. *P. tricornutum,* and *T. pseudonana* that are involved in nutrient processing and cell division. Those genes that may be exclusively expressed in mat-forming or non-mat-forming *D. geminata.*

Assessing growth rates and gene expression of mat-forming *D. geminata* will contribute to knowledge of stalk formation. Frequency of dividing cells data has demonstrated that *D. geminata* is a phosphorus-limited organism. *D. geminata* increases cell division rate when SRP levels are >0.002 mg/L; it reduces division rate, while increasing stalk production, under lower SRP levels (Bothwell and Kilroy 2011). The ability to form stalks under low SRP is unique to *D. geminata*, as other closely related diatoms in the Cymbellaceae and Gomphonemataceae families form the same branched sulfated xylogalactan stalks under higher nutrient conditions (Huntsman and Sloneker 1971, Wang et al. 2000, Gretz 2006 et al., Gretz 2008, Khan-Bureau et al. 2014). Bothwell and Kilroy demonstrated a link between SRP and FDC in New Zealand. Showing the same relationship in Western Canada where *D. geminata* is likely a native

invader, in addition to differences in gene expression, may lend additional support to the low SRP hypothesis globally.

It is expected that SRP and FDC will be significantly higher at the non-mat-forming site, while SRP and FDC should be lower at the mat-forming site. Predictions are based on the aforementioned studies in New Zealand (Bothwell and Kilroy 2011, Kilroy and Bothwell 2012). FDC from Alberta and British Columbia mat-forming samples should be statistically similar if *D. geminata* is behaving the same physiologically regardless of geographical location. However, SRP concentrations may be slightly higher in rivers on Vancouver Island compared to those in the Canadian Rocky Mountains, which are usually oligotrophic (Lamontagne et al. 1994, British Columbia Ministry of Environment 2011, British Columbia Ministry of Environment 2014). Higher SRP may result in higher cell division in the British Columbia samples.

It can be expected that gene products of *P. tricornutum* and *T. pseudonana* will be expressed in mat-forming and non-mat-forming *D. geminata. D. geminata* cells that are forming stalks undergo different molecular and physiological processes as compared to those that are not. Non-stalk forming *D. geminata* cells are predicted to have higher frequencies of cell division. Accordingly, different genes may be expressed resulting in proteins necessary to accomplish stalk growth and cell division. If the low SRP theory is correct, genes expressed in the mat-forming sample may include phosphohydrolases. Phosphohydrolases hydrolyze phosphate groups and are known to be activated under low phosphorus conditions (Bosse and Kock 1998). Genes expressed exclusively in the mat-forming sample may also include those known to be present in phosphorus starved *P. tricornutum.* Examples include genes coding for superoxide dismutase (SOD), an

antioxidant, phosphomannomutase (PMM), involved in fructose and mannose metabolism, plastid-lipid associated protein (PAP), activated by stress in plants, and flavonol synthase 2 (FLS2), involved in flavonoid biosynthesis (Dyhrman et al. 2012). Additional gene products found in *P. tricornutum* under low phosphorus conditions include alkaline phosphatase D (phoD), carbonic anhydrase (CA), and ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (rbcS) genes (Duff et al. 1991, Dyhrman et al. 2012, Feng et al. 2015).

Cells that are not forming stalks are more likely to be dividing and expressing genes coding for products involved in cell division. Proteins involved in cell division in diatoms include kinetochore protein ZW10, chromokinesins, and extra-spindle pole-like proteins, which are involved with chromosome segregation (De Martino et al. 2009). Diatom spindle kinesin 1 and dyneins are involved in spindle assembly and elongation during mitosis (De Martino et al. 2009). Cyclin dependent kinases and cyclins control the progression of cell division in diatoms (Bowler et al. 2008, Huysman et al. 2010). Rab family small GTPase Sec4 protein is involved in cytokinesis (Walworth et al. 1989) and syntaxins regulate membrane fusion in the plane of cell division (Jantsch-Plunger and Glotzer 1999). Genes coding for the aforementioned proteins have been characterized in *P. tricornutum* and *T. pseudonana* (Armbrust et al. 2004, Bowler et al. 2008, Tanaka et al. 2015), and, therefore, may be identified as exclusively expressed in non-mat-forming *D. geminata*.

There may be similarities in genes expressed during phosphorus starvation and stalk formation between *D. geminata* and *C. crescentus,* as both form stalks under low inorganic phosphorus conditions. Genes may include those in the phosphate (Pho)

regulon, which is triggered under low phosphorus conditions and plays a role in stalk elongation in *C. crescentus* (Gonin et al. 2000).

The objectives of this study are to 1) compare FDC and SRP between a mat-forming and non-mat-forming site on the Bow River; 2) compare FDC between mat-forming British Columbia and Alberta *D. geminata* samples; 3) sequence the RNA of periphyton from mat-forming and non-forming sites; 4) generate a list of *T. pseudonana*, *P. tricornutum* and *C. crescentus* genes that are exclusively expressed at mat-forming and non-mat-forming *D. geminata* sites and identify those that could be involved in stalk production and cell division.

This study is novel as FDC, and the link to low SRP, is explored in Western Canada rivers where *D. geminata* is likely native. It is also the first to investigate gene expression and identify possible candidate genes involved in stalk formation in *D. geminata*. If results of this study support the low SRP hypothesis, then it may be beneficial to shift the focus of global management of *D. geminata* to phosphorus addition programs rather than the current approach of felt-soled wader bans and educational campaigns to stop the spread of cells.

Materials and Methods

Study Sites

Periphyton was collected during the summers of 2013, 2014, and 2015. British Columbia samples were collected from mat-forming sites on the Koksilah (KOKS), Cowichan (COWN), and Little Qualicum (QUAL) Rivers on Vancouver Island. Alberta

mat-forming sites for FDC comparison were Edna Lake Outflow (EDNA), Bow River at Edworthy Park (EDWR), and Louise Creek (LOUC) (Figure 3). Samples for RNA analysis and FDC and SRP comparison between a mat-forming and non-mat-forming site were collected from the Bow River at EDWR (mat-forming) and Douglasdale (DOUG) (non-mat-forming) (Figure 4).

The Little Qualicum River is a fourth order river that flows from Cameron Lake on Vancouver Island into the Georgia Straight near the town of Qualicum Beach. The river is easily accessed for recreation and angling opportunities and supports many trout and Pacific salmon species. The surrounding land is used for agriculture, logging and recreation. Total phosphorus near the sampling site has ranged from 0.8-2.74 mg/L between 2004 and 2011 (British Columbia Ministry of Environment 2014). Recent water quality assessments have not measured SRP.

The Cowichan and Koksilah Rivers are located near Duncan, British Columbia and surrounded by forestry, agriculture, and residential areas. They are both frequently used for recreation, including angling. The Cowichan River flows from Lake Cowichan, while the Koksilah River originates in the mountains southwest of Shawnigan Lake. Both rivers terminate at the Cowichan/Koksilah estuary. Total phosphorus measured in 2008 near sampling sites on the Cowichan and Koksilah Rivers averaged 0.008 mg/L and 0.006 mg/L respectively (British Columbia Ministry of Environment 2011).

Edna Lake Outflow and Louise Creek originate in low nutrient Rocky Mountain lakes in Jasper and Banff national parks. Both creeks are shallow and located near major highways.

The Bow River runs east from its origin at Bow Glacier and Bow Lake in the Rocky Mountains through Calgary, Alberta, Canada. The Bow River supports many species of fish and boasts a world-renowned recreational trout fishery. The Bow River is used frequently for other recreational activities including kayaking, canoeing, and rafting. Due to treated wastewater and run-off, nutrient concentrations increase from upstream to downstream within the City of Calgary. The median dissolved phosphorus concentration in the Bow River in Calgary between 2004-2011 was 0.001 mg/L (CPP-Hutchinson 2013). EDWR is located upstream from City of Calgary wastewater treatment plants and therefore should experience lower nutrient concentrations than DOUG, which is downstream from Bonnybrook wastewater treatment plant (BRBC 2005).

Sample Collection

Samples were collected from British Columbia and Alberta mat-forming sites for FDC comparison in spring and summer of 2013. A probe, scalpel, and/or forceps were used to remove periphyton, including *D. geminata* mat material, from rocks. Samples were placed in 125 mL borosilicate Qorpak glass bottles with 70% ethanol and stored at 4°C.

On September 10, 2014, periphyton was collected from rocks for RNA analysis using a probe, scalpel and/or forceps. On September 10, 2014, EDWR had visible *D. geminata* mat formation, while DOUG was a non-mat-forming site (no visible stalk material). Periphyton was placed in 15 mL Falcon tubes containing RNA*later* and placed on dry ice immediately. At the laboratory, a small sample from each tube was viewed

under the microscope to confirm the presence of *D. geminata* cells (Leica DM IRB inverted scope at 200x). Tubes were stored at -80°C until processing.

Periphyton was collected from the Bow River for the analysis of FDC in summer 2015. Stalk-forming *D. geminata* was present at EDWR for all 2015 sample dates and at DOUG on August 11, 2015. *D. geminata* at EDWR produced longer stalks (10-30 cm), while stalks at DOUG were ~2 cm. Periphyton was collected from both sites on July 7, 21 and August 11, 2015. A probe, scalpel or forceps were used to remove periphyton from submerged rocks. Samples were placed in 125 mL borosilicate Qorpak glass bottles with 70% ethanol and stored at 4°C.

Water samples were also collected at the sites on July 7, 21, and August 11, 2015 to measure SRP concentrations. Three 1 L Nalgene bottles were filled per site on each sampling date and place into a 4°C cooler immediately in the field. Upon return to the laboratory, water samples were stored in the dark at 4°C until processing, which occurred the day of collection.

Bow River discharge and Calgary precipitation data for 48 hours before sampling were obtained from Calgary monitoring stations from the Government of Canada website in order to explain any sudden increases in SRP caused by rain or flooding.

Molecular and Bioinformatic Methods

RNA was extracted from periphyton obtained at EDWR and DOUG, stored in RNA*later* at -80°C, using E.Z.N.A. RNA Isolation Kit (Omega Bio-Tek, Georgia, USA). Library preparation and sequencing were performed according to Illumina protocols. Bioinformatic analyses were performed on the 4 Gb of metatranscriptome data that was

obtained. Transcriptomes from EDWR and DOUG samples were assembled individually using Trinity (Grabherr et al. 2011, Haas et al. 2013). After assembly, MegaBLAST was used to reveal genera predominately present in each sample based on sequence similarity in Phylum Bacillariophyta (Morgulis et el. 2008). RNA extraction, sequencing, transcriptome assembly, and MegaBLAST analyses were performed by Funomics Global Inc. in Saskatoon, Canada. Putative gene products from *P. tricornutum* and *T. pseudonana,* that were exclusively found in EDWR or DOUG transcriptome assemblies, were identified using DIAMOND (Buchfink et al. 2015). In addition, transcriptome assemblies from EDWR and DOUG were aligned to *C. crescentus* reference sequences using DIAMOND (Buchfink et al. 2015). Gene products from *C. crescentus* that were exclusively found in the EDWR transcriptome were identified.

Frequency of Dividing Cells

Approximately 1 ml of 70% ethanol, containing *D. geminata* cells, was removed from each sample of interest using a Pasteur pipette and deposited onto a plastic Petri dish in a "zigzag" pattern. Using a Leica DM IRB inverted scope at 200x, the "zigzag" was scanned visually from one end to the other for *D. geminata* cells. Each cell encountered was either classified as dividing or not dividing using the method described in Bothwell and Kilroy, 2011. Three replicates of 200 cells were counted for each date, for comparison between EDWR and DOUG, or site, for comparison between AB and BC. By dividing the number of dividing cells by the total number of cells counted, FDC was obtained as a percentage.

Soluble Reactive Phosphorus

All plastics and glassware were washed in 10% hydrochloric acid, triple rinsed with distilled water and air dried prior to use. SRP was measured the same day samples were collected. A simplified method for SRP analysis was followed (Eisenrich et al. 1975). Forty-seven millimeter 0.45 micron Pall cellulose-acetate membrane filters were used to filter water samples. Absorbance was measured at 885 nm using a spectrophotometer (Shimadzu 120V UV-1800 UV).

Statistical Analyses

Mean and standard error values were plotted and visually compared for British Columbia and Alberta mat-forming *D. geminata*. An ANOVA and post hoc Tukey HSD test were performed to compare mean FDC from each site.

FDC data from the Bow River were analyzed using two sample t-tests. Analyses compared mean FDC between EDWR and DOUG for three sample dates: July 7, July 21, and August 11, 2015. Mean and standard error of SRP, where values were above the lowest detectable limit, were plotted for visual analysis. All statistical tests were performed using the statistical package RStudio (v1.1.419; RStudio Team, 2016).

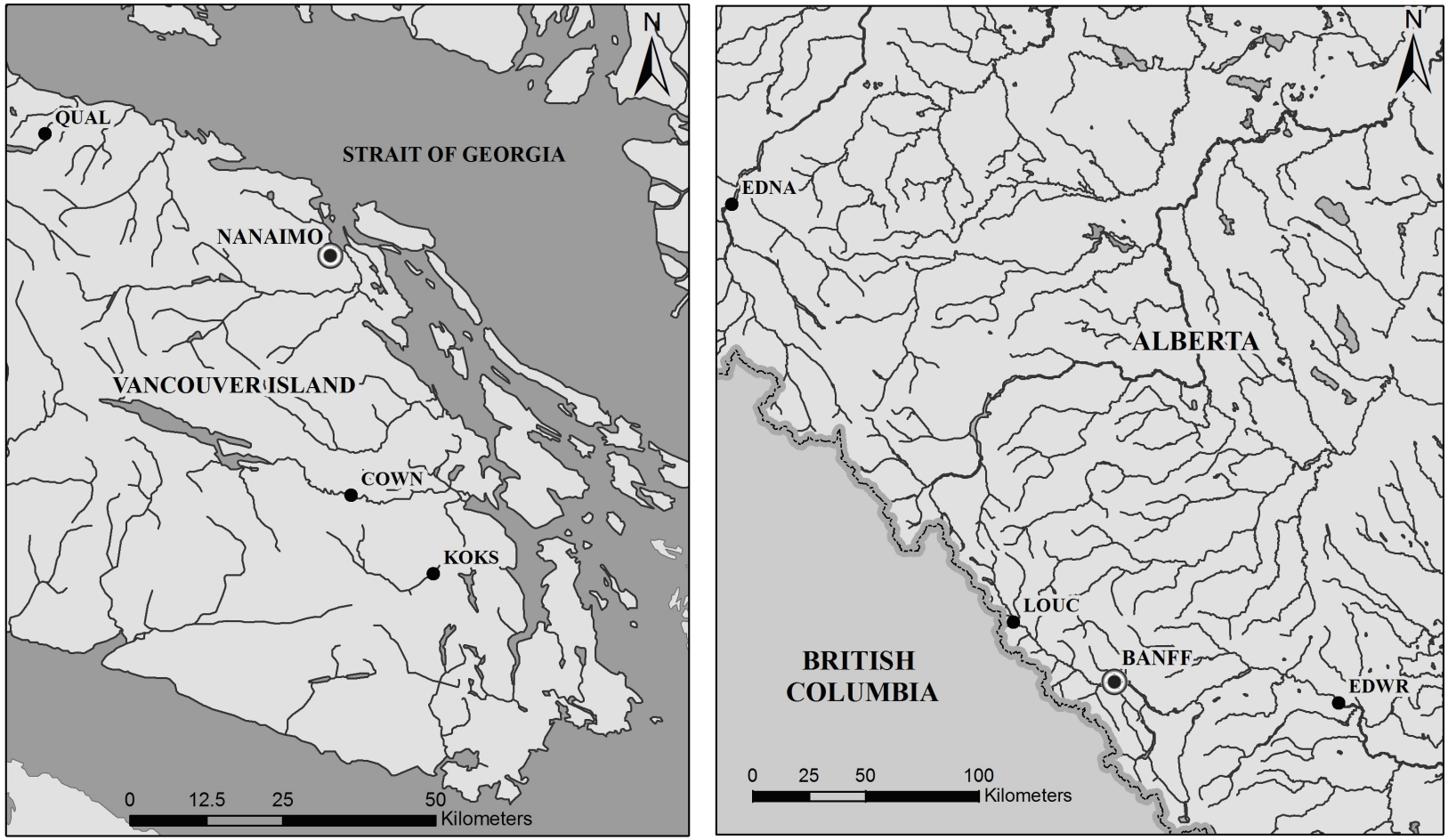

Figure 3. Maps of Vancouver Island, British Columbia and Western Alberta sites sampled in 2013, 2014, and 2015 for FDC comparison. Sites are indicated by solid black circles.

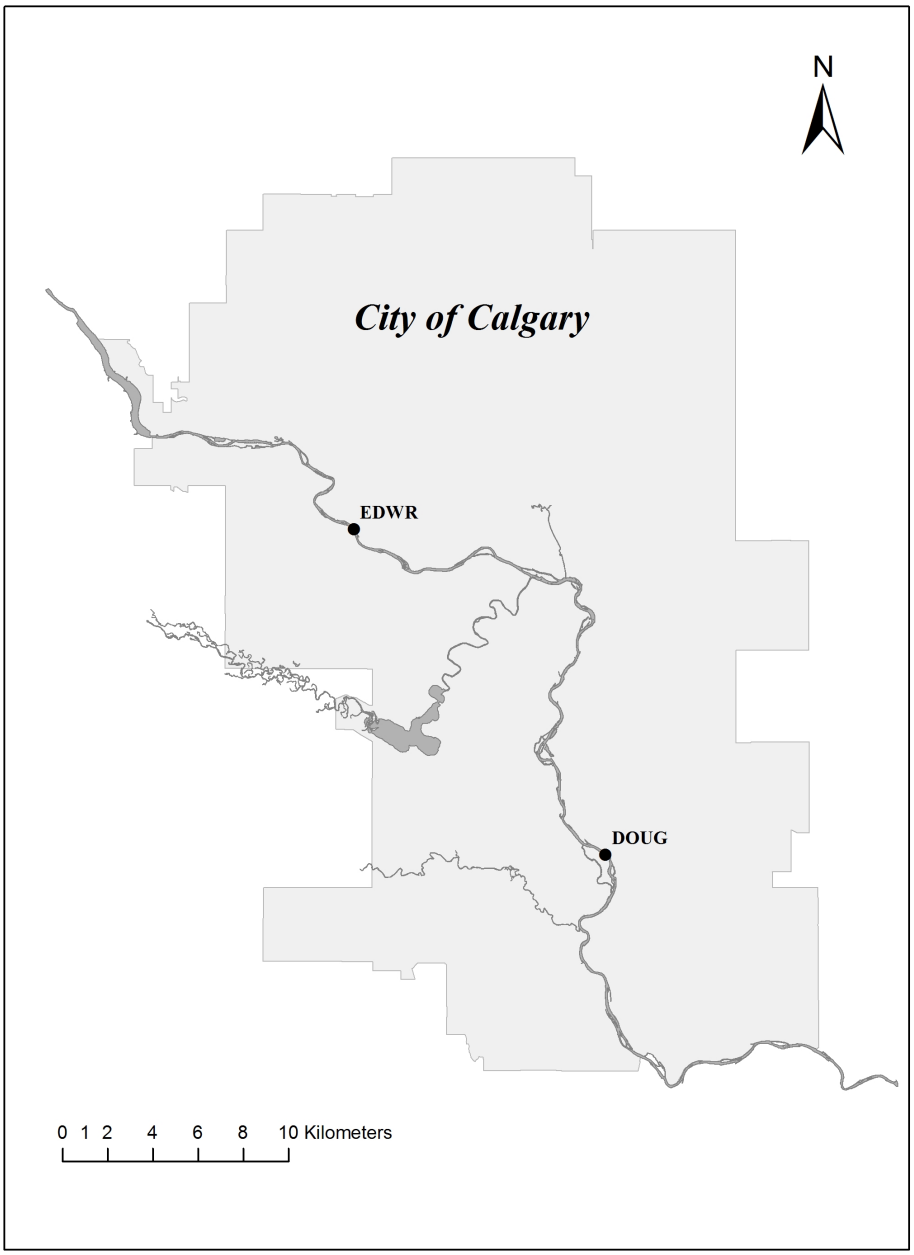

Figure 4. Map of 2015 sampling sites on the Bow River, AB. Light grey outline shows limits of the City of Calgary. Black circles indicate sampling sites at EDWR and DOUG.

Results

FDC was significantly higher at DOUG (mean = 3.33 ± 0.60) compared to EDWR (mean = 1.17 ± 0.17) on July 7, 2015 (two sample t-test, df = 4, t = 3.47, p = 0.025; Figure 5). FDC was also significantly higher at DOUG (mean = 4.17 ± 0.73) compared to EDWR (mean = 1.17 ± 0.33) on July 21, 2015 (two sample t-test, df = 4, t = 3.75, p = 0.020; Figure 5). Finally, FDC on August 11, 2015 was also higher at DOUG (mean = 3.33 ± 0.33) compared to EDWR (mean = 1.50 ± 0.29), and this difference was significant (two sample t-test, df = 4, t = 4.16, p = 0.014; Figure 5).

SRP was below the lower limit of detection of 0.002 mg/L (Eisenreich et al. 1975) for all samples taken at EDWR. Consequently, a reliable statistical analysis could not be performed to compare mean SRP between EDWR and DOUG. However, mean SRP at DOUG for each sample date was >0.002 mg/L. DOUG means were between 0.004 mg/L and 0.0099 mg/L (Figure 6). The standard curve for SRP produced r^2 values between 0.9972-0.9985.

Bow River volumetric flow rates were below average, and close to historical lows, on all sampling dates (Table 1) (Government of Canada Water Office). Precipitation measured at the Calgary International Airport on July 5, 6, and 7 (before sampling) was 0.3 mm, 2.2 mm, and 0 mm respectively. July 19-21 and August 9-11 experienced no precipitation (Table 1) (Government of Canada Weather and Climate Historical Data).

When FDC at various Alberta and British Columbia sites were compared, significant differences were found (ANOVA, df = 5,12, F = 5.46, p = 0.008; Figure 7). A post hoc Tukey test showed that mean FDC was significantly higher at COWN (3.5 ±

0.29) compared to all Alberta sites (Tukey HSD, $p<0.05$). LOUC had the lowest mean FDC (1.17 ± 0.17) (Figure 7).

D. geminata was visually confirmed to be present in EDWR and DOUG samples collected for RNA analysis. Paired end read assemblies were generated for EDWR and DOUG RNA-seq data. There were 193,097 contigs generated from the EDWR sample and 407,048 contigs from the DOUG sample. MegaBLAST analysis showed that several phyla were present in both samples including Bacillariophyta, the diatoms (Morgulis et el. 2008). When the Bacillariophyta phylum was further analyzed, it revealed that genetic material from EDWR and DOUG was similar to several diatom genera (Table 2). In both samples, *Pseudo-nitzschia* and *Nitzschia* made up at least 43% of all diatoms with similar sequences. *Navicula, Gomphonema, Thalassoisira, Sellaphora* and *Phaeodactylum* were also in the top 6 diatoms with sequences similar to those found in the periphyton of EDWR and DOUG. *D. geminata* was also confirmed to be present in the samples as genetic material showed 90%-99% similarity to known *D. geminata* 18S rDNA and chloroplast sequences.

Twenty-five gene products, known from *P. tricornutum* and *T. pseudonana,* were found exclusively in EDWR, while 172 were found in DOUG (Table 3, Table 4). The alignment of EDWR and DOUG transcriptome assemblies to *C. crescentus* sequences resulted in the identification of 41 gene products found only in EDWR (Table 5).

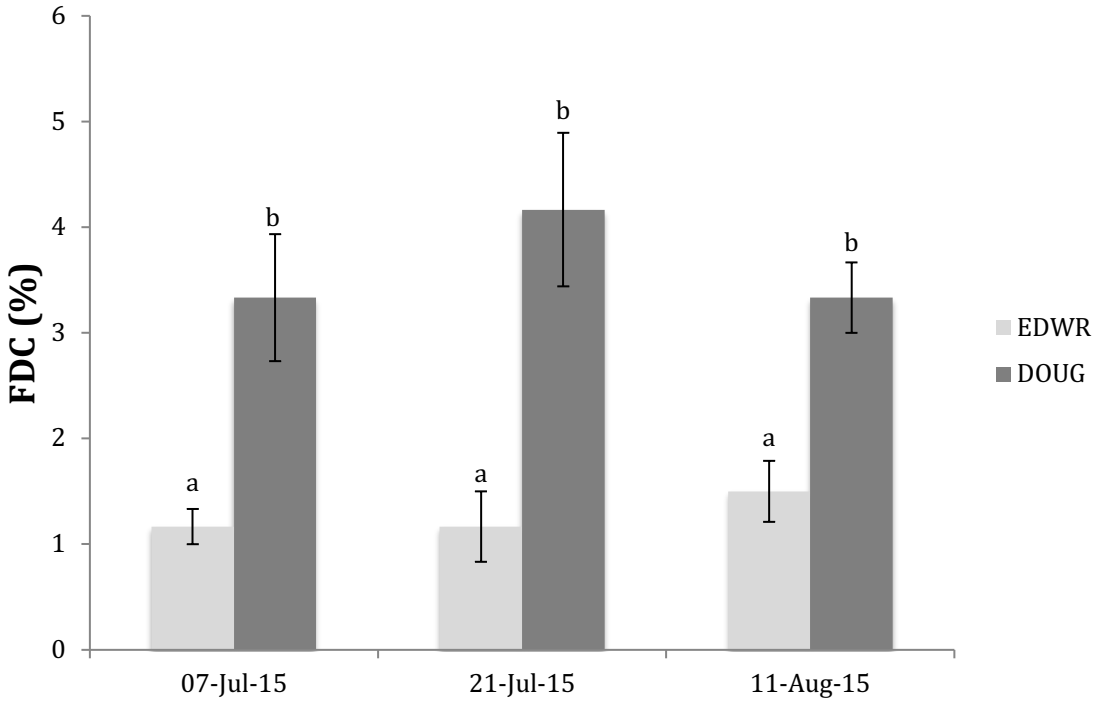

Figure 5. Mean and standard error (n=3) of FDC, as a percentage, at EDWR and DOUG sampled on July 15, July 21, and August 11, 2015. For each date, means with different letters (a, b) are significantly different from each other (two sample t-test, $p<0.05$).

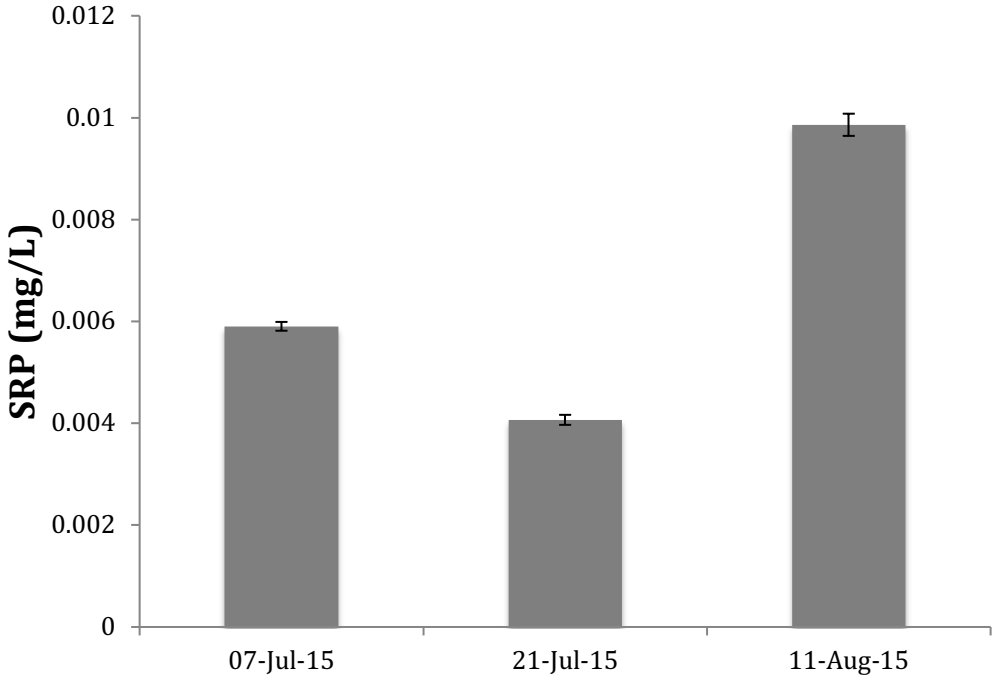

Figure 6. Mean and standard error (n=3) of SRP, in mg/L, at DOUG sampled July 15, July 21, and, August 11, 2015.

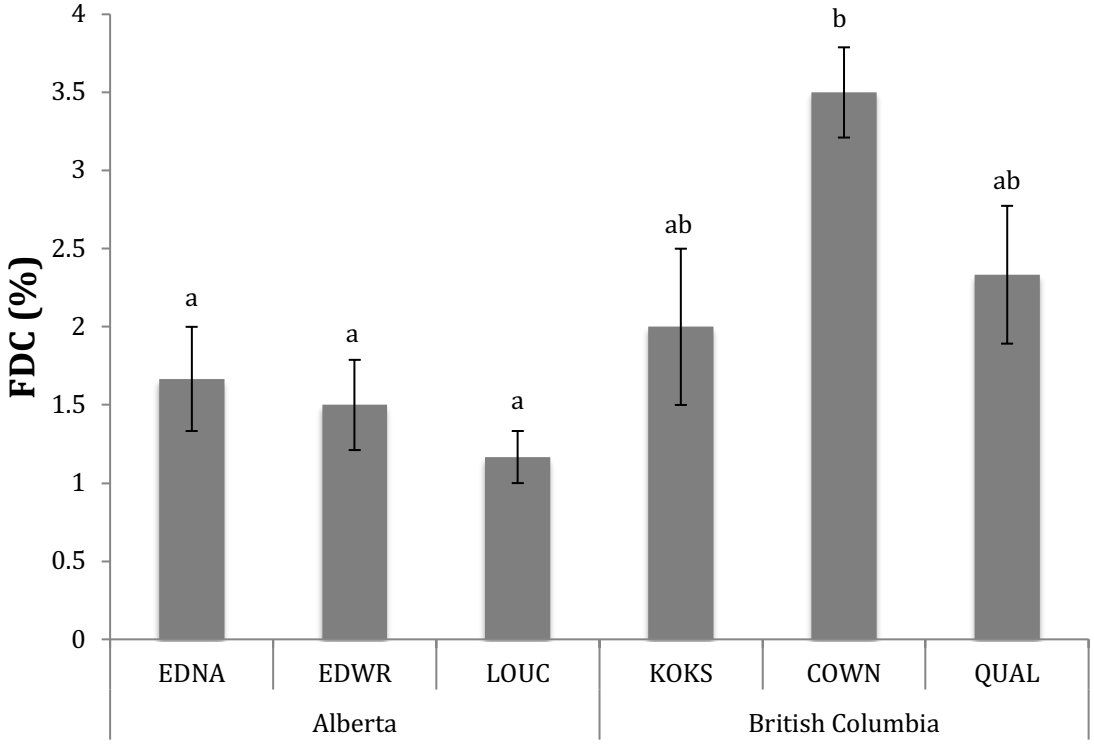

Figure 7. Mean and standard error (n=3) of frequency of dividing *D. geminata* cells as a percentage for EDNA, EDWR and LOUC in Alberta and KOKS, COWN, and QUAL in British Columbia. All sites were mat-forming at time of sample collection. Letter symbols represent significant differences (ANOVA, $p<0.05$).

Table 1. Discharge and precipitation for 2015 sampling dates and their preceding 48 hours from the Calgary Bow River station and Calgary International Airport weather station (Government of Canada Water Office, Government of Canada Weather and Climate Historical Data).

Date	Discharge (m^3/s)	Precipitation (mm)
07/05/15	113.0	0.3
07/06/15	105.0	2.2
07/07/15	106.0	0.4
07/19/15	96.6	0
07/20/15	96.0	0
07/21/15	96.4	0
08/09/15	77.1	0
08/10/15	69.0	0
08/11/15	64.9	0

Table 2. Percentages of genera predominately present in each sample based on sequence similarity in Phylum Bacillariophyta.

Genus	**EDWR %**	**DOUG %**
Pseudo-nitzschia	22	35
Nitzchia	21	10
Thalassiosira	8	8
Phaeodactylum	7	8
Gomphonema	8	6
Navicula	0	14
Sellaphora	7	5
Pinnularia	6	3
Chaetoceros	4	3
Fragilaria	4	3
Cylindrotheca	0	5
Frustulia	0	5
Ulnaria	4	0

Table 3. Putative *D. geminata* gene products found in EDWR sample exclusively.

Reference Organism	Gene ID	Description
P. tricornutum	48229	Carboxy-lyase, mRNA
P. tricornutum	298800	Iron starvation induced
P. tricornutum	577280	Beta chain succinyl-coa
P. tricornutum strain	1492950	PsbC (psbC) gene, partial
P. tricornutum	1640846	Iron-sulphur assembly protein
P. tricornutum strain	2266543	Palmitoyl-CoA delta
P. tricornutum	3621095	Predicted protein (COPdelta)
P. tricornutum	4217157	Histone deacetylase 1 isoform
P. tricornutum strain	4694144	PsbC (psbC) gene, partial
P. tricornutum	5084034	Synthase of glutamate synthase
P. tricornutum	5211513	Predicted protein (Hsp70_1)
P. tricornutum	7504336	Predicted protein (Sec4)
P. tricornutum	23425010	Biotin synthase, mRNA
P. tricornutum	23653550	Oligosaccharyl transferase
P. tricornutum	24260912	RAD23 (hRad23), mRNA
P. tricornutum	25185908	Gdp-mannose 4, 6-dehydratase
P. tricornutum	25588781	Reductase of ferredoxin
P. tricornutum	25654586	Beta-glucan elicitor receptor
T. pseudonana	1742019	CCMP1335 polyadenlyte binding protein
T. pseudonana	7083079	Peptidyl-prolyl cis-trans isomerase
T. pseudonana	23294364	Dtdp-glucose 4, 6-dehydratase
T. pseudonana	24483455	Rieske iron-sulfur protein
T. pseudonana	26093047	Peptidyl-prolyl cis-trans isomerase
T. pseudonana	26214102	Vacuolar membrane proton pump
T. pseudonana	27486727	Eukaryotic peptide chain release

Table 4. Putative *D. geminata* gene products found in DOUG sample exclusively.

Reference Organism	Gene ID	Description
P. tricornutum	9974371	Pyruvate kinase (PK3) gene, complete
P. tricornutum	8292853	1-deoxy-d-xylulose-5-phosphate reductoisomerase
P. tricornutum	4455999	1-hydroxy-2-methyl-2-(E)-butenyl-4-diphsphate synthase
P. tricornutum	23767788	2-oxoglutarate dehydrogenase
P. tricornutum	16483704	2-phosphoglycolate phosphatase
P. tricornutum	12559219	3-dehydroquinate synthase
P. tricornutum	39121443	3-deoxy-7-phosphoheptulonate
P. tricornutum	53850810	3-hydroxyacyl-coenzyme
P. tricornutum	16182690	3-isopropylmalate dehydrogenase
P. tricornutum	17375159	3-oxoacyl-[acyl-carrier-protein] synthase
P. tricornutum	4456032	Putative chloroplast 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase
P. tricornutum	28818951	6-phosphogluconate dehydrogenase
P. tricornutum	36544563	Acetylornithine aminotransferase
P. tricornutum	15447936	Acyl desaturase, mRNA
P. tricornutum	7953306	Adenylosuccinate lyase
P. tricornutum	29556681	Agmatinase mRNA
P. tricornutum	6359274	Glyoxlate aminotransferase
P. tricornutum	8565744	Bifunctional 6-phosphofructo-2-kinase
P. tricornutum	33218672	Calreticulin, mRNA
P. tricornutum	40876430	Chromosome 3 delta 12 fatty
P. tricornutum	20241871	COP9 SigNalosome subunit
P. tricornutum	25052434	Coproporphyrinogen III
P. tricornutum	2235548	CPS III, carbamoyl-phosphate
P. tricornutum	11325448	Cry-dash, cryptochrome/photolyase
P. tricornutum	5006747	CULlin protein 3 (CUL1_1)
P. tricornutum	40887078	Cystosolic class II aldolase
P. tricornutum	53538924	Delta 1-pyrroline-5-carboxylate
P. tricornutum	45269611	Desaturase delta 9
P. tricornutum	27521110	Det3-like protein, mRNA
P. tricornutum	60302244	Dihydrolipoyl dehydrogenase
P. tricornutum	54401614	Early light induced proteins
P. tricornutum	11183692	Enoyl-acp reductase (FABI)
P. tricornutum	27014352	Delta 12 fatty acid desaturase, mRNA
P. tricornutum	51798920	FeS assembly protein suf
P. tricornutum	62004038	ptSLC4-6 mRNA for solute carrier protein
P. tricornutum	27986456	ptbZIP16 mRNA for bZIP transcription
P. tricornutum	61554821	Partial vppl gene for proton-translocating
P. tricornutum	12554732	GLNA glutamine synthase

P. tricornutum	59168374	Glutamyl-t-rna reductase
P. tricornutum	54000907	Glycine decarboxylase t-protein
P. tricornutum	12691919	H3.3, mRNA
P. tricornutum	56223112	Histone acetyltransferase
P. tricornutum	49384287	Homeobox protein, mRNA
P. tricornutum	5321058	Hydroxymethylbilane synthase
P. tricornutum	9974342	Pyruvate kinase
P. tricornutum	50837883	L-galactono-1,4-lactone
P. tricornutum	13580618	Succinate-coa ligase
P. tricornutum	29014901	Lipoamide dehydrogenase
P. tricornutum	50614836	Lutein deficient 1-like
P. tricornutum	967769	Mannose-6-phosphate isomerase
P. tricornutum	13782862	Mitotic checkpoint protein
P. tricornutum	45269636	Delta-9-desaturase (d9) mRNA, complete
P. tricornutum	5192167	Nuclear-encoded-like protein
P. tricornutum	5997756	Oxidoreductase, mRNA
P. tricornutum	7544629	Beta-tubulin gene, partial cds
P. tricornutum	53749152	Peroxiredoxin, mRNA
P. tricornutum	17743732	Phosphatase, mRNA
P. tricornutum	13266179	Phosphoserine transaminase
P. tricornutum	10789902	Phytoene dehydrogenase
P. tricornutum	34822876	Plastidic ribulose-phosphate
P. tricornutum	23738786	Prohibitin-like protein
P. tricornutum	18570885	Pyrophosphate-dependent
P. tricornutum	968240	Pyrroline-5-carboxylate
P. tricornutum	52799055	Pyruvate kinase 1 (PK1)
P. tricornutum	56067807	Rad51 DNA recombination/repair
P. tricornutum	18566377	Regulatory proteasome non-atpase
P. tricornutum	10237299	Short-chain dehydrogenase/reductase
P. tricornutum	26989031	Transketolase, mRNA
P. tricornutum	42395101	Ubiquitin conjugating enzyme
P. tricornutum	25982386	Udp-n-acetylglucosamine
P. tricornutum	14504812	Urea transporter, mRNA
P. tricornutum	5243827	Zeta-carotene desaturase
T. pseudonana	44586858	Delta-11 fatty acid desaturase (desN)
T. pseudonana	39734341	Silicon transporter
T. pseudonana	18185897	ABC transporter, mRNA
T. pseudonana	10436586	Vascular plant like serine/threonine
T. pseudonana	8549731	Oxidoreductase, mRNA
T. pseudonana	22168406	SRP54, signal recognition particle
T. pseudonana	4332814	Cysteinyl-trna synthetase (CTS1)
T. pseudonana	14250638	U5 small nuclear ribonucleoprotein
T. pseudonana	9893482	Microtubule-associated protein
T. pseudonana	9516839	Transketolase (TKT2), mRNA
T. pseudonana	17200611	Acyl desaturase/hydroxylase
T. pseudonana	3199100	Zeaxanthin epoxidase, mRNA

T. pseudonana	6161217	Triose or hexose phosphate
T. pseudonana	17767649	2-dehydro-3-deoxyphosphoheptonate
T. pseudonana	18017634	Atp-dependent RNA DEAD/DEAH
T. pseudonana	3055989	Metalloprotease (ftsH), mRNA
T. pseudonana	18316494	Cyclophilin-type peptidyl-prolyl
T. pseudonana	5626696	Lipoic acid synthetase, mRNA
T. pseudonana	28608843	Phosphoribosyl pyrophosphate
T. pseudonana	24467159	Serine threonine phosphatase
T. pseudonana	12204074	Oliosaccharyl transferase (STT3b)
T. pseudonana	21015149	Rab-like rab-type small G protein
T. pseudonana	30654265	Ubiquitin activating enzyme
T. pseudonana	12582212	Cyclophillin-type peptidyl-prolyl
T. pseudonana	5343997	Nucleolar gtp-binding protein
T. pseudonana	4623790	Glycine decarboxylase p-protein
T. pseudonana	47170751	Leucyl-trna synthetase (LEUS)
T. pseudonana	23987758	Serine threonine protein phosphatase
T. pseudonana	17240649	Clathrin adaptor complex subunit
T. pseudonana	46916551	Nitrate transporter (NRT1)
T. pseudonana	13920254	RAB geranylgeranyl transferase
T. pseudonana	4144530	Imidazoleglycerol-phosphate
T. pseudonana	42704713	Ribonucleotide reductase large
T. pseudonana	4815687	Alanine racemase, mRNA
T. pseudonana	29677391	Phosphofructokinase (PFK2)
T. pseudonana	6739775	CPSase, mRNA
T. pseudonana	49930091	Coatomer protein subunit beta
T. pseudonana	7966114	Exportin1, mRNA
T. pseudonana	49529937	Phosphoglucomutase (PGM2)
T. pseudonana	38057567	Serine hydroxymethyltransferase
T. pseudonana	59750399	Histone deacetylase 1 HDAC
T. pseudonana	8458320	Cyclin putative, mRNA
T. pseudonana	3283968	Probable dihydroxyacid dehydratase
T. pseudonana	45249374	Vacuoler ATPase (VAT1), mRNA
T. pseudonana	55280696	Cation transporting ATPase
T. pseudonana	57162451	3-oxoacyl-synthase, mRNA
T. pseudonana	5846828	Histidyl-trna synthetase, mRNA
T. pseudonana	843225	Atpase-like protein, mRNA
T. pseudonana	33586517	V-type h-atpase, mRNA
T. pseudonana	39734299	Silicic acid transporter, silicon
T. pseudonana	6116243	Probable bifunctional purine
T. pseudonana	20378908	T-complex chaperonin protein
T. pseudonana	23299958	Nucleoside diphosphate kinase
T. pseudonana	20045296	Cdc37 protein, mRNA
T. pseudonana	4079622	Cryptochome/photolyase family
T. pseudonana	9063629	Histone deacetylase 1p, mRNA
T. pseudonana	13495554	Probable malate synthase, mRNA
T. pseudonana	3058141	Methionyl-trna formyltransferase

T. pseudonana	51209899	Trypsin-like serine protease
T. pseudonana	20449339	Luminal binding protein (BIP3)
T. pseudonana	23025534	Lysyl-trna synthetase, mRNA
T. pseudonana	15083672	Sulfolipid biosynthesis protein
T. pseudonana	25438860	Glycine decarboxylase t-protein
T. pseudonana	40150701	Thiamin biosynthesis protein
T. pseudonana	58471813	Adenosuccinate synthase (PURA)
T. pseudonana	14263680	Catalytic subunit of clp protease
T. pseudonana	19851706	DNA topoisomerase, mRNA
T. pseudonana	53771683	Topoisomerase (TOP2), mRNA
T. pseudonana	54677128	Myo-inositol-1-phosphate synthase-like
T. pseudonana	4881977	5-methyltetrahydrofolate-homocysteine
T. pseudonana	40155796	Coatomer protein subunit gamma
T. pseudonana	53947844	Ubiquitin protein ligase, mRNA
T. pseudonana	23023600	Cation transporting ATPase
T. pseudonana	41220745	Calcium transporting rt-atpase
T. pseudonana	19004902	DnaJ protein, mRNA
T. pseudonana	23958168	ATP sulfurylase, mRNA
T. pseudonana	29201880	Sterol-c-methyltransferase
T. pseudonana	44114613	Udp-d-glucose 6-dehydrogenase
T. pseudonana	19718838	Cystathionine beta-lyase (CBL1)
T. pseudonana	17717855	Cyclin-dependent kinase (cdk1)
T. pseudonana	28032896	Serine hydroxymethyltransferase
T. pseudonana	17269083	Nuclear vcp-like protein
T. pseudonana	9076027	ArfGAP, mRNA
T. pseudonana	46471086	3-isopropylmalate dehydratase
T. pseudonana	54768878	Glutamate synthase (glsF)
T. pseudonana	37749070	Precursor of synthase (CSN1)
T. pseudonana	25100895	Pt17531-like protein (ACT1)
T. pseudonana	12449720	Actin-like protein (ACT1)
T. pseudonana	17541397	Serine threonine protein phosphatase
T. pseudonana	61062332	Importin alpha 1 subunit-like
T. pseudonana	9491672	Hypothetical protein (GLNN)
T. pseudonana	14023269	Chromosome 7 glutathione reductase
T. pseudonana	56672641	Dead box family RNA helicase
T. pseudonana	60083154	Precursor of hydrogenase lipoamide
T. pseudonana	48359434	Phosphoglycerate kinase precursor
T. pseudonana	16517199	Glucose-6-phosphate isomerase
T. pseudonana	47194196	ABC-transporter family protein
T. pseudonana	7953339	Adenylosuccinate lyase (ADL)
T. pseudonana	46324284	Beta subunit of clathrin adaptor

Table 5. Putative *D. geminata* gene products from *Caulobacter crescentus* found in EDWR sample exclusively.

Gene ID	Description
221235104	Glutathione S-transferase
594551898	Phosphate transport ATP-binding
221236290	Acylamino-acid-releasing enzyme
221234390	Electron transport complex I subunit, NADH-ubiquinone oxidoreductase
594552235	MutX/nudix family phosphohydrolase
221236117	Alcohol dehydrogenase
221234713	Ribonuclease D
221235065	Acyl-CoA dehydrogenase
231234937	SSU ribosomal protein S2P
221233312	ATP synthase A chain
16127201	Sensory box histidine kinase/response
221233385	Chemotaxis receiver domain protein
221234165	Pyrazinamidase/nicotinamidase
221236749	Intermediate filament-like cell shape determinant creS
221235170	Signal peptidase I
221234214	FAD-linked oxidoreductase
221235966	Cytochrome c2
221234711	Phosphoribosylglycinamine formyltransferase
221233855	Peptide release factor-glutamine N5-methyltransferase
221234170	Haloalkane dehalogenase
221235635	Guanine deaminase
221234836	DNA polymerase III, delta subunit
221234783	Proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase
221235519	Phosphoribosylformylglycinamidine synthase I
594552183	Quinone oxidoreductase
221234819	SMP-30/gluconolaconase/LRE-like protein
594551911	Aminomethyltransferase family protein
221233455	LSU ribosomal protein L25P
221235515	Cytochrome P450
221234000	UDP-perosamine 4-acetyl transferase
221234865	Conserved ACT domain protein
594552221	N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase
221232943	Dephospho-CoA kinase
221233172	UDP-N-acetylglucosamine 4,6-dehydratase
221236625	Alanine dehydrogenase
221234116	3'-phosphoadenosine 5'-phosphosulfate sulfotransferase
221235108	Nucleoside permease nupC
594552373	Aspartate racemase
221233908	Hydroxyacylglutathione hydrolase
221235271	Inositol monophosphatase family
221234984	Glutamine synthetase

Discussion

Mean FDC was consistently significantly higher at DOUG where SRP >0.002 mg/L, compared to EDWR where SRP was consistently below the detection limit. Thus, *D. geminata* cells in a supposedly native geographical location, Southern Alberta, divide under similar SRP conditions as cells in New Zealand where *D. geminata* may be a non-native invader. A positive relationship exists between FDC and SRP in *D. geminata* in New Zealand and cell division is phosphorus limited (Bothwell and Kilroy 2011, Kilroy and Bothwell 2012). The present research supports the findings of research conducted in New Zealand. Results obtained in this study also support the hypothesis that *D. geminata* will have a significantly higher FDC at SRP >0.002 mg/L. A major challenge for field-based studies of of *D. geminata* is that the proposed 0.002 mg/L threshold for stalk formation is also the limit of detection for SRP (Eisenreich et al. 1975). Therefore, the threshold for stalk formation may be <0.002 mg/L SRP under some circumstances.

The difference in FDC between DOUG and EDWR was likely due to higher SRP at DOUG caused by wastewater effluent. Temperature, light and nutrient availability may each affect cell division in diatoms (Furnas 1978, Vaulot et al. 1987, Brzezinski et al. 1990, Lockhart 2013). However, light and temperature were likely similar at EDWR and DOUG as they were sampled on the same date, ~25 km apart, and nitrate is proven not to limit *D. geminata* cell division (Bothwell and Kilroy 2011). SRP can increase due to increases in precipitation and discharge (Honisch et al. 2002). Precipitation and discharge remained steady for 48 hours before sampling at EDWR and DOUG and were close to historical lows, so were likely not responsible for any increases in SRP (Table 1).

With the exception of one sampling date, *D. geminata* at DOUG was non-mat-forming under higher SRP concentrations while EDWR was mat-forming with undetectable SRP concentrations. These results concurred with research conducted in New Zealand, which determined that stalk formation is associated with phosphorus limitation and that cell division rates are phosphorus limited in *D. geminata* mats (Bothwell and Kilroy 2011). In phosphorous deficient systems, *D. geminata* may form stalks to lift cells into the water column where more nutrients are available (Taylor and Bothwell 2014). The one exception occurred on August 11, 2015 when DOUG *D. geminata* was mat-forming with mean SRP of 0.00986 mg/L. Stalks at DOUG on August 11, 2015 were ~2 cm long compared to 10-20 cm at EDWR. The observation of stalks at DOUG provides some evidence that low SRP may not be the only trigger of stalk formation.

Mean FDC at British Columbia sites was not significantly different than mean FDC at Alberta sites. A single exception was COWN, where FDC was significantly higher than all Alberta sites. A brief spike in SRP at COWN, possibly from upstream point sources such as a fish hatchery or wastewater treatment plant, may have caused a significant increase in cell division at the time of sampling (British Columbia Ministry of Environment 2011).

Collectively, the results of this study demonstrate the same link between FDC, SRP and stalk formation as previous studies (Bothwell and Kilroy 2011, Kilroy and Bothwell 2012). With the exception of the observation at DOUG on August 11, 2015, *D. geminata* is behaving similarly at mat-forming and non-mat-forming locations in Alberta,

British Columbia and New Zealand and, therefore, may respond well to a single management strategy, such as phosphorus addition, regardless of location.

As hypothesized, gene products from *P. tricornutum* that are associated with extracellular compounds were identified in the mat-forming EDWR sample. GDP-mannose 4,6-dehydratase (GMD) and oligosaccharyl transferase (OST) were found in the EDWR sample. GMD is an enzyme that is part of a biosynthetic pathway that synthesizes the sugars fucose and rhamnose (Webb et al. 2004). Fucose and rhamnose are present in diatom EPS, with rhamnose being one of the most abundant sugars (Hoagland et al. 1993, Wustman et al. 1997). OST is a membrane protein complex involved in an important step in N-linked glycosylation pathway. N-linked glycosylation is a prevalent form of glycosylation that is important for cell-extracellular matrix attachment (Uematsu et al. 2014). Accordingly, it would appear that both OST and GMD may be crucial to *D. geminata* stalk formation.

Beta glucan elicitor receptor protein was also expressed in the EDWR sample. Beta glucan is a component of bacterial cell walls. Beta glucan elicitor receptors are present on plant cell membranes to detect beta glucan elicitors and trigger the appropriate defence response to the threat of fungi or bacteria. The trigger of a defence mechanism occurs through the octadecanoid pathway and leads to production of the jasmonic acid hormone that induces defence genes (Davies 1995). Diatoms and bacteria engage in many interactions including those that threaten diatoms (Amin et al. 2012). It is possible that *D. geminata* forms stalks to raise cells into the water column away from the threat of bacteria. Further, it has been shown that bacteria are drivers of the secretion of EPS in diatoms (Bruckner et al. 2011).

Putative *D. geminata* gene products found only in the EDWR mat-forming sample, using the reference organism *C. crescentus,* include Pho regulon products sensory box histidine kinase sensor protein and phosphate transport ATP-binding protein. The phosphate (Pho) regulon is made up of a group of genes responsible for inorganic phosphorus management in bacteria (Wanner and Chang 1987). It is critical in low phosphate conditions for uptake and storage of the essential nutrient. Sensory box histidine kinase sensor protein is one of two components controlling the Pho regulon by signaling inorganic phosphorus starvation (Wanner and Chang 1987, Santos-Beneit 2015). Phosphate transport ATP-binding protein transports phosphate across the cell membrane and is responsible for uptake of phosphate under nutrient stress (Qi et al. 1997, Adams et al. 2008). Similar genes may exist in diatoms as they share many genes with bacteria and relatively little research has been conducted on the molecular mechanisms behind phosphate starvation in diatoms (Dyhrman et al. 2012). Phosphohydrolase and creS genes were also expressed in the mat-forming EDWR sample. Phosphohydrolases can be activated under inorganic phosphorus limited conditions to access internal phosphate supply (Duff et al. 1991, Bosse and Kock 1998). The creS gene influences cell shape in bacteria and may play a role in stalk elongation (Ausmees et al. 2003, Wagner et al. 2005).

As hypothesized, many putative *D. geminata* gene products found in the DOUG, non-mat-forming sample, are related to the higher cell division rate present at the non-mat-forming site including 1) RAD51, which repairs DNA in preparation for cell division (Shinohara et al. 1992); 2) ribonucleotide reductase, an enzyme that catalyzes the reaction to synthesize deoxyribonucleotides, which regulate DNA synthesis during cell division

(Elledge et al. 1992); 3) histone deacetylase, which is important for accurate cell division as it allows histones to wrap DNA tightly and condense into chromosomes (Yang 2002); 4) mitotic checkpoint proteins that keep the duplicated chromosomes attached until they are appropriately affixed to the mitotic spindle; 5) importin alpha, which regulates spindle assembly (Kohler et al. 1999); 6) valosin containing proteins, which help to disassemble the spindle (Nicklas 1997, Cao et al. 2003); 7) DNA topoisomerases, which are active in mitosis to prevent nondisjunction (Holm et al. 1989, Champoux 2001); and 8) cyclins and cyclin dependent kinases, which regulate the cell cycle (Galderisi et al. 2003).

As hypothesized, sequences coding for silicon transporters or silicic acid transporters (SITs) were only found in the DOUG sample where dividing cells have an increased need for silicon. Diatoms require silicon in the form of silicic acid for frustule formation (Lewin 1962). SITs are membrane proteins found in diatoms that are responsible for transporting silicic acid into the cell (Hildebrand et al. 1998).

As hypothesized, gene products related to stalk formation were identified in the sample from the low SRP, mat-forming site (EDWR) and genes related to cell division were found in the sample from the higher SRP, non-mat-forming site (DOUG). Therefore, the metatranscriptomic approach was successful in identifying expressed genes that differed between periphyton with, *D. geminata* cells, at the two sites. Genes that link inorganic phosphorus starvation directly to stalk formation are those coding for sensory box histidine kinase sensor protein, phosphate transport ATP-binding protein and phosphohydrolase.

As the aforementioned gene products are limited to those present in the reference organisms, caution should be taken when interpreting the gene expression results in this study. In addition, only one sample from each site was sequenced and samples contained genetic material from several organisms present in periphyton. As such, genes from non-target organisms may have been identified as exclusively expressed at the mat or non-mat-forming site. However, this investigation was important as it proposes genes that may be directly involved in problematic *D. geminata* stalk formation under low SRP conditions and may inform future phosphorus addition management strategies.

Conclusion

The results show that, for all sampling dates, FDC is significantly higher at the non-mat-forming site on the Bow River with higher SRP. *D. geminata* at EDWR and DOUG reacted similarly to low SRP as populations in New Zealand where it is supposedly invasive. The results support the low SRP hypothesis except for on August 11, 2015 when mat-forming *D. geminata* was observed at DOUG with a mean SRP >0.002 mg/L. However, the observation of stalk formation at a site with SRP >0.002 mg/L only occurred once. Collectively, the results support the low SRP hypothesis but also indicate that low SRP may not be the only cause of stalk formation.

Mean FDC at Alberta sites was lower than at British Columbia sites. There was no significant difference other than at COWN, which had a significantly higher mean FDC than all Alberta sites. FDC at COWN may have been increased on the sampling date due to a short-lived increase in SRP from an up-river point source. The findings demonstrate that British Columbia and Alberta mat-forming *D. geminata* have relatively

low FDC compared to the non-mat-forming site, DOUG. The low SRP hypothesis is supported by the results in this study, as values are similar to those found in New Zealand (Kilroy and Bothwell 2012). Further collection of SRP and FDC data from more rivers in *D. geminata*'s native and supposedly invasive ranges would help to confirm the SRP hypothesis. In addition, a method for measuring SRP concentrations below the current limit of detection (0.002 mg/L) is needed. A more sensitive method to measure SRP would allow the threshold for *D. geminata* stalk formation to be further resolved.

Genes that were identified from *P. tricornutum*, *T. pseudonana* and *C. crescentus* as expressed only in the mat-forming sample include those coding for proteins required during phosphorus starvation, which lends support to the low SRP hypothesis for cause of mat formation. Genes responsible for silica processing and mitosis were expressed in the non-mat-forming sample. As expected, cells that are not forming stalks have a higher rate of cell division. Future studies would benefit to explore gene expression in an increased number of samples and geographical locations to provide further knowledge of the relationship between FDC, SRP, and stalk formation, and the potential relationship between stalk formation and bacteria.

CHAPTER 3: PERIPHYTON PRESERVATION AND DNA EXTRACTION COMPARATIVE ANALYSIS

Introduction

Benthic algal mats, formed by the stalk-producing diatom *Didymosphenia geminata*, have been increasingly documented in streams and rivers around the world since the late 1990s (Kilroy 2004, Bothwell et al. 2009, Whitton et al. 2009, Gillis and Chalifour 2010, Reid et al. 2012). Collectively, stalks form thick nuisance mats, which are now found in North America, Asia, Europe, and Southern Hemisphere countries including New Zealand, Chile and Argentina (Kilroy 2004, Kirkwood et al. 2007, Spaulding and Elwell 2007, Bothwell and Spaulding 2008, Reid et al. 2012). Nuisance mats have negative economic and ecological consequences (Larson 2007, Shearer et al. 2007, Spaulding and Elwell 2007, Bickel and Closs 2008, Kilroy et al. 2009, Gillis and Chalifour 2010). The cause of excess stalk formation is unknown, but proposed to be low SRP (soluble reactive phosphorus) concentrations or a mat-forming genetic variant (Kirkwood et al. 2007, Spaulding and Elwell 2007, Whitton et al. 2009, Kilroy and Bothwell 2011, Bothwell et al. 2014).

The proposal of a mat-forming genetic variant of *D. geminata* being spread globally resulted in a call for molecular studies by many researchers, yet few have been published, partly due to difficulties with extraction of genetic material (Kirkwood et al. 2007, Spaulding and Elwell 2007, Bothwell and Spaulding 2008, Blanco and Ector 2009, Bothwell et al. 2009, Whitton et al. 2009). Uyua et al. (2014) extracted *D. geminata* DNA using various methods. One third of DNA samples obtained did not show bands on agarose gels. Further, Uyua et al. (2014) assessed the quality of DNA extracted, from the

methods that obtained the highest yields, and found that only 29.1% of samples had a 260/280 absorbance ratio between 1.7 and 1.9. A 260/280 absorbance ratio of ~1.8 is considered ideal. A lower ratio is an indication of protein or phenol contamination or due to very low DNA yield (Sambrook and Russel 2001). The inability to consistently isolate high quality *D. geminata* DNA remains a barrier to further molecular studies.

Molecular studies on *D. geminata* have been inhibited by the periphyton matrix in which the cells are found, lack of techniques for culturing *D. geminata* in the laboratory, and presence of polysaccharide stalks (Kuhajek and Wood 2014, Uyua et al. 2014). Polysaccharide stalks inhibit enzymes involved in PCR and may also inhibit those used for DNA isolation (Pandey et al. 1996, Monteiro et al. 1997). Collectively, the aforementioned factors present unique challenges for studies that aim to integrate molecular methods to understand the ecology and evolution of *D. geminata*. For additional molecular studies to occur, protocols must be developed that circumvent these challenges and consistently result in high quality and quantity of DNA.

A critical step to prepare DNA for extraction is sample preservation that reduces oxidation and degradation of DNA by enzymes (Yagi et al. 1996, Adams et al. 1999, Prendini et al. 2002). The preservation buffer and storage temperature that most effectively prevent degradation should produce the highest quality and quantity of DNA from samples. Ethanol is the most commonly used preservative in zoological studies, but has also been used for periphyton and diatoms at concentrations of 70% and 95% (Corse et al. 2010, Nagy 2010, Uyua et al. 2014). Sterile water has also been used to preserve periphyton at -20°C and -80°C (Sherwood et al. 2008, Sanli et al. 2015). RNA*later* is a suitable preservative for DNA and RNA, but few studies have used RNA*later* for DNA

preservation due to the high cost (Vink et al. 2005, Nagy 2010). Experimental comparisons of preservation methods have not been done for *D. geminata,* but are important to ensure extraction results in the highest possible yield of DNA (Michaud and Foran 2011, Moreau et al. 2013).

Selecting the extraction method that will provide the highest yield of DNA is also important. *D. geminata* DNA has been extracted using commercial kits, cetyltrimethyl ammonium bromide (CTAB), and organic extraction (OE) methods resulting in varied yield amounts up to 3180 ng (Coyne et al. 2001, Cary et al. 2007, Uyua et al. 2014). Kit methods specifically have been tested before, for *D. geminata* and other freshwater diatoms, with variable results. In a comparison of six commercially available kits, only one produced PCR amplifiable DNA from all three freshwater diatoms tested (Nguyen et al. 2010). It is important to be able to reliably obtain required amounts of DNA for downstream applications such as PCR and NGS, which typically require nanograms and micrograms of DNA respectively.

The objective of this study was to conduct a comparative analysis for storing periphyton and subsequently extracting DNA. Although *D. geminata* DNA has been extracted successfully studies have shown that quality and quantity of DNA can vary depending on the buffers and temperatures used to store samples prior to extraction (Dillon et al. 1996, Dawson et al. 1998, Mtambo et al. 2006). However, the effectiveness of different storage buffers or temperatures on *D. geminata* DNA extraction has not been assessed.

Materials and Methods

Study Site

Periphyton was collected from the left bank, facing downstream, of the Bow River near the community of Bowness within the City of Calgary on June 3, 2015 (51.086428, -114.172566). The Bow River runs east from its origin at Bow Lake in the Rocky Mountains through Calgary, Alberta, Canada. Nutrient concentrations increase from upstream to downstream within the city of Calgary due to treated wastewater input, storm sewers and lawn run-off (Sosiak 2002). The Bow River supports a variety of fish populations and is known world-wide for exceptional angling opportunities (Berry 1997).

Sample Collection

D. geminata stalk material in periphyton was collected from submerged rocks using forceps, briefly rinsed in river water to remove excess sediments, and placed into 25 mL Falcon tubes containing various storage buffers (Figure 8). Four tubes each of RNA*later*, 70% EtOH, 95% EtOH and ddH_2O were used to contain the periphyton for a total of 16 Falcon tubes. To keep samples as close to their respective experimental temperature as possible during transport, half of the them (two of each storage buffer) were immediately placed in a cooler with dry ice at -78.5°C, while the others were temporarily stored in a cooler containing ice packs at 1°C.

Upon arrival at the laboratory, a small sample from each tube was inspected visually under the microscope (Leica DM IRB inverted, 200x) to confirm the presence of *D. geminata* cells. Tubes stored on dry ice were transferred to a -80°C freezer, while the tubes stored on ice packs were moved to a -20°C freezer. Tubes were stored at their

respective temperatures for a maximum of two months, until DNA extraction occurred. Starting material was assessed quantitatively by removing 100 mg wet weight of periphyton from each tube and counting diatoms from all taxa present. Other materials present, such as sediment and detritus, were qualitatively described.

Molecular Methods

Three different DNA extraction protocols were performed on collected stalk material stored in each storage buffer (RNA*later*, 70% EtOH, 95% EtOH, ddH_2O) and temperature (-20°C, -80°C) combination. Triplicate extractions were performed for each extraction protocol: Qiagen DNeasy® Plant Mini Kit Handbook Instructions (kit), a modified version of the Qiagen DNeasy® Plant Mini Kit protocol (modified) (Vanderzwan, unpublished) and an organic extraction (OE) protocol (Sambrook and Russel 2001, Uyua et al. 2014). Total DNA yield from each sample along with DNA quality, as a 260/280 absorbance ratio, were compared.

Samples for all DNA extraction protocols were prepared by using forceps to transfer 100 mg wet weight of periphyton from each Falcon tube to 2 ml microcentrifuge tubes. Twenty-four microcentrifuge tubes were prepared per extraction method for a total of 72 microcentrifuge tubes of stalk material. Stalk material had been stored in one of four storage buffers and at one of two different temperatures (Figure 8). Microcentrifuge tubes were centrifuged at 15,000 rpm for 4 minutes. Excess buffer was pipetted off the top of the stalk material. To remove any remaining buffer, 1 ml ddH_2O was added and each tube was vortexed. Tubes were centrifuged again at 15,000 rpm for 4 minutes.

Excess water was removed using a pipette. The stalk material was rinsed again in the same manner.

Lysis buffer (Qiagen AP1 buffer) was added to each sample that would undergo the kit and modified protocols. All samples were vortexed for 3 minutes with a 3 mm tungsten carbide bead to mechanically break up the silica frustules of the diatoms. Instructions given in the Qiagen DNeasy® Plant Mini Kit Handbook were followed exactly for the kit protocol. The modified protocol included variations from the kit protocol. Namely, during the incubation period at 65°C, tubes were inverted every 5 minutes. Incubation times, as listed in the handbook were doubled throughout the protocol. Each elution was performed twice with 20 µl of elution buffer (Qiagen AE buffer).

An established OE protocol (Sambrook and Russell 2001) was used with slight modifications according to Uyua et al. 2014. The obtained DNA pellets were washed with 1 ml 70% EtOH. The DNA pellet was air dried for 15 minutes and resuspended in 10 µl ultrapure DNase free water. DNA was stored at 4°C.

All extracted DNA was quantified and tested for quality, indicated by the ratio of absorbance at 260 nm and 280 nm, using a Qubit 2.0 Fluorometer and Nanodrop ND-1000 Spectrophotometer respectively. Life Technologies Qubit dsDNA BR Assay Kit reagents were used for Qubit quantifications. Stock concentration values were multiplied by elution volume to give total DNA yield values.

Statistical Analyses

All concentration readings provided by the Qubit 2.0 Fluorometer as "<1 ng/µl" were recorded as 1 ng for analytical purposes. Data were visually analyzed by plotting means and standard error for DNA yield obtained in nanograms and absorbance ratio (260/280) for each extraction protocol.

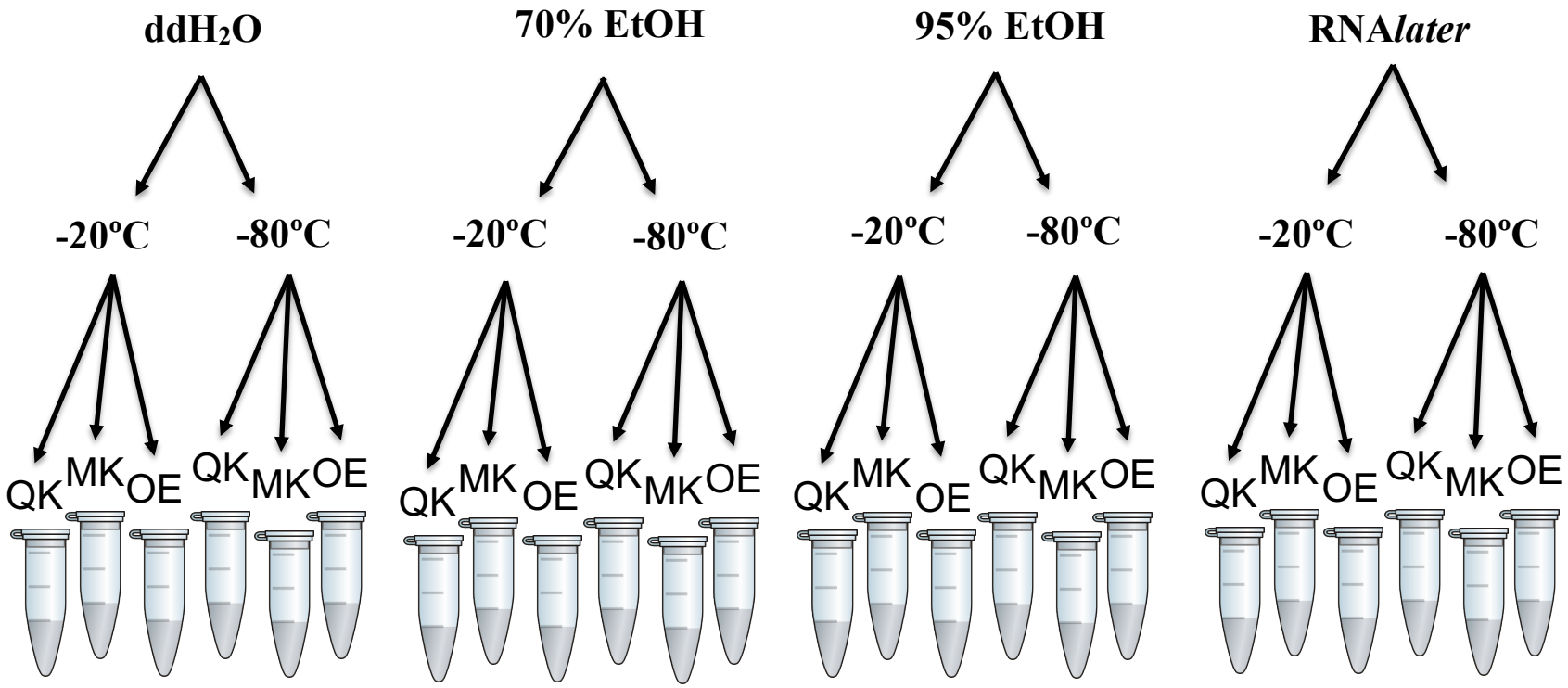

Figure 8. Periphyton preservation and DNA extraction experimental setup. Four storage buffers (ddH_2O, 70% EtOH, 95% EtOH, RNA*later*), two storage temperatures (-20°C, -80°C), and three extraction methods (Qiagen kit (QK), modified kit (MK), organic extraction (OE)) were used for a total of 24 treatments. Triplicate extractions were performed for each treatment combination.

Results

D. geminata cells were visually confirmed to be present in all samples used for DNA extraction. *D. geminata* cells and stalk material made up the majority of each sample. There was a mean of 117.63±6.61 *D. geminata* cells found per 100 mg wet weight of periphyton (Table 6). There were no diatoms attached to the *D. geminata* stalks, however, samples contained other diatom taxa (Table 6). Grains of sand and detritus were present in small amounts.

Unmodified kit extractions produced a mean of 21.54±4.77 ng of DNA. Periphyton stored in RNA*later* at -80°C had the highest mean yield of extracted DNA (77.27±5.51 ng). Samples stored at -20°C produced a mean of 2±0 ng, except for the sample stored in 70% EtOH which yielded 23.4±12.13 ng. Periphyton stored in 95% EtOH at -80°C also produced a mean yield of 2 ng. All treatments with a mean yield of 2 ng had a standard error of zero (Figure 9).

Mean yield produced from the modified extractions was 295.55±44.34 ng. Periphyton stored in ddH_2O at -80°C had the highest mean yield of extracted DNA (491.60 ng), but also the largest standard error (366.70 ng). Samples stored in RNA*later* at -20°C produced 419.07±167.13 ng of DNA. Periphyton stored in ddH_2O at -20°C produced the smallest mean yield of DNA (118.53±114.53 ng).

All ddH_2O and EtOH storage treatment combinations extracted with the OE method had mean yields below 33.00 ng and as low as 1.00 ng. DNA amounts obtained from samples stored in RNA*later* and extracted using the organic method were higher than all other samples and by a magnitude of at least 7. Periphyton stored in RNA*later* at

-20°C and -80°C provided mean yields of 243.67±167.12 ng and 262.67±46.56 ng respectively (Figure 9).

The modified method also consistently produced higher mean yields of DNA across all samples than the OE method. The kit method was generally the least successful. Samples stored at -80°C consistently produced more DNA than those stored at -20°C except for the sample stored in RNA*later* and extracted using the modified protocol.

Overall, DNA extracted using the kit and modified protocols, was of higher quality than DNA extracted using the OE method. Kit and modified extracted DNA had mean 260/280 absorbance ratio values between 1.56–1.86 while OE produced DNA with mean 260/280 absorbance ratios between 1.24–1.46 (Figure 10).

Table 6. Diatom cells in periphyton samples. Mean of cells counted in eight samples of 100 mg wet weight.

Diatom	**Mean number of cells ± SEM**
Didymosphenia geminata	117.63±6.61
Cymbella spp.	6.13±1.13
Brachysira spp.	1.25±0.62
Amphora spp.	2.25±0.65

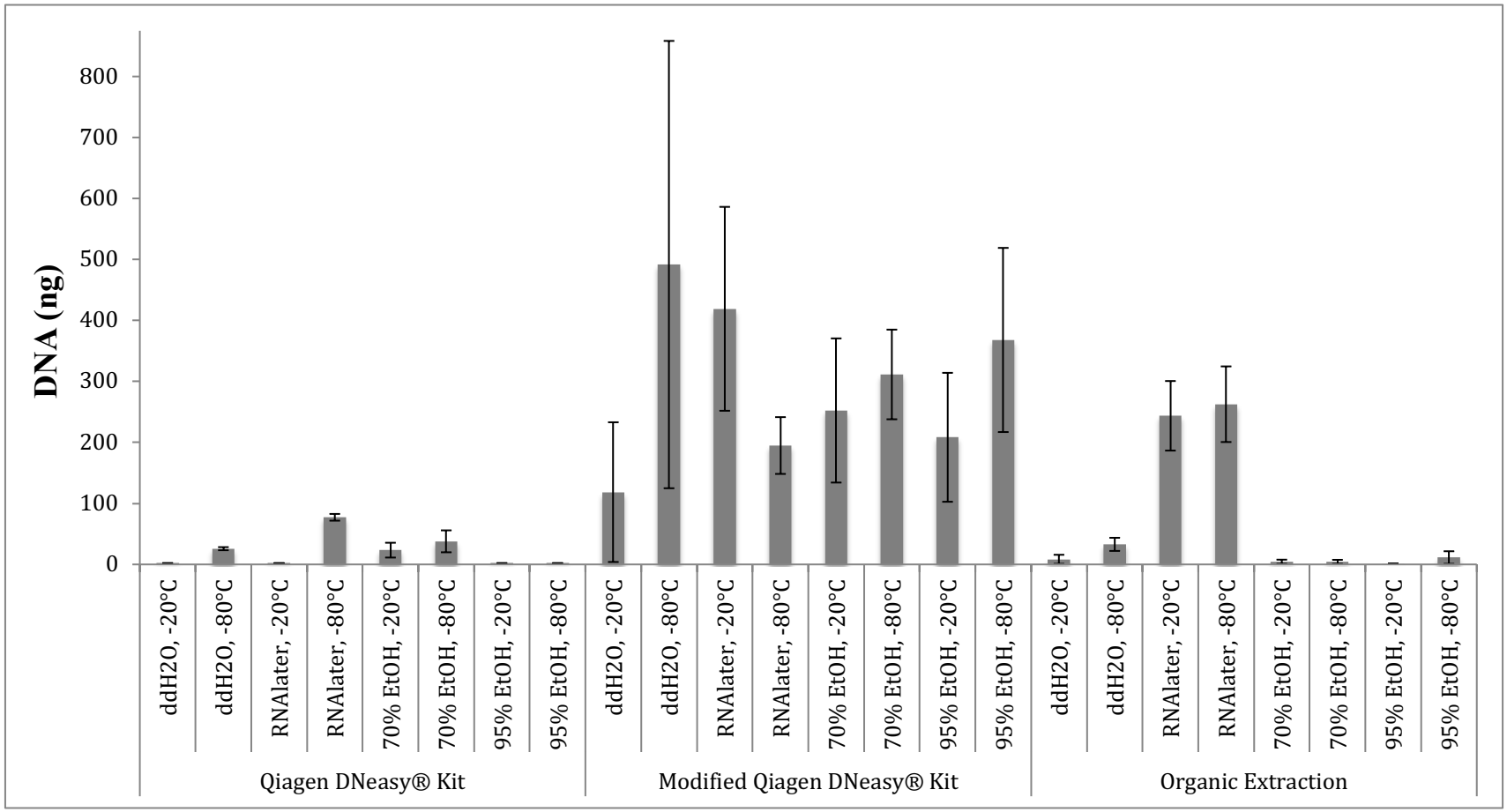

Figure 9. Mean ± SEM total DNA yield obtained using the Qiagen DNeasy® Plant Mini Kit, the modified Qiagen DNeasy® Plant Mini Kit, and organic extraction protocols for each of the 4 storage buffers at -20°C and -80°C. Triplicate extractions were performed, each from 100 mg wet weight of periphyton.

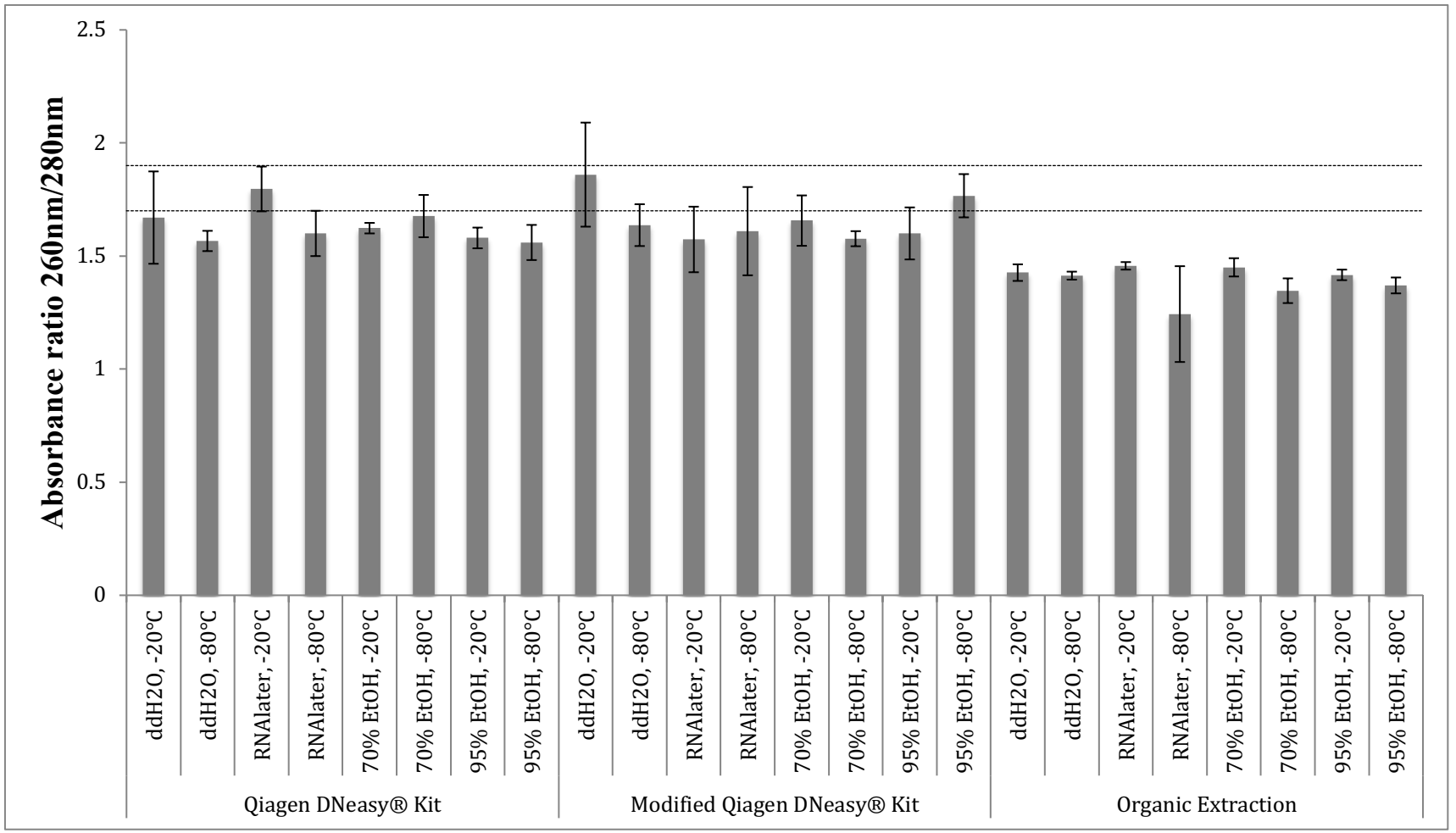

Figure 10. Mean ± SEM absorbance ratio (260nm/280nm) obtained using the Qiagen DNeasy® Plant Mini Kit, the modified Qiagen DNeasy® Plant Mini Kit, and OE protocols for each of the 4 storage buffers at -20°C and -80°C. Triplicate extractions were performed. Dashed lines encompass the ideal range of 1.7-1.9 260/280 absorbance ratio.

Discussion

The results of this study indicate that DNA extracted from periphyton stored in 95% EtOH at -80°C, using the modified kit method, had the best combination of quantity (368±150.96 ng) and quality (260/280 absorbance ratio of 1.77). Overall, the modified kit method produced more, higher quality, DNA than the kit or OE methods. Further, extraction method had a greater impact on DNA yield and quality than preservation buffer and temperature. The exception to this was for the OE results, where RNA*later* preserved samples performed better than the others.

Fourteen of the obtained mean DNA yields would satisfy recommended requirements for PCR, while none would satisfy recommended requirements for NGS. For PCR, 25–200 ng of template DNA is commonly used and Illumina recommends >1 μg for NGS. As many of the DNA yields obtained here would not satisfy the recommended yield requirements for PCR or NGS, this study confirms that extracting *D. geminata* DNA is difficult, likely due to the presence of stalk material. However, research has shown promise for successful PCR and NGS from picogram amounts of DNA (Mutter and Boynton 1995, Taberlet et al. 1996, Parkinson et al. 2012)

DNA extracted using the OE method generally had lower quality, measured by 260/280 absorbance ratio, than DNA extracted using the other methods. Ideally, the 260/280 absorbance ratio should fall between 1.7−1.9 (Sambrook and Russel 2001) for downstream applications. A value less than 1.7 was obtained for all OE extractions and indicates contamination from proteins or reagents used in the extraction process (Sambrook and Russel 2001). It is possible that protein or phenol contamination remained in the samples despite the use of proteinase K and the precaution of using

additional chloroform to remove excess phenol. Organics, such as detritus in the starting material, may have also caused contamination. Organics are removed differently in different methods. Commercial kits remove organics using a charged column while the organic extraction protocol separates nucleic acids from other materials using chemical phases of different polarity.

The kit methods may have an advantage over the OE method due to the spin column with built in filter, designed to remove unwanted debris such as polysaccharides (DNeasy® Plant Handbook 2012). Removal of debris is important as samples of mat-forming *D. geminata* contain a high proportion of stalk biomass relative to cell biomass (Larned et al. 2006). Stalks contain sulfated polysaccharides and have high uronic acid content (Gretz 2008). Acidic polysaccharrides can interfere with enzymes used in molecular protocols and can be precipitated with the extracted DNA causing issues in downstream applications (Monteiro et al. 1997 and Pandey et al. 1996). The presence of a high proportion of stalks in mat samples could explain the relatively low yields from the OE extractions and, in general, contribute to the difficulty of extracting DNA from *D. geminata*.

Liberating DNA from the frustule could be seen as another challenge. However, cell walls were assumed to be broken by the tungsten carbide beads used in the kit and modified protocols, and the organic method has been shown to liberate DNA (Uyua et al. 2014).

Low yields of DNA were obtained using the organic extraction method, except from samples preserved in RNA*later*. Both RNA*later* preserved samples produced mean DNA yields similar to those obtained by the modified kit method of extraction. The

composition of RNA*later* may be responsible for its superior performance as a storage buffer in the organic extractions. RNA*later* is a saturated ammonium sulfate solution (Lader 2001). Ammonium sulfate is known for its ability to change the solubility of proteins (Taylor 1953). *D. geminata* stalks are composed of polysaccharides and proteins (Gretz 2008). Therefore, stalks may have experienced partial degradation and precipitation when in contact with RNA*later,* which could have altered stalk integrity and reduced the amount of interference from stalk material in the extraction process. RNA*later* did not have the same obvious performance advantage in the kit extractions, possibly due to stalk material being removed by the spin column filter.

The obtained results for mean DNA yield, extracted using the modified and OE methods, were similar to previous studies on *D. geminata* DNA extraction (Uyua et al. 2014). In contrast to those results, this study found more consistent success with the modified kit method, while Uyua et al. (2014) were able to obtain yields between 200−300 ng, from samples stored in 70% EtOH at -20°C, using the same OE method used here. DNA yields obtained in the present study, from kit and modified protocol extracted samples, and the RNA*later* preserved samples from the OE method, are also similar to other studies dealing with samples containing large amounts of EPS, iron compounds and sediments, which are present in *D. geminata* mats (Bey et al. 2010, Sharma et al. 2014). DNA yields obtained with the kit were higher than those obtained in a recent study, which compared six other extraction kits for use on freshwater diatoms. All kits assessed in the study produced lower than detectable amounts of DNA (Nguyen et al. 2010).

Molecular studies on *D. geminata*, such as population genomic studies, may benefit from pooling DNA from multiple samples (Pool-Seq) if DNA yields for NGS are difficult to obtain. The Pool-Seq method can effectively estimate allele frequencies and bioinformatic tools have been designed to analyze Pool-Seq data (Gautier et al. 2010, Kofler et al. 2011). Pool-Seq may address the issue of low yield in some cases and has been used to explore adaptive divergence in ecotypes of prickly scupin (*Cottus asper*) and periwinkles (*Littorina saxatilis*) (Gautier 2015, Dennenmoser et al. 2016).

One major caveat of this study is that the extracted DNA likely included genetic material from organisms other than *D. geminata*. According to a Megablast search of RNA sequences from mat-forming *D. geminata* from the Bow River, 21% of genetic material was consistent with bacterial sequences. Even so, this study contributes to the improvement of *D. geminata* DNA extraction techniques. Moreover, extracting DNA from periphyton that contains *D. geminata* cells may be beneficial for metagenomic and metabarcoding approaches. For example, DNA extracted from periphyton may be compared to barcode markers for *D. geminata* to identify its presence before mat formation occurs.

Future studies should aim to improve on molecular techniques for *D. geminata* in multiple ways. First, the amount of *D. geminata* DNA present in genetic material extracted from *D. geminata* mats should be quantified. *D. geminata* specific primers could be used in PCR, followed by gel electrophoresis using mass ladder to quantify the amount of *D. geminata* DNA present. Future studies should also aim to obtain higher yields and quality of *D. geminata* DNA. Commercial kit protocols could be altered further to improve performance for *D. geminata*. For example, using buffers with higher

salt content may be beneficial to remove some of the polysaccharides present (Porebski et al. 1997). Future studies could ensure that all extracted DNA is from *D. geminata* cells. *D. geminata* cells may be physically separated from the remainder of the periphyton sample to ensure that all extracted DNA is from *D. geminata* cells. Due to *D. geminata*'s relatively large size, physical separation of *D. geminata* cells may be possible by manual sorting or by using filters. In addition, extracting DNA from isolated cells may minimize downstream interference caused by polysaccharide mats. However, manual sorting is time consuming and may result in low DNA yields. Finally, DNA from multiple extractions may be pooled for some downstream applications. Collectively, addressing the aforementioned objectives would greatly advance molecular knowledge and protocol development for *D. geminata.*

Conclusion

To obtain the highest possible yield and quality of DNA, out of the methods tested in this study, samples of *D. geminata* mat material with attached cells should be placed in 95% EtOH and stored at -80°C as soon as possible after collection. The modified kit method, which was developed to optimize yield and quality of *D. geminata* DNA, should be used for extraction (Vanderzwan, unpublished). In the modified kit method tubes were inverted every 5 minutes during the 65°C incubation period, incubation times were doubled throughout the protocol, and each elution was performed twice with 20 µl of elution buffer (Qiagen AE buffer). Although DNA quantities extracted from periphyton samples in this study likely include genetic material from non-target organisms, the results contribute to the development of a reliable method for

storing *D. geminata* stalk material and extracting *D. geminata* DNA. Further experiments should be carried out to establish a reliable method for DNA extraction from stalk forming *D. geminata* cells in benthic periphyton.

CHAPTER 4: GENERAL CONCLUSION

Invasive species are detrimental to ecosystems as they have the ability to quickly colonize new geographical locations and decrease the biodiversity of native organisms (Elton 1958, Kolar and Lodge 2000). *Didymosphenia geminata* is likely both a native and non-native invader (Kilroy 2004, Blanco and Ector 2009, Reid et al. 2012). Through stalk formation, *D. geminata* increases periphyton abundance to nuisance levels that interfere with invertebrates, fish, and anthropogenic river uses (Larson 2007, Shearer et al. 2007, Spaulding and Elwell 2007, Kilroy et al. 2009, Gillis and Chalifour 2010). Excess stalk material produced by *D. geminata* is a global issue. Studies have suggested that growth of excess stalk material is triggered by low SRP (soluble reactive phosphorus) caused by decreased concentrations in rivers worldwide (Kilroy and Bothwell 2011, Bothwell et al. 2014). A second hypothesis states that mat-forming *D. geminata* is the result of a genetic variant that has been transported by anglers (Kirkwood et al. 2007, Spaulding and Elwell 2007, Whitton et al. 2009). Despite the establishment of hypotheses for nuisance mat formation, the cause remains unknown.

The objectives of this thesis were to compare FDC (frequency of dividing cells), SRP, and gene expression between a non-mat-forming and mat-forming site on the Bow River, compare FDC between sites in British Columbia and Alberta, and perform a DNA storage and extraction comparative experiment. The relationship between FDC and SRP has only been studied in New Zealand where *D. geminata* is likely a non-native invader. Studying FDC and SRP in additional geographical locations will further test the low SRP hypothesis. This is the first study to examine gene expression in *D. geminata.* An

established DNA extraction technique for *D. geminata* does not exist despite the hypothesis of a mat-forming variant and calls for molecular studies.

To investigate the relationship between SRP, FDC, and stalk-formation, I measured FDC and SRP at a mat-forming and non-mat-forming site on the Bow River within Calgary, Alberta. As expected, FDC and SRP were lower at the mat-forming site. Interestingly, stalk formation was observed at DOUG on the third sampling date when SRP and FDC were still relatively high compared to the mat-forming site, EDWR, suggesting that SRP concentrations may not be the only trigger of stalk formation. FDC was also counted at three mat-forming sites on Vancouver Island, British Columbia and in Alberta. As hypothesized, there was no significant difference between mean FDC at British Columbia and Alberta sites mat-forming sites, except for COWN which had a significantly higher FDC than all Alberta sites. Increased FDC may have been caused by a brief increase in SRP from an upstream point source. The findings of this study mainly support the low SRP hypothesis and research done in New Zealand on the relationship between SRP, FDC and mat formation. However, further inquiry should focus on this relationship in additional geographical locations to confirm the low SRP hypothesis.

To identify putative gene products found exclusively in *D. geminata* at a mat-forming or non-mat-forming site, I compared RNA sequences obtained from periphyton to reference sequences from *P. tricornutum*, *T. pseudonana,* and *C. crescentus*. Genes that were identified as expressed only in the mat-forming sample included those coding for proteins required during phosphorus starvation, which lends support to the low SRP hypothesis for cause of mat formation. As expected, genes that were identified as expressed only in the non-mat-forming sample, with a higher rate of cell division,

included those responsible for silica processing and mitosis. Unexpectedly, beta glucan elicitor receptor protein was expressed in the EDWR sample. Beta glucan elicitor receptors are involved in defence response against bacteria (Davies 1995). Diatoms and bacteria are part of a complex benthic periphyton community and routinely interact in a variety of ways (Amin et al. 2012). Further, bacteria can increase EPS production in diatoms (Bruckner et al. 2011). It is possible that *D. geminata* forms stalks to raise cells into the water column away from the threat of bacteria. This thesis offers new insight on gene expression that could contribute to future research on interactions between bacteria and *D. geminata* that may contribute to stalk formation.

A caveat of the present study is lack of replication, as gene expression was analyzed from one mat-forming and one non-mat-forming site. However, despite the challenges involved in molecular studies on *D. geminata,* this study discovered gene products that may be involved in stalk formation. Future studies would benefit from exploring gene expression from an increased number of mat-forming and non-mat-forming sites to provide more data on FDC, SRP, stalk formation and gene expression. Differential gene expression studies of *D. geminata* in artificial channels, where replication could be performed and phosphorus concentrations could be manipulated, would also be of value.

To compare DNA extraction methods, I extracted DNA from periphyton samples stored in a variety of storage buffer and temperature combinations using a commercial kit method, a modified version of that method and an organic extraction method. As I hypothesized, the modified method produced the highest quantity and quality DNA. All storage buffers and temperatures tested for that method produced enough DNA for

standard template amounts (25-200 ng) used in PCR. Interestingly, DNA from samples stored in RNA*later* using the OE method also yielded relatively high quantities of DNA, likely due to stalk material being precipitated by the RNA*later* in the OE method. The kit methods used a spin column filter to remove stalks. The kit method was not successful likely due to shorter incubation times and one fewer elution step. The results of this study confirm that *D. geminata* DNA extraction can be performed successfully. However, a consistent method remains to be found.

Efforts to curb nuisance *D. geminata* mat formation are currently focused on stopping the spread of cells on anglers' gear. New Zealand and other countries have attempted to stop the spread of cells by banning felt soled waders (Invasive Species Action Network 2014, Fish and Game New Zealand 2016). However, recent research that supports the low SRP hypothesis for mat formation challenges previous views that invasive *D. geminata* is being spread globally on fishing gear. If the low SRP hypothesis for mat formation is continually supported by empirical and molecular research, then management practices should shift resources from felt-soled wader bans toward phosphorus addition programs, which have shown promise (James et al. 2015).

In conclusion, this thesis supports the low SRP hypothesis, yet also suggests that SRP is unlikely to be the only trigger for stalk formation. Stalks were observed at a non-mat-forming site on the Bow River with SRP >0.002 mg/L. Additional accounts of *D. geminata* mats in higher nutrient waters have been documented in Europe (Kawecka and Sanecki 2003, Spaulding and Elwell 2007). The above noted observations, and the lack of data supporting a proposed global SRP decrease in rivers, suggest that the SRP hypothesis for mat formation has yet to be proven conclusively. Further, a mat forming

genetic variant has not been ruled out. Globally, further studies on *D. geminata* are needed in order to test hypotheses for mat formation, including low SRP, a mat-forming genetic variant, and interactions with bacteria. Molecular studies could reveal the cause of mats by identifying genes responsible for stalk formation, genetic differences indicative of a variant, or bacterial species that are consistently present in environmental DNA associated with mat-forming *D. geminata.*

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