THESIS

WATER QUALITY AND SURVIVABILITY OF *DIDYMOSPHENIA GEMINATA*

Submitted by

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ABSTRACT

WATER QUALITY AND SURVIVABILITY OF *DIDYMOSPHENIA GEMINATA*

Didymosphenia geminata or *Didymo* has become a world-wide invasive aquatic species. During blooms, the algae can form thick mats covering entire reaches of stream bottom, which in turn creates negative aesthetic, ecologic, and economic impacts. Although *Didymo* is historically present in the United States, it is spreading quickly into areas that were previously free of it, and is even growing in waters that were thought not ideal habitat for *Didymo*. Previous research on how water quality affects *Didymo* growth and spreading appear to be influenced by streamflow rates and water pH levels. Other water quality parameters have not been fully tested on *Didymo,* which would contribute to a better understanding of what controls *Didymo* growth. The first goal of this study was to colonize *Didymo* in an artificial stream within a laboratory setting. The second goal was to evaluate the survivability of *Didymo* by exposing it to different water quality parameters.

Artificial stream configurations with various light intensity and duration, water temperature and velocity, source water chemistry, and different growth media were used. In all attempts colonization of *Didymo* was unsuccessful as *Didymo* slowly deteriorated and became covered by other algae that were more successful in the artificial conditions.

Didymo survivability as affected by a 60 minute exposure to different water quality parameters followed previously determined results in that known algaecides did affect cell viability, while other non-toxic parameters showed no effect on *Didymo*. Nitrate, nitrite, phosphate, chloride, calcium, and magnesium did not affect *Didymo* survivability. Ammonia also did not affect *Didymo* but signs of cells lysis were observed and possible mortality may occur with longer exposure times.

Copper, zinc, chlorine, and pH affected *Didymo* survivability. Copper showed the greatest affect on *Didymo* survivability with the median lethal concentrations (LC_{50}) for copper at 9.3°C and 13.0°C being 3.3 mg/L and 5.4 mg/L respectively at pH 7.7. For copper toxicity in waters with a lower pH (6.7) the resulting LC_{50} was 33 mg/L. Generally, both colder water temperature and higher pH increased copper toxicity on *Didymo*. The affect of temperature on copper toxicity was shown to be statistically significant (p-value 0.02). However, there was no statistically significant affect of pH on copper toxicity (p-value 0.07). The LC_{50} could also not be determined for all three zinc tests but the highest zinc concentration of 40 mg/L had on average 56% of *Didymo* cells surviving. No apparent trend on the affect of temperature to zinc toxicity on *Didymo* could be determined; however, the interaction of temperature on zinc toxicity was statistically significant (p-value 0.02). Chlorine at temperatures of 11.5°C and 17.3°C had LC_{50}s of 5.67 and 8.46 mg/L respectively. The affect of temperature on chlorine toxicity was statistically significant (p-value <0.001). *Didymo* survivability was affected in water with pH 4.3 but not in water with pH 5.9 and 6.9. Cell lysis was occurring in water with pH 10.7 but no sign of any affect on *Didymo* survivability was found in water with pH 9.9.

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1. INTRODUCTION

1.1 *Didymosphenia geminata*

In 2004, the discovery of *Didymosphenia geminata*, or *Didymo*, in New Zealand, a non-native area, resulted in new studies about this little known diatom (Kilroy 2004; Kilroy *et al.* 2005a; Kilroy *et al.* 2006a;b; Duncan *et al.* 2007; Larned *et al.* 2007a; Sutherland *et al.* 2007). This diatom, or single-celled algae, is usually found in oligotrophic waters where it produces extracellular stalk material which adheres to substrates and merges to form thick mat-like structures along the river bottom (Spaulding and Elwell 2007). It only takes one cell to be transferred into new water for *Didymo* to colonize as the cell divides through vegetative reproduction. As the cells produce stalk material, both the cells and stalks can continue to divide, forming mature colonies (Whitton *et al.* 2009).

Recently scientists have documented a spread in distribution and growth in blooms of *Didymosphenia geminata*, now making it a world-wide nuisance species, causing negative effects aesthetically, ecologically, and economically (Spaulding and Elwell 2007). Major blooms of *Didymo* can cause a decrease in aquatic invertebrates which can lead to a decline in fish populations (Kilroy 2004). *Didymo* has been found to block water intakes and completely engulf water diversion canals, resulting in expensive clean-ups (Kilroy 2004). Although there has been an increase in research on *Didymo*, there has been little study on how water quality affects the survivability of *Didymo*. Survivability is defined here as percentage of *Didymo* cells still viable after a 60 minute exposure to varying concentrations of a single water quality parameter. The importance of studying the effect of water quality on *Didymo* was suggested earlier (Kumar *et al.* 2009). By defining the water quality ranges that *Didymo* can survive in, researchers and managers will have a better idea of what controls the spread of this nuisance species.

Research on *Didymo* began shortly after 2003 when *Didymo* was unknowingly transported to New Zealand, possibly from a visiting angler's felt-soled boots, where it has quickly spread (Kilroy 2004;

Kilroy *et al.* 2005b; Kilroy *et al.* 2006a;b; Duncan *et al.* 2007; Larned *et al.* 2007a;b; Sutherland *et al.* 2007). Some studies looked at water quality data, but because of the wide range of concentrations of various parameters that *Didymo* has been found in, they were unable to determine any one parameter, besides pH, as having an affect on *Didymo* survivability (Kilroy *et al.* 2005b; Kilroy *et al.* 2006c). Other studies tested various algaecides to determine the most toxic to *Didymo* (Jellyman *et al.* 2006). From there the algaecide was tested on a reach of river with mixed results, and the possibility of creating additional short-term and long-term negative impacts on the aquatic environment (Shearer *et al.* 2008). So with no conclusive controls yet on how to rid *Didymo* from the rivers, it is important to continue searching for more water quality parameters that may affect *Didymo's* survivability.

1.2 Water quality parameters

Uncertainty exists on how most water quality parameters affect *Didymo*, but certain parameters have been identified that may affect its survivability.

1.2.a Streamflow

One controlling factor on *Didymo's* spread and growth is streamflow (Spaulding and Elwell 2007). The annual peak flows of mountain rivers may scour the streambed and scour *Didymo* growths. As streamflow increases there is enough stream power to mobilize bed material, essentially scraping the *Didymo* from the river bottom. In the River Tees in the United Kingdom, growths of *Didymo* were much larger when scouring floods did not occur (Whitton and Crisp 1984). On Vancouver Island, Canada there was a higher abundance of *Didymo* when no winter flooding occurred (Rieberger 1991). *Didymo* blooms have been found to occur more frequently in rivers where dams regulate flow (Kawecka and Sanecki 2003; Larson 2007). Other research found that large floods can reduce *Didymo* biomass by inducing bed mobilization and the resulting scour (Spaulding and Elwell 2007).

1.2.b pH, temperature, water clarity, and light

The only water quality parameter known to adversely affect *Didymo* is pH. The pH levels that are preferred by diatoms including *Didymo* is between 6.4 and 9.0, but was found to survive between pH 4.0 and 9.5 (Kilroy *et al.* 2006b).

Besides streamflow and pH, other factors such as temperature, water clarity, and light may affect *Didymo* survivability (Kilroy *et al.* 2005a; Kilroy *et al.* 2006b; Larned *et al.* 2007b). *Didymo* is found in greater abundance in areas with high intensity light and low temperatures (Kawecka and Sanecki 2003), although it has been found in temperatures ranging from 0.1°C to 27°C (Whitton *et al.* 2009). Water clarity may play a role in where *Didymo* grows as it was not found in any peat stained rivers (Noga 2003). In addition it has been found that waters with low turbidity have a greater *Didymo* presence (Kirkwood *et al.* 2007).

1.2.c Nutrients

Although *Didymo* is predominantly found in oligotrophic waters, phosphorus and nitrogen are essential nutrients to *Didymo* growth and higher concentrations of either may increase growth rates of *Didymo* (Spaulding and Elwell 2007). The ability of *Didymo* to hydrolyze organic phosphorus within its stalks allows the cells to utilize the inorganic phosphorus and possibly out-compete other periphyton (Ellwood and Whitton 2007); especially for waters with low total phosphorus concentrations, but that have a high ratio of organic to inorganic phosphorus (Whitton *et al.* 2009). It has also been found that *Didymo* is able to trap phosphorus in the dense mats it forms allowing it to sequester the phosphorus when needed (Sundareshwar *et al.* 2011). This explains why *Didymo's* unique mat structure allows it to thrive in low nutrient rivers while other algae without this ability are limited in their growth.

1.3 Source water chemistry

Differences in water chemistry between three rivers and their spring-fed tributaries in New Zealand was identified as a possible cause to why *Didymo* was killed there (Sutherland *et al.* 2007). No one parameter could be identified as the cause of death, however, the differences in water chemistry showed that the spring fed creeks had higher conductivity, alkalinity, calcium, nitrate, and magnesium concentrations. Calcium is known to be part of the process linking *Didymo* and the substrate they grow on (Geesey *et al.* 1999). Other water quality parameters mentioned to be of interest in the spring-fed creek study were elevated concentrations of nitrite and ammonia as they are toxic to certain algae (Sutherland *et al.* 2007).

1.4 *Didymo* occurrence and measured water quality parameters

In order to gain a better understanding of what water quality parameters may control the occurrence of *Didymo,* a literature review was conducted. Each parameter has been broken down into three classes of concentrations where *Didymo* has been found in abundance (red line), less-frequently or low numbers (yellow line), and absent sites (green line) (Figure 1.1 and Table 1.1). Although no differences in concentration values between the three classes for any one parameter stands out, the charts proved useful in defining the ranges that *Didymo* was found.

Already some water quality parameters have been identified as lethal to *Didymo*. These include copper, zinc sulfate, chlorine, and quaternary ammonium compounds (Kilroy *et al.* 2006b). Although these disinfectants cause *Didymo* mortality they may not be suitable to use as management practices in the stream itself. The use of GEMEX™, a chelated copper based algaecide, has been tested in a New Zealand stream and monitored for its effectiveness at killing *Didymo* (Shearer *et al.* 2008). Results were

Figure 1.1 - Range of concentrations for different water quality parameters that *Didymosphenia geminata* was present. Red bars indicate substantial amounts of *Didymo*, yellow bars show less frequent or lower numbers of *Didymo* present, and green bars specify no presence of *Didymo*.

Figure 1.1 (continued) - Range of concentrations for different water quality parameters that *Didymosphenia geminata* was present. Red bars indicate substantial amounts of *Didymo*, yellow bars show less frequent or lower numbers of *Didymo* present, and green bars specify no presence of *Didymo*.

Table 1.1 –Previous studies cited to form the range of concentrations charts in Figure 1.1 along with which water quality parameters were sampled for within each study.

Reference	Year	Study Site	Parameters Sampled (mg/L)
Bhatt et al.	2007	Indian Himalayan Rivers	D.O., hardness, NO_3, pH, PO_4, temperature
Kara & Sahin	2001	Degirmendere River, Turkey	ph and temperature
Kawecka & Sanecki	2003	Southern Poland	B.O.D.$_5$, Ca, Cl_2, C.O.D., conductivity, D.O., K, Mg, Na, NH_4, NO_3, pH, PO_4, SO_4, temperature
Kawecka & Sanecki	2003	River San, Poland	alkalinity, B.O.D.$_5$, Ca, Cl_2, C.O.D., conductivity, D.O., K, Mg, Na, NH_4, NO_3, pH, PO_4, SO_4, temperature
Kilroy et al.	2006	General Statement	pH
Kirkwood et al.	2007	Bow and Red Deer Rivers, Alberta, Canada	conductivity, pH, P, temperature
Larson	2007	Rapid Creek, South Dakota, USA	alkalinity, bicarbonate, Ca, carbonate, Cl_2, conductivity, D.O., hardness, K, Mg, Na, NH_4, NO_2, NO_3, pH, P, SO_4, Si, T.D.S., temperature
Noga	2003	Czarna Orawa River, Poland	B.O.D.$_5$, Ca, Cl_2, C.O.D., conductivity, D.O., Mg, NH_4, NO_2, NO_3, pH, PO_4, SO_4, temperature
Shelby	2006	White River, Arkansas, USA	alkalinity, aluminum, arsenic, B.O.D.$_5$, carbonate, Cl_2, copper, D.O., hardness, K, Na, NH_4, pH, PO_4, SO_4, T.D.S., temperature, T.S.S.
Sherbot & Bothwell	1993	Vancouver Island, Canada	NO_3, pH
Spaulding & Elwell	2007	General Statement	NO_3, pH, P, temperature
Sutherland et al.	2007	New Zealand	alkalinity, Ca, Cl_2, conductivity, K, Na, NO_3, pH, SO_4, temperature

mixed as fish and macro-invertebrates showed no sign of long-term effect with a single dose of GEMEX™ however, with *Didymo* not being fully eliminated from the stream, multiple doses may be needed and this could have adverse effects on other aquatic organisms. For example using multiple doses over an extended period could create a build-up of copper in the stream sediment, which many invertebrates process as a food source, leading to possible bio-accumulation in the food chain (Demirak *et al.* 2006).

So although copper and perhaps some other parameters have been identified as toxic to *Didymo*, the practicality of using them is hampered by both short-term and long-term negative effects to the environment. Therefore, the search must continue for other water quality parameters that are toxic to *Didymo* but that do not adversely affect the aquatic environment.

1.5 Hypothesis and study objectives

Although parameters such as streamflow and pH can affect *Didymo*, there is little knowledge on *Didymo's* survivability with exposure to other water quality parameters. Based on a literature review water quality parameters were identified as having a possible affect on *Didymo*. Therefore the hypothesis was ammonia, calcium, chloride, chlorine, copper, magnesium, nitrate, nitrite, phosphate, and zinc concentrations would affect *Didymo* survivability.

Specific objectives were:

1) To colonize *Didymo* in an artificial stream in a laboratory setting to determine how temperature, light, and nutrient regimes affect *Didymo* survivability.

2) To expose *Didymo* to the aforementioned water quality parameters to assess their affect on its survivability.

2. METHODS

2.1 Colonizing *Didymo* in a laboratory using an artificial stream setting

An artificial stream was built at the Colorado Division of Wildlife, Aquatic Toxicology Laboratory in Fort Collins, Colorado. A temperature controlled water bath was set up with troughs and a water pump with vinyl tubing for water circulation and flow. Troughs were made by cutting five inch diameter vinyl plastic fence posts in half. Caps for the fence posts were cut in half and glued to the end of the troughs with vinyl glue and then sealed with non-toxic aquarium silicon. The vinyl glue was not used inside of the trough to avoid any possible glue toxicity. Substrate materials, some colonized by *Didymo*, were collected from the Cache La Poudre River and placed in the troughs staggered to test varying flows and depths.

Initially, dechlorinated Fort Collins municipal tap water was used in the troughs but it was decided to instead collect water from the Cache La Poudre River at the *Didymo* sampling site and use this as source water for the troughs. F/2 medium™, was added to the water to provide essential nutrients needed to colonize algae (Brinkman, Personal Communication, 2009). Silicate was also added since it is necessary for diatoms as it makes up their cell walls (Spaulding and Elwell 2007). To replicate sunlight 48 inch long T12 aquatic plant grow lights were placed approximately 18 inches above the troughs. Various temperature, light, and flow regimes were used to try and find the ideal setup for *Didymo* to grow (Table 2.1). Varying concentrations of F/2 medium and silicate were also used.

2.1a Shallow troughs

The initial two trough setups were 2.5 inches deep with a Taam Rio+ 800™ aquarium pump with a flow capacity of 211 gal/hr making average velocities in the trough approximately 0.14 ft/s. Both

Table 2.1 – Matrix of different experimental trough configurations for attempting to colonize *Didymo* in an artificial stream setting within a laboratory.

	Trough depth	Substrate	Water Pump Flow Capacity	Temperature (°C)	Lighting (48" bulbs)	Light Exposure Time	F/2 Medium™	Sodium Silicate nonahydrate
Setup #1	**2.5 in**	**rocks, terra cotta, and tiles**	211 gal/hr	21	2 - General Electric Plant and Aquarium™ T12 40 watt	12 hrs on 12 hrs off	132 µL/L Part A 132 µL/L Part B	30 mg/L
	2.5 in	**rocks, terra cotta, and tiles**	211 gal/hr	10	2 - General Electric Plant and Aquarium™ T12 40 watt	12 hrs on 12 hrs off	132 µL/L Part A 132 µL/L Part B	30 mg/L
Setup #2	**2.5 in**	**rocks, terra cotta, and tiles**	211 gal/hr	12	2 - General Electric Plant and Aquarium™ T12 40 watt	12 hrs on 12 hrs off	132 µL/L Part A 132 µL/L Part B	30 mg/L
	2.5 in	**rocks, terra cotta, and tiles**	211 gal/hr	12	2 - Coralife Nutri-Grow™ T12 40 watt	12 hrs on 12 hrs off	132 µL/L Part A 132 µL/L Part B	30 mg/L
Setup #3	**2.5 in**	**rocks and tiles**	422 gal/hr	12	1 - General Electric Plant and Aquarium™ T12 40 watt 1 - Coralife Nutri-Grow™ T12 40 watt	12 hrs on 12 hrs off	132 µL/L Part A 132 µL/L Part B	30 mg/L
Setup #4	**2.5 in**	**rocks and tiles**	422 gal/hr	12	1 - General Electric Plant and Aquarium™ T12 40 watt 1 - Coralife Nutri-Grow™ T12 40 watt	16 hrs on 8 hrs off	132 µL/L Part A 132 µL/L Part B	30 mg/L
Setup #5	**2.5 in**	**rocks and tiles**	422 gal/hr	8	1 - General Electric Plant and Aquarium™ T12 40 watt 1 - Coralife Nutri-Grow™ T12 40 watt	16 hrs on 8 hrs off	132 µL/L Part A 132 µL/L Part B	30 mg/L
Setup #6	**2.5 in**	**tiles colonized with didymo**	422 gal/hr	8	1 - General Electric Plant and Aquarium™ T12 40 watt 1 - Coralife Nutri-Grow™ T12 40 watt	12 hrs on 12 hrs off	80 µL/L Part A 80 µL/L Part B	30 mg/L
Setup #7	**4.75 in**	**rocks and tiles**	980 gal/hr	8	1 - General Electric Plant and Aquarium™ T12 40 watt 1 - Coralife Nutri-Grow™ T12 40 watt	12 hrs on 12 hrs off	132 µL/L Part A 132 µL/L Part B	30 mg/L
Setup #8	**4.75 in**	**rocks and tiles**	980 gal/hr	8	1 - General Electric Plant and Aquarium™ T12 40 watt 1 - Coralife Nutri-Grow™ T12 40 watt	12 hrs on 12 hrs off	264 µL/L Part A 264 µL/L Part B	30 mg/L
Setup #7	**4.75 in**	**rocks and tiles**	980 gal/hr	5	1 - General Electric Plant and Aquarium™ T12 40 watt 1 - Coralife Nutri-Grow™ T12 40 watt	12 hrs on 12 hrs off	132 µL/L Part A 132 µL/L Part B	30 mg/L

troughs were filled with river water, substrate from the river with and without *Didymo*, broken pieces of terra cota, and biomedical tiles. Terra cota was used to for its roughness and the same biomedical tiles were previously used to successfully to colonize *Chladophora* (Brinkman, Personal communication, 2009). Eventually, only substrate from the river and the tiles were used as the terra cota was deemed redundant.

Lighting was provided by four General Electric Plant and Aquarium™ T12 40 watt bulbs, which radiated 1900 lumens and had a color temperature of 3100K, that were used in fixtures placed a foot above the water surface. As the testing continued, two Coralife Nutri-Grow™ T12 40 watt bulbs, which had a color temperature of 6500K, were used to determine if bulb type was affecting *Didymo* growth. The lights were initially set on a 16 hour on-off cycle but were then reduced to a 12 hour cycle after bubbles appeared in the *Didymo* mats which could be attributed to high photosynthetic processes (Stevenson *et al.* 1996).

The standard recipe for the media added to the troughs was 30mg/L of sodium silicate nonahydrate, 132µL/L of F/2 medium Part A, and 132µL/L of F/2 medium Part B mixed in river water (Brinkman, Personal Communication, 2009). The media was then placed in 2L bottles, which were held upright at the end of the troughs with the cap removed and a hole cut in the neck, to maintain the water level in the trough while adding the media slowly over time.

2.1.b Deep trough

In order to attain higher water velocities a five inch deep trough was made by cutting just the top side of the vinyl plastic fence post. A Rainbow Lifegard Quiet One™ 4000 HH aquarium pump with flow capacity of 980 gal/hr was used to increase average velocities in the trough to approximately 0.29 ft/s. All other parameters were the same as the shallow troughs.

2.1.c Brick applications

A final configuration was designed to increase the water velocities within one of the shallow troughs while still using the same water pump. Vinyl plastic sheets were cut and screwed onto the top of four concrete bricks. Tiles were glued onto the vinyl sheets using Loctite Aqua Marine Epoxy™. The bricks were placed in the Cache La Poudre River at the sampling site, where *Didymo* was abundant, to allow for the colonization. After 12 days, two of the bricks were removed with a slight cover in *Didymo*. The bricks were taken back to the lab in a cooler filled with river water, where the vinyl sheets were unscrewed from the bricks and placed into the trough. More tiles were placed along the bottom of the trough to allow for colonization. All other parameters were the same as the shallow trough setup.

2.2 Water quality and its effect on *Didymo* survivability

To determine how different water quality parameters affect *Didymo*, samples were collected from the main stem of the Cache La Poudre River in Colorado (Figure 2.1). The sites are areas that under lower streamflows have an abundance of *Didymo*. Methods of collecting and analyzing *Didymo* followed previously established protocol (Kilroy *et al.* 2006b). Rocks with healthy colonies of *Didymo* were collected in one liter containers with river water and transported in a cooler to the lab. Average time from the end of collection to the first rock placement in the exposure chambers was approximately 30 minutes.

Test water was dechlorinated Fort Collins Municipal water which is maintained by a conductivity controller to keep hardness near 45 mg/L as $CaCO_3$, which is approximately the same as found in the Cache La Poudre River (Brinkman and Johnston 2008). A continuous flow serial diluter was used to deliver a control and five different concentrations with a 50% dilution ratio (Benoit *et al.* 1982). Sample rocks with *Didymo* were placed in their exposure chambers (Figure 2.2) which are equipped with an air

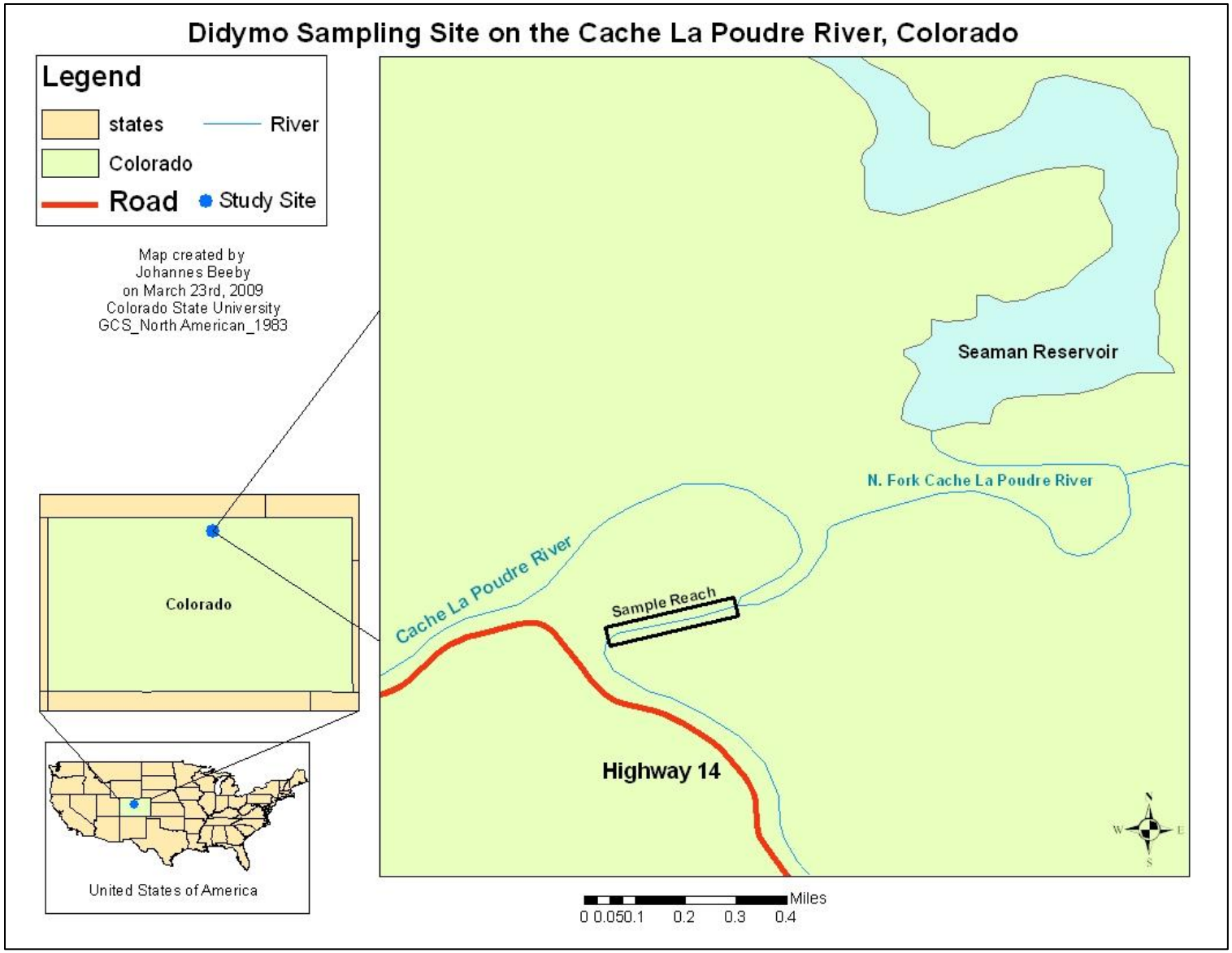

Figure 2.1 - Locational map of *Didymo* sampling sites on the Cache La Poudre River in Colorado.

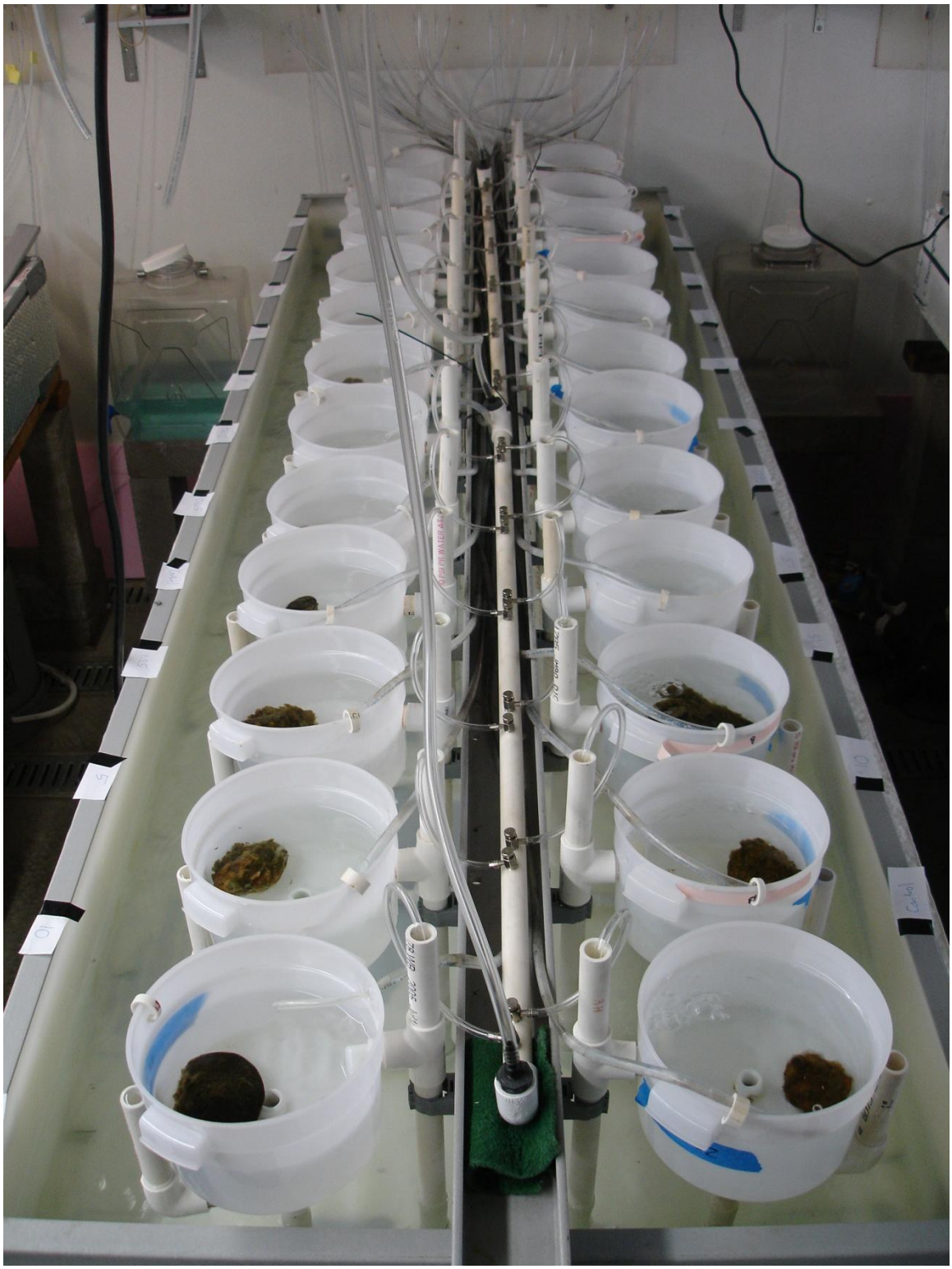

Figure 2.2 – Exposure chamber setup for testing different water quality parameters on *Didymo* at the Colorado Division of Wildlife Aquatic Toxicology Laboratory, Fort Collins, CO.

lift system to maintain flowing water to copy natural river flow. Four treatment blocks were used to create four replicates of each treatment and control. Individual rocks were selected and placed every 10 minutes into their varying treatments for a total of 60 minutes in the exposure chamber. Rock selection and treatment number were done randomly. The exposure time of 60 minutes was chosen to best replicate the use of a spike concentration by managers in the field to control algae growth.

After 60 minutes had passed for each rock, a tuft of healthy white *Didymo* from the center of the colony was taken and placed into 20mL of 5% solution of Neutral Red stain, a biological assay which can only be taken up by live cells, and for which the procedure has been proven for its use with determining *Didymo* cell viability (Kilroy *et al.* 2006b; Clearwater *et al.* 2007). The samples were then shaken vigorously by hand to ensure stain penetration, and then left to stand for 10 minutes. A microscope set at 200X magnification was used to count viability of the first 100 *Didymo* cells viewed (Kilroy *et al.* 2006b). The number of live cells in the control was counted both at the start of testing and again after the cell viabilities for each concentration within the test block were counted to ensure time since removal from the river did not affect the results. For the various parameters that did affect *Didymo* cell viability follow up tests at different temperatures were performed to determine if temperature also had an effect on the parameters ability to kill *Didymo*. A two-way ANOVA with replication was run to determine if there was any statistical significance at a 95^{th} confidence level between the results from the different temperature tests. Data were square root transformed before analysis to make them normally distributed with equal variances.

The data were also tested using the Trimmed Spearman-Karber Method, Version 1.5, which calculated the median lethal concentration (LC_{50}) (EPA 2006). In certain circumstances the LC_{50} could not be calculated because more than 50% of the data had to be trimmed which does not allow for a resulting LC_{50} to be determined. The minimum required trim value is calculated and then cut from the

upper and lower extremes of the data in order to determine the resulting LC_{50} in tests that do not have both a 0% and 100% mortality result.

For each parameter that showed an effect on *Didymo*, four replicates were tested for statistical significance. Three replicates were tested for parameters that showed no affect on *Didymo* cell. Water samples were also taken at the end of testing to confirm water quality concentrations used of each parameter. Regression analysis was performed in Microsoft Excel™ to test for any significant trends of cell viability for each water quality parameter.

2.2.a pH

A pH stock solution of 37% hydrochloric acid was used to make test solutions of pH 4.3, 5.9, 6.9, and 7.4 (Table 2.2). An Oakton™ pHTestr 2 was used to measure pH levels in the exposure chambers. The average water temperature was 11.1°C. Testing was conducted on April 14th, 2010.

Sodium hydroxide was used to create an alkaline pH stock solution and the resulting exposure chamber pH levels were 7.7, 9.9, and 10.7. An Oakton™ pHTestr 2 was used to measure pH levels in the exposure chambers. The average water temperature was 11.3°C. Testing was conducted on April 16th, 2010.

2.2.b Copper

Four separate tests, each at a different temperature or concentration range, were run using a stock solution of copper sulfate on *Didymo*. The initial test had nominal concentrations of 0.63, 1.3, 2.5, 5.0, and 10. mg/L plus a control. The range of concentrations used was chosen based on results from previous copper toxicity studies on *Didymo* which found 5 mg/L killed 94% of *Didymo* cells after a 60 minute exposure (Jellyman *et al.* 2006).

Table 2.2 – Test matrix of water quality parameters tested along with the nominal concentrations ranges and temperatures of which the tests were conducted. All tests were run for 60 minutes. A calcium chloride stock solution was used to determine the effect of calcium and chloride on *Didymo* survivability.

	Low pH	**High pH**	**Copper**	**Zinc**	**Chlorine**	**Ammonia**	**Nitrate**	**Nitrite**	**Phosphate**	**Chloride**	**Calcium**	**Magnesium**
Chemical compound used	Hydrochloric Acid	Sodium Hydroxide	Copper Sulfate	Zinc Sulfate	Sodium Hypochlorite	Ammonium Sulfate	Calcium Nitrate	Sodium Nitrite	Potassium Phosphate	Calcium Chloride		Magnesium Chloride
	HCl	HCl	$CuSO_4$	$ZnSO_4$	ClNaO	NH_4SO_4	$Ca(NO_3)_2*4H_2O$	$NaNO_2$	KH_2PO_4	$CaCl_2*2H_2O$		$MgCl_2*6H_2O$
Nominal concentrations												
Treatment #1 (mg/L)	4.3	10.7	40	40	10	25	10	10	2.5	178	100	10
Treatment #2 (mg/L)	5.9	9.9	20	20	5.0	13	5.0	5.0	1.3	89	50	5.0
Treatment #3 (mg/L)	6.9	8.9	10	10	2.5	6.3	2.5	2.5	0.75	45	25	2.5
Treatment #4 (mg/L)	7.3	7.7	5.0	5.0	1.3	3.1	1.3	1.3	0.38	31	13	1.3
Treatment #5 (mg/L)	7.4		2.5	2.5	0.63	1.6	0.63	0.63	0.19	17	6.3	0.63
Average Temperature (°C)	11.1	11.3	9.3 13.0 16.3	9.6 14.1 15.7	9.9	9.4	9.5	10.4	9.9	11.5 17.3	9.9	10

The final tests were conducted at three different temperatures (9.3°C, 13.0°C, and 16.3°C) to determine temperature effect on copper toxicity to *Didymo*. All three tests had nominal concentrations of copper in the exposure chambers at 2.5, 5.0, 10., 20., and 40.mg/L plus a control. The increase in copper concentrations from the previous tests was done in attempt to achieve 100% mortality of *Didymo*.

All water samples taken for copper concentrations were filtered using a 0.45 micron filter and immediately acidified with Ultrex™ nitric acid to a pH less than 2.0. The samples were analyzed using the atomic absorption method by the Colorado Division of Wildlife Aquatic Toxicology Laboratory. These tests were conducted on January 27th, March 28th, and April 5th, 2010.

2.2.c Copper at different pH levels

In order to test copper toxicity at low pH levels a water tank fed with dechlorinated Fort Collins Municipal water was held at a pH 6.2 by using a pH controller which added a stock solution of hydrochloric acid to the tank. This was then used as the source water for the diluter system (Table 2.3). Testing occurred on April 21st, 2010. For the copper toxicity at high pH levels the same methodology was used but a stock solution of sodium hydroxide was added to the water tank to keep the pH at 9.7 (Table 2.3). Testing occurred on April 17th, 2010. Alkalinity measurements were taken for comparative purposes.

2.2.c Zinc

Zinc toxicity tests were conducted at three different temperatures (9.6°C, 14.1°C, and 15.7°C). A stock solution of zinc sulfate was prepared and nominal zinc concentrations of 2.5, 5.0, 10., 20., and 40.mg/L were prepared. The range of concentrations chosen were the same as used for the later copper tests in order to determine if one metal was more toxic to *Didymo* than the other. Collected water

Table 2.3 – Average water quality characteristics for toxicity tests of copper at varying pH values on *Didymo*.

Low pH Test	Copper concentration (mg/L)	pH	Alkalinity (mg/L)	Temperature (°C)
Treatment #1	40	6.0	5.7	11.0
Treatment #2	20	6.5	5.7	11.0
Treatment #3	10	6.7	5.1	11.2
Treatment #4	5.0	6.7	5.3	11.1
Treatment #5	2.5	6.7	4.9	11.1
Control	0.0	6.7	7.2	11.2
High pH Test	**Copper concentration (mg/L)**	**pH**	**Alkalinity (mg/L)**	**Temperature (°C)**
Treatment #1	40	6.7	82.2	11.5
Treatment #2	20	8.2	92.7	11.4
Treatment #3	10	8.6	92.1	11.6
Treatment #4	5.0	9.5	90.7	11.4
Treatment #5	2.5	9.7	90.0	11.2
Control	0.0	10.1	87.8	11.4

Samples were immediately acidified to less than pH 2.0 with Ultrex™ nitric acid. Samples were analyzed using the atomic absorption method by the Colorado Division of Wildlife Aquatic Toxicology Laboratory. These tests occurred on April 2- 4, 2010.

2.2.d Chlorine

A sodium hypochlorite stock solution was used to make concentrations of chlorine were 0.63, 1.3, 2.5, 5.0, and 10. mg/L. The range of concentrations chosen were the same as used for the initial copper tests in order to determine which was more toxic to *Didymo*. Two tests were done at different temperatures to determine if temperature had an effect on chlorine toxicity to *Didymo*. Water samples were analyzed immediately using N, N-diethyl-p-phenylenediamine sulfate (DPD method), with a Hach™

2100 Spectrophotometer (American Public Health Association. *et al.* 1985). The average water temperatures were 11.5°C and 17.3°C. The tests were conducted on April 17th and 18th, 2010.

2.2.e Nitrogen species

A stock solution of ammonium sulfate was used to prepare nominal concentrations of ammonia of 1.6, 3.2, 6.3, 12, and 25 mg/L. The range of concentrations used was high enough to affect *Didymo* cell viability based on previous studies at the CDOW on the chronic toxicity of ammonia on juvenile rainbow trout (Brinkman, Personal Communication, 2009). Average water temperature was 9.9°C and the average pH less than 7.5. After testing water samples were immediately acidified to a pH below 2.0 by adding sulfuric acid. Testing occurred on March 12th, 2010.

A stock solution of calcium nitrate was used to make nominal concentrations of nitrate of 0.63, 1.3, 2.5, 5.0, and 10. mg/L. The range of concentrations used were high enough to affect *Didymo* cell viability based on the average nitrate concentration of 1.5 mg/L in the spring-fed tributaries where *Didymo* did not survive (Sutherland *et al.* 2007). Water samples were kept at 4°C and analyzed within 24 hours using the cadmium reduction method with a Hach™ 2100 Spectrophotometer (American Public Health Association. *et al.* 1985). The average water temperature was 9.4°C. The test was conducted on February 28th, 2010.

A stock solution of sodium nitrite was used to make test solutions of nitrite of 0.63, 1.3, 2.5, 5.0, and 10. mg/L. The range of concentrations used were high enough to affect *Didymo* cell viability based on the highest concentration of nitrite in the literature where *Didymo* was not found to be growing was 0.36 mg/L (Noga 2003). Water samples were kept at 4°C and analyzed within 24 hours using the ferrous sulfate method with a Hach 2100 Spectrophotometer (American Public Health Association. *et al.* 1985). The average water temperature was 9.5°C. Testing occurred on March 31st, 2010.

2.2.f Phosphate

Potassium phosphate stock solution was used to make test solutions of phosphate of 0.16, 0.31, 0.63, 1.3, and 2.5 mg/L. The range of concentrations used was high enough to affect *Didymo* cell viability based on the highest concentration of phosphate in the literature where *Didymo* was not found to be growing was 0.10 mg/L (Kawecka and Sanecki 2003). Water samples were kept at 4°C and analyzed within 24 hours using the PhosVer 3 (ascorbic acid) method with a Hach™ 2100 Spectrophotometer (American Public Health Association. *et al.* 1985). The average water temperature was 10.4°C. The test was conducted on March 7th, 2010.

2.2.g Chloride

A stock solution of calcium chloride was made to have nominal concentrations of 11, 22, 45, 89, and 180 mg/L. The range of concentrations used was high enough to affect *Didymo* cell viability if it was indeed toxic based on the highest concentration of chloride in the literature where *Didymo* was not found to be growing was 32 mg/L (Kawecka and Sanecki 2003). Water samples were kept at 4°C and analyzed within 24 hours using the mercuric thiocyanate method with a Hach™ 2100 Spectrophotometer (American Public Health Association. *et al.* 1985). The average water temperature was 9.9°C. Testing occurred on February 17th, 2010.

2.2.h Calcium

A stock solution of calcium chloride was used to make concentrations of 6.3, 12, 25, 50., and 100 mg/L. The range of concentrations used was high enough to affect *Didymo* cell viability if it was indeed toxic based on the highest concentration of calcium in the literature where *Didymo* was not found to be growing was 85 mg/L (Kawecka and Sanecki 2003). Water samples were immediately reduced to a pH <2 by adding sulfuric acid and analyzed at the Colorado Division of Wildlife Aquatic Toxicology Laboratory

using the atomic absorption method. The average water temperature was 9.9°C. Testing occurred on February 17th, 2010.

2.2.i Magnesium

A stock solution of magnesium chloride was used to make concentrations of 0.63, 1.3, 2.5, 5.0, and 10. mg/L. The range of concentrations used was high enough to affect *Didymo* cell viability if it was indeed toxic based on the highest concentration of calcium in the spring-fed tributary study in New Zealand was approximately 8 mg/L. Water samples were immediately reduced to a pH <2 by adding sulfuric acid and analyzed at the Colorado Division of Wildlife Aquatic Toxicology Laboratory using the atomic absorption method. The average water temperature was 10.0°C. The test was conducted on February 26th, 2010.

3. RESULTS

3.1 Can *Didymo* be colonized in a laboratory using an artificial stream setting

Substrate covered with *Didymo* was placed into troughs where water pumps were creating flow velocities, within the range that *Didymo* is found in rivers, in order to allow for substrate free of *Didymo* to become colonized. Lighting and essential nutrients were provided at levels that would promote algal growth based on previous studies and best professional judgment.

The colonization of *Didymo* in an artificial stream was unsuccessful. Numerous variations of light, water velocity, growth medium, water source, and temperature were combined. However, in all various configurations *Didymo* was unable to survive and soon became covered by other algae growth in the troughs. Therefore, the diluter system methodology was used instead to assess how different water quality parameters affected *Didymo*.

3.2 Water quality parameters and their affect on *Didymo* survivability

Overall, only a few of the parameters tested proved to affect *Didymo* cell viability. All of the parameters that did affect *Didymo* were previously recognized as algaecides. Best fit line regressions were conducted on each parameter along with the resulting standard error and p-value.

3.2.a pH

Only the lowest pH level of 4.3 showed any sign of having an effect on *Didymo*. The mean number of live cells at a pH 4.3 was 89% compared to 99% for the higher pH levels of 5.9, 6.9, and 7.4. A logarithmic function best fit the data and the standard error for the regression was 0.011 with a p value of <0.001 (Figure 3.1).

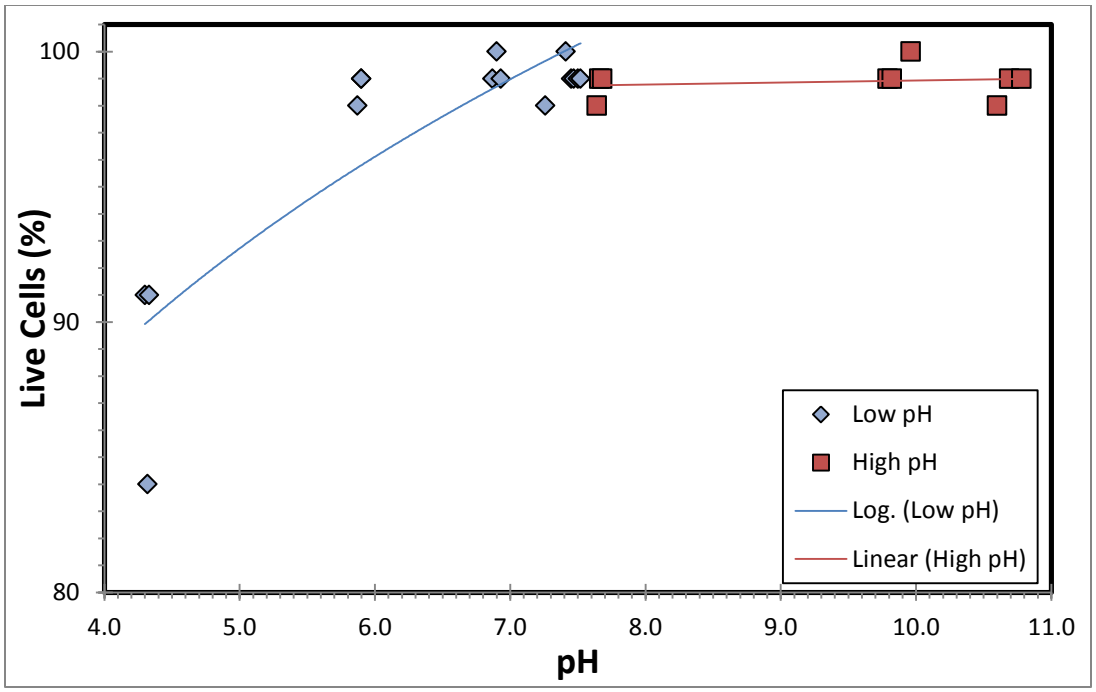

Figure 3.1 - *Didymo* survivability after a 60 minute exposure to differing low pH values, by using hydrochloric acid, and differing high pH values by using sodium hydroxide. Average water temperatures were 11.1°C and 11.3°C respectively. The y-axis is exaggerated to show 80 to 100% of live cells.

No significant decrease in *Didymo* survivability occurred at any of the high pH levels. All pH levels had at least 99% of cells remain alive after a 60 minute exposure. However, cell lysis appeared at pH 10.7, but since the cells still took up the Neutral Red Stain they were considered alive. A linear function best fit the data and the standard error for the regression was 0.63 with a p value of 0.66 (Figure 3.1).

3.2.b Copper

Didymo had decreased cell viability with increased copper concentrations. Temperature influenced copper toxicity to *Didymo* with colder temperatures having higher toxicity. A logarithmic

function best fit the different temperature test data all with p values <0.001 (Figure 3.2). Results from ANOVA analysis showed that there was a statistically significant difference between the interactions of the temperature tests with a p-value of 0.02.

3.2.c Influence of pH on copper toxicity

As copper concentrations increased *Didymo* cell viability decreased at all pH levels (Figures 3.3a and b). Copper toxicity was greatest for all concentrations in the control pH 7.7 conditions compared to the pH 6.2 and pH 9.7 tests. Copper toxicity was higher in the pH 9.7 conditions than at pH 6.2 for all copper concentrations except for in the 40 mg/L test. At this concentration the pH 6.2 had on average 36% live cells remaining and the pH 9.7 had 54% of cells still alive. Measured concentrations tested were on average within 68% of the nominal concentrations and differences are discussed later. Results from ANOVA analysis showed that there was no statistically significant difference between the interactions of the pH tests with a p-value of 0.07.

3.2.d Zinc

Increased zinc concentrations decreased *Didymo* cell viability (Figure 3.4). Measured concentrations tested were on average within 6.7% of the nominal concentrations (Table 3.4). Temperature's affect on zinc toxicity to *Didymo* was ambiguous and will be discussed in the next chapter. Linear functions best fit the data and the standards errors starting with 9.6°C up to 15.7°C were 14, 6.9, and 7.6 all with p values <0.001. Results from ANOVA analysis showed that there was a statistically significant difference between the interactions of the temperature tests with a p-value of 0.01.

Table 3.1 – Nominal and measured concentrations of copper sulfate for the different temperature tests. Temperature and average pH are also shown. Alkalinity values were approximately 36 from water quality tests conducted just prior to the tests on *Didymo*. Large discrepancies in nominal vs. measured concentrations are reviewed in the Discussion section.

Date	Temperature (°C)	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	pH	Live Cells (%)
4/5/2010	16.3	40	27		33
		20	13		39
		10	6.9		21
		5.0	3.5		43
		2.5	1.7		48
		Control	0.02		97
1/27/2010	13.0	40	7.4	6.2	24
		20	3.7	6.6	30
		10	3.0	7.0	26
		5.0	1.4	7.3	50
		2.5	0.74	7.2	47
		Control	0.02	7.7	93
3/28/2010	9.3	40	6.4		4
		20	3.2		23
		10	1.6		26
		5.0	0.82		23
		2.5	0.44		62
		Control	0.00		94

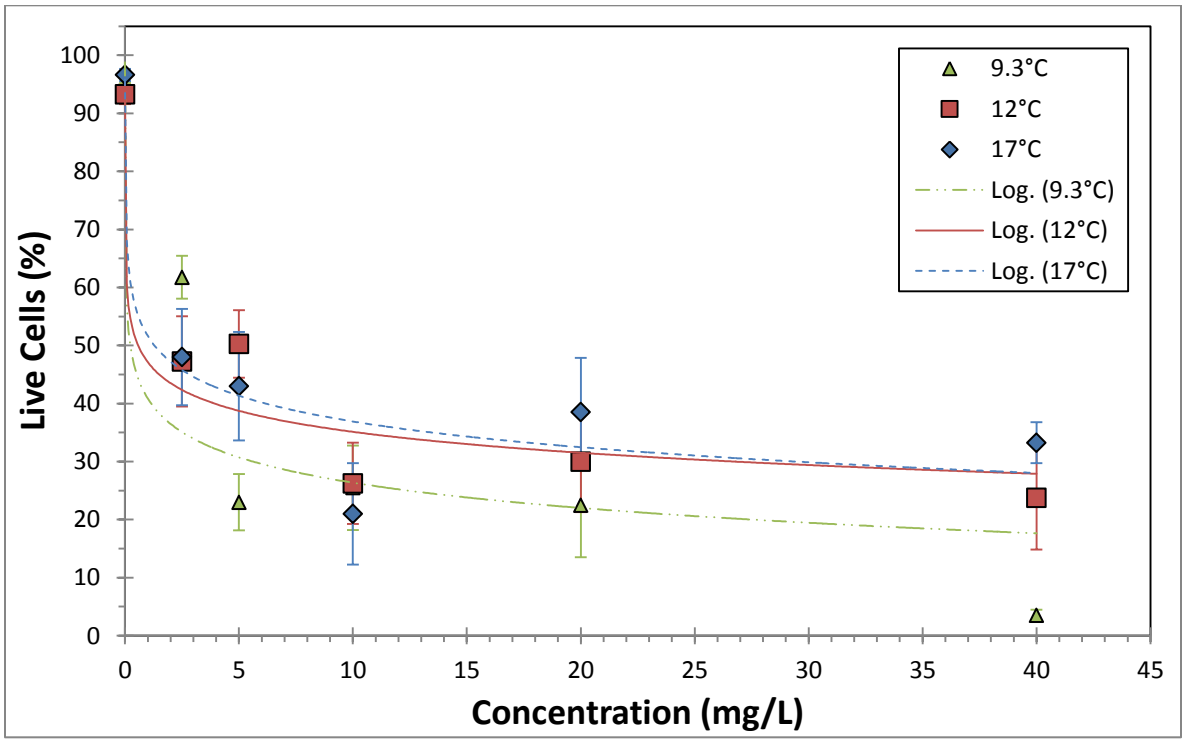

Figure 3.2 - *Didymo* survivability after a 60 minute exposure to 2.5, 5.0, 10., 20., 40. mg/L of copper at three different temperatures. Error bars represent the standard error for the percent of live cells at each concentration.

Table 3.2 – Results from copper toxicity tests at different temperatures on *Didymo*. Numbers of live cells for each concentration are presented.

Results	Percent Live Cells		
Concentration	9.3°C	13.0°C	16.3°C
Control	94	93	97
2.5	62	47	48
5.0	23	50	43
10	26	26	21
20	23	30	39
40	4	24	33

Table 3.3 - Results from copper toxicity tests in low and high pH source water on *Didymo*. Number of live cells for each concentration are presented.

Results	Number of Live Cells		
Concentration	pH 6.2	pH 7.7	pH 9.7
Control	99	93	98
2.5	98	47	80
5.0	98	50	88
10	95	26	79
20	85	30	74
40	36	24	54

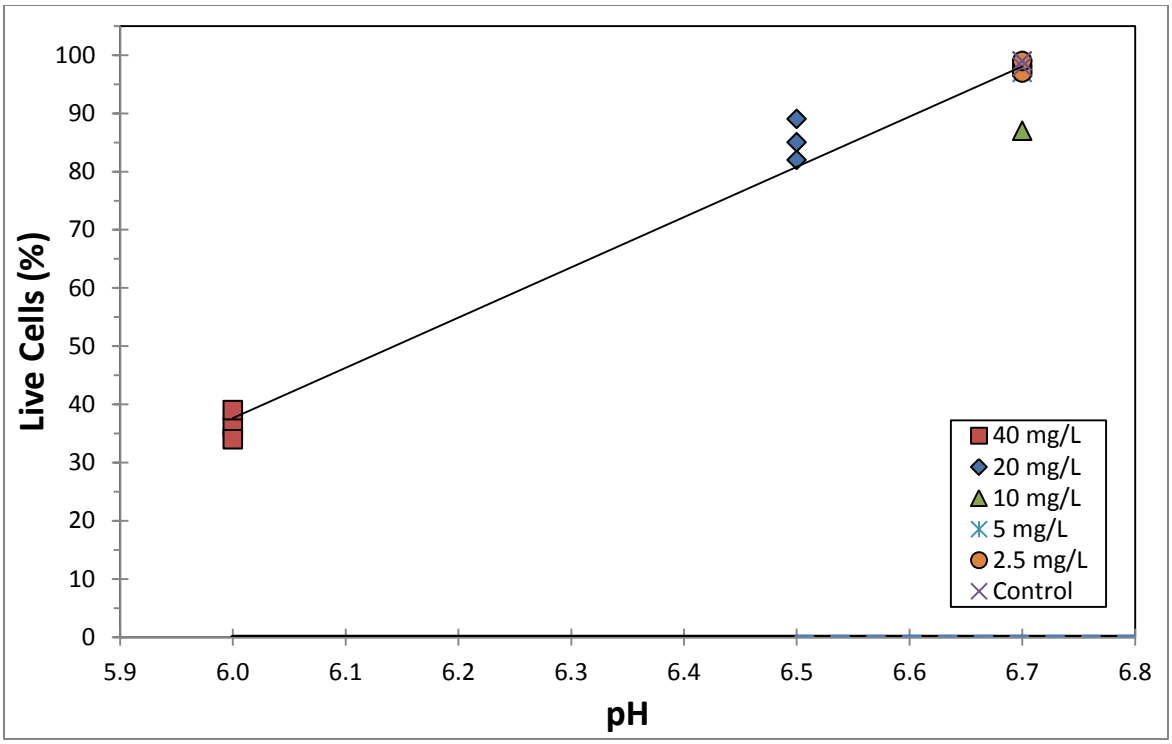

Figure 3.3a - *Didymo* survivability after a 60 minute exposure to 2.5, 5.0, 10., 20., 40. mg/L of copper in low pH source water.

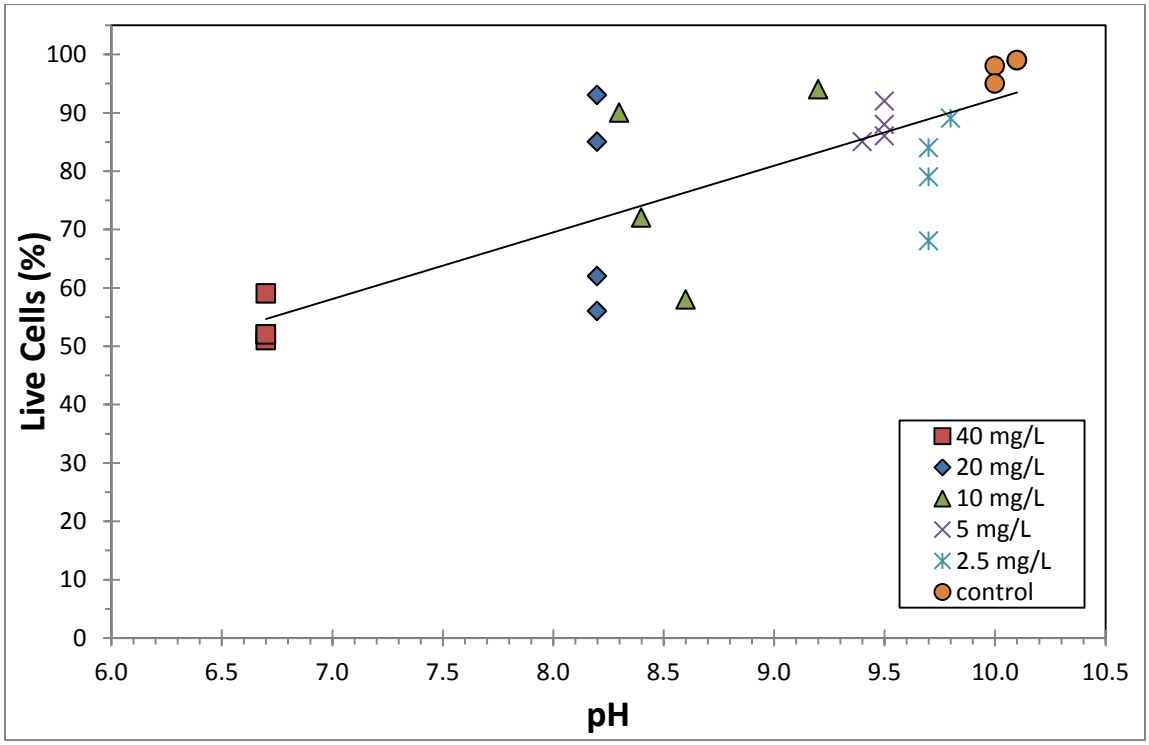

Figure 3.3b - *Didymo* survivability after a 60 minute exposure to 2.5, 5.0, 10., 20., 40. mg/L of copper in high pH source water.

Table 3.4 – Nominal and measured concentrations of Zinc.

Date	Temperature (°C)	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	Live Cells (%)
4/2/2010	9.6	40	40	67
		20	21	53
		10	11	82
		5.0	5.5	91
		2.5	2.9	87
		Control	0.00	95
4/3/2010	14.1	40	40	58
		20	19	79
		10	9.3	94
		5.0	4.4	95
		2.5	2.1	97
		Control	0.00	98
4/4/2010	15.7	40	42	42
		20	20	65
		10	10	93
		5.0	4.7	92
		2.5	2.3	91
		Control	0.00	96

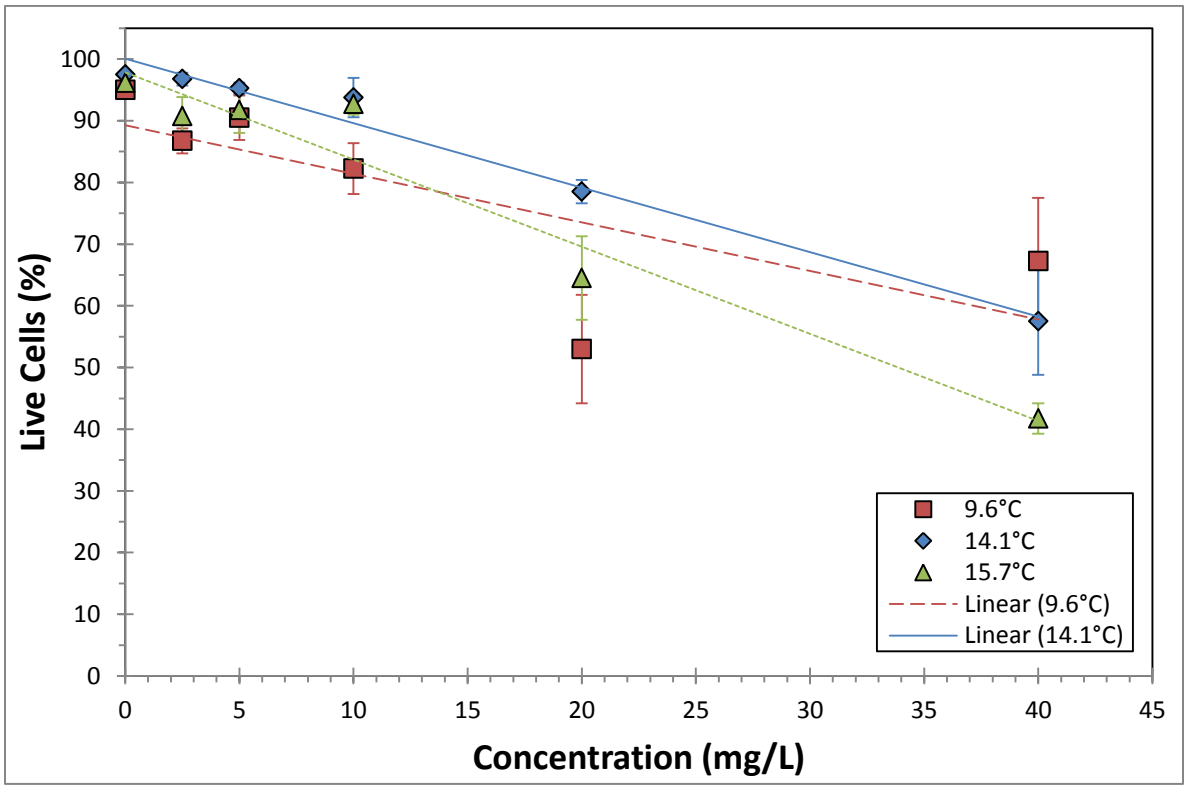

Figure 3.4 - *Didymo* survivability after a 60 minute exposure to 2.5, 5.0, 10., 20., 40. mg/L of zinc at three different temperatures. Error bars represent the standard error for the percent of live cells at each concentration.

3.2.e Chlorine

Chlorine concentrations affected *Didymo* viability. Temperature had an effect on the toxicity of chlorine on *Didymo*, as colder water temperature increased the toxicity of chlorine on *Didymo*. Actual chlorine concentrations were on average 85% of nominal concentrations (Table 3.5). Although large, the discrepancy between actual and nominal concentrations occurred in both tests, and will be discussed further in the next chapter. Only two temperature tests were conducted as peak flows from snowmelt runoff did not allow for *Didymo* sample collection. Linear functions best fit the data and the standard errors for the 11.5°C and 17.3°C tests were 7.9 and 10., all with p values <0.001 (Figure 3.5). Results from ANOVA analysis showed that there was a statistically significant difference between the interactions of the two temperature tests with a p-value of <0.001.

3.2.f Nitrogen Species

Ammonia did not affect *Didymo* cell viability. However, cell lysis was observed at the end of the 60 minute exposure. On average measured ammonia concentrations were within 13% of nominal concentrations (Table A.1). A linear function best fit the data and the standard error was 2.4 with a p value of 0.96 (Figure 3.6). Nitrate did not affect *Didymo* cell viability. All concentration levels (5.1, 6.4, 7.1, 9.1, 11.3, 17.3 mg/L) of nitrate tested resulted in less than 5% of the *Didymo* cells dying. Final concentrations of nitrate

averaged 43% of nominal concentrations and discussed later (Table A.1). A linear function best fit the data and the standard error was 1.9 with a p value of 0.16 (Figure 3.6).

Didymo was not affected by nitrite concentrations. Measured nitrite concentrations on average were within 25% of nominal concentrations (Table A.1). A linear function best fit the data and the standard error was 1.5 with a p value of 0.97 (Figure 3.6).

Table 3.5 - Nominal and measured concentrations of chlorine.

Date	Temperature (°C)	Nominal Concentration (mg/L)	Measured Concentration (mg/L)
4/17/2010	11.5	10	14
		5.0	1.1
		2.5	0.10
		1.3	0.04
		0.63	0.05
		Control	0.03
4/18/2010	17.3	10	16
		5.0	0.78
		2.5	0.06
		1.3	0.05
		0.63	0.05
		Control	0.03

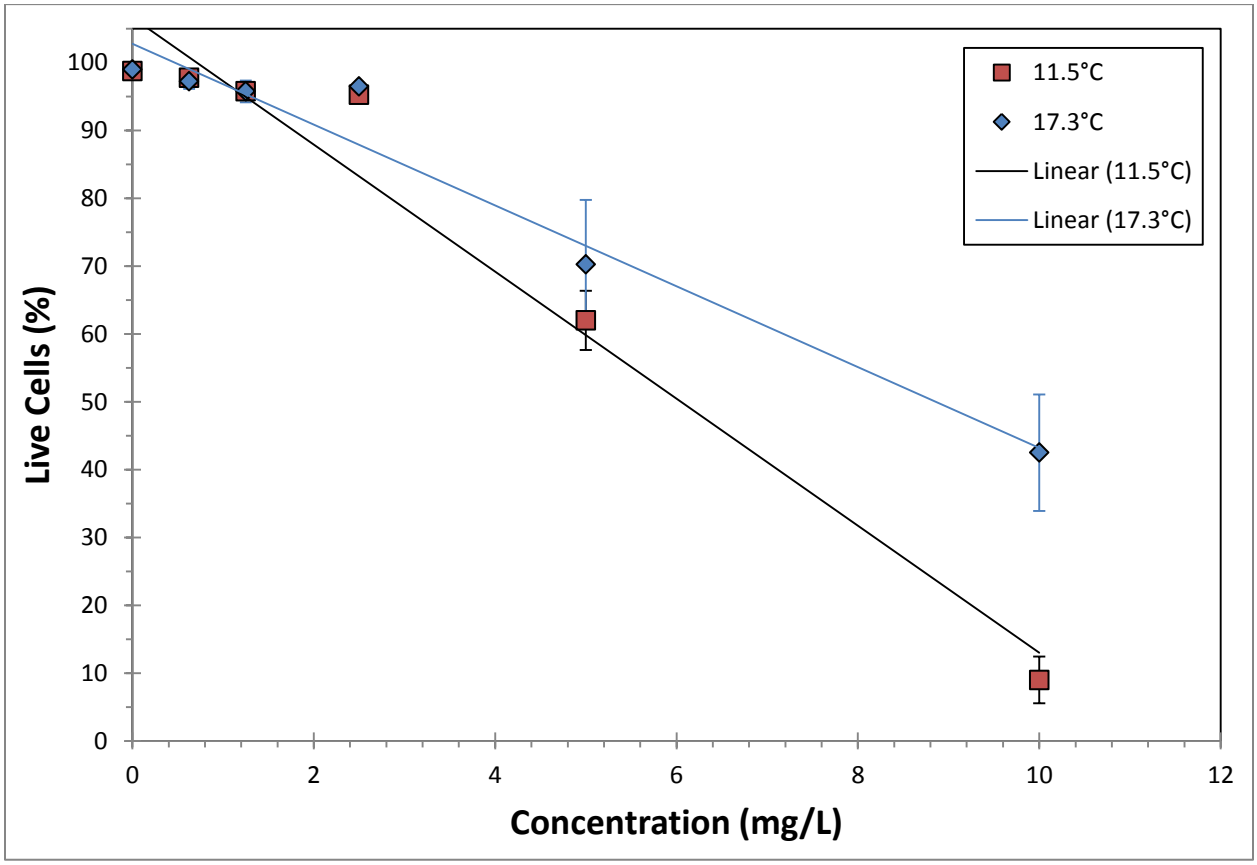

Figure 3.5 - *Didymo* survivability after a 60 minute exposure to varying concentrations of chlorine at two different temperatures. Error bars represent the standard error for the percent of live cells at each concentration.

Table 3.6 - Results from chlorine toxicity tests at different temperatures on *Didymo*. Number of live cells for each concentration are presented.

Results	Number of Live Cells	
Concentration	**11.5°C**	**17.3°C**
Control	99	99
0.63	98	97
1.3	96	96
2.5	95	97
5.0	62	70
10	9	43

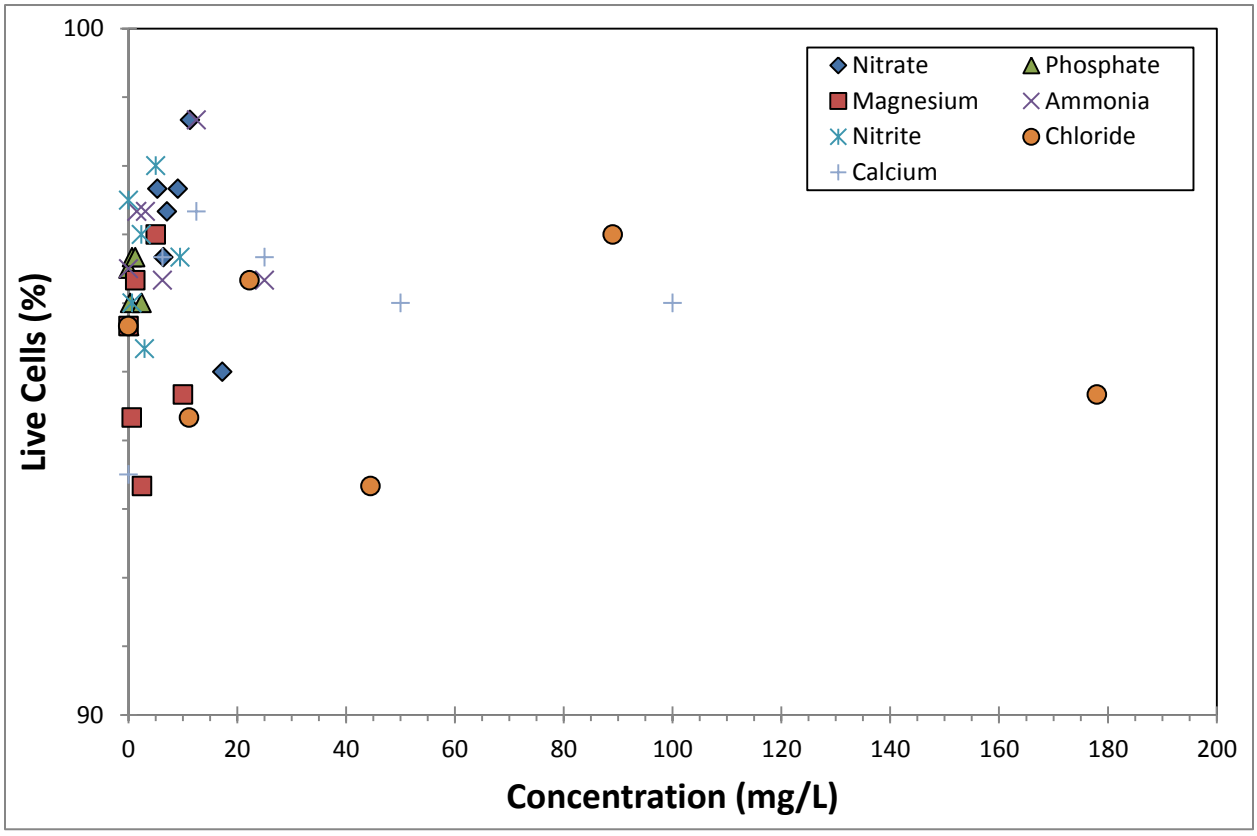

Figure 3.6 - *Didymo* survivability after a 60 minute exposure to varying concentrations of non-toxic parameters. The y-axis is exaggerated to shoe 90 to 100% of live cells.

3.2.g Phosphate

Didymo cell viability was not affected by concentrations of phosphate. Measured and nominal phosphate concentrations on average were within 4.8% of each other (Table A.1). A linear function best fit the data and the standard error was 1.6 with a p value of 0.94 (Figure 3.6).

3.2.h Chloride

Chloride had no effect on *Didymo* cell viability. The percent of live cells remaining after each chloride treatment was greater than 94%. Measured chloride concentrations (11, 22, 45, 89, 178 mg/L) on average were within 130% of nominal concentrations (Table A.1). The discrepancy between the measured and nominal concentrations will be discussed in the next chapter. A linear function best fit the data and the standard error was 2.7 with a p value of 0.45 (Figure 3.6).

3.2.i Calcium

Concentrations of calcium (6.3, 13, 25, 50., 100 mg/L) did not affect *Didymo* cell viability. All tests had greater than 94% of the *Didymo* cells remaining alive after the exposure treatments. On average nominal concentrations of calcium were within 28% of nominal concentrations (Table A.1). A linear function best fit the data and the standard error was 1.9 with a p value of 0.45 (Figure 3.6).

3.2.j Magnesium

Didymo cell viability was not affected by magnesium concentrations (0.63, 1.3, 2.5, 5.0, 10. mg/L). After exposure, the percent of live cells remained above 94% for each treatment. Measured concentrations of magnesium on average were within 6.5% of nominal concentrations (Table A.1). A

linear function best fit the data and the standard error of the regression was 2.0 with a p value of 0.87 (Figure 3.6).

3.3 LC_{50} results

The LC_{50} for copper could not be calculated for the highest temperature test due to having to trim greater than 50% of the data which makes it not possible to calculate the LC_{50}. However, the resulting LC_{50} for the test at 9.3°C was 3.3 mg/L as compared to the test conducted at 13.0°C which was 5.4 mg/L (Table 3.7).

For pH effects on copper toxicity the resulting LC_{50} for the pH 6.2 conditions was 33 mg/L, while the pH 7.7 conditions had a LC_{50} of 5.4 mg/L (Table 3.7). A LC_{50} for the pH 9.7 test could not be calculated as 55% of the data had to be trimmed. Since more than 50% of the data had to be trimmed in order to calculate the LC_{50} for zinc, it was not possible to come up with a resulting LC_{50} for the three different temperature tests (Table 3.7). The resulting LC_{50} for the two temperature tests of chlorine were 5.7 mg/L at 11.5°C and 8.5 mg/L at 17.3°C (Table 3.7).

Table 3.7 – Resulting median lethal concentrations (LC_{50}) from the Trimmed Spearman-Karber test for copper at varying temperatures and pH, zinc, and chlorine.

Copper	**9.3°C**	**13.0°C**	**16.3°C**
LC_{50}	3.3	5.4	-
95% Lower Confidence	2.9	4.0	-
95% Upper Confidence	3.7	7.1	-
Percent Trim	34%	48%	51%
Copper	**pH 6.2**	**pH 7.7**	**pH 9.7**
LC_{50}	33	5.4	-
95% Lower Confidence	30	4.0	-
95% Upper Confidence	37	7.1	-
Percent Trim	36%	48%	55%
Zinc	**9.6°C**	**14.1°C**	**15.7°C**
LC_{50}	-	-	-
95% Lower Confidence	-	-	-
95% Upper Confidence	-	-	-
Percent Trim	63%	59%	68%
Chlorine	**11.5°C**	**17.3°C**	
LC_{50}	5.7	8.5	
95% Lower Confidence	5.2	6.9	
95% Upper Confidence	6.2	10	
Percent Trim	9.1%	43%	

4. DISCUSSION

Colonization of *Didymo* within a laboratory setting has yet to be accomplished and represents one of the difficulties in determining the factors that limit *Didymo's* growth and survival. Looking at possible reasons of why the colonization attempt did not work can give insight for future colonization efforts.

4.1 Can *Didymo* be colonized in a laboratory using an artificial stream setting?

Despite that *Didymo* could not be successfully colonized in an artificial stream setting there is knowledge to be gained. Previous efforts were also unable to grow *Didymo* outside of its natural habitat (Rieberger 1991). The difficulty of colonizing algae depends on the species and can range from easy to difficult. In order to colonize algae, certain requirements must be met such as correct light intensity and duration, necessary growth nutrients, water source and temperature, and suitable substrate media for the algae to colonize (James 1978). Since *Didymo* is mostly found in flowing rivers suitable water velocities seem important to colonizing *Didymo.*

Given that *Didymo* inhabits clear rivers, with the highest biomass occurring in areas with greater exposure to sunlight, there has been speculation about light being a key parameter for *Didymo* growth (Kawecka and Sanecki 2003; Kilroy 2004; Whitton *et al.* 2009). Therefore the type of lighting, and on-off timing cycle used over the troughs, were manipulated throughout the experiments to determine a preferred setup. Despite *Didymo* not growing, other algae species flourished under the lighting used. Perhaps, the illumination used in the troughs was not the controlling factor on *Didymo* growth.

Water source can play the biggest role in whether colonization of algae is successful (James 1978). Initial trough setups used dechlorinated municipal tap water as this was found to be suitable for colonization of other algae (Brinkman, Personal Communication, 2009). However, to ensure that the

source water would not limit *Didymo* growth it was switched to Cache la Poudre River water taken at the same location and time that the *Didymo* samples were collected. Since the same water was being used as where *Didymo* was growing in the river, the water source was not the reason for the unsuccessful colonization in the laboratory.

Water temperatures in the troughs were held within *Didymo's* survivable range of water temperatures, from 0.1-27°C, and were 4-8°C warmer than water temperatures present in the Cache La Poudre River at the time of *Didymo* collection. Since the water temperatures within the troughs were within the range that *Didymo* is found in the natural environment, it was not believed to be the reason for *Didymo* not growing in the artificial stream setting.

Since *Didymo* relies on flowing waters to replenish its nutrient requirements it was important to have flow velocities within the trough that would mimic river flow velocities. Furthermore it has been shown that some algae in laboratory settings sustained greater growth in a current of at least a 0.5 ft/sec which was attributed to the removal of a film near the algae surface which increased material exchange between the algae and its environment (Whitford 1960). Average near bed velocities of 45 sites with high *Didymo* biomass was 1.64 ft/s but *Didymo* was found in water velocities from barely any movement up 3.28 ft/s (Kilroy *et al.* 2006a). Average velocities in the troughs were estimated to range from 0.14-.29 ft/s, depending on the experimental setup, which is slower than the water flow velocities that the *Didymo* was collected in. However, the troughs were placed at a slope to increase flow velocities and the fastest flow velocities were visually estimated to be greater than the 0.5 ft/s mentioned previously. It is then presumed that a flow velocity of 0.5 ft/s was present at some point in the troughs. Furthermore, both trough setups grew other algae that were present where *Didymo* was found in the Cache la Poudre River. Therefore, the flows would have been adequate for *Didymo* growth since the algae are found next to each other in the natural environment.

The substrate materials for the troughs were taken directly from the river where *Didymo* was observed. Previous studies found that *Didymo* preferred to colonize rocks with greater surface roughness and intact biofilms (Bergey *et al.* 2009). Initially, rocks collected to use in the troughs were soaked in hydrogen peroxide and scrubbed. This method was quickly abandoned as it has been determined that diatoms are usually not the first colonizers of bare substrate but rather colonize after a biofilm has been formed (Bergey *et al.* 2009). It was then decided to collect rocks from the *Didymo* sample sites on the Cache la Poudre river with intact biofilms to better allow for the colonization of *Didymo*. Biomedical tiles were also placed in the troughs since *Didymo* colonized the brick applications that were placed in the river therefore proving the tile surface was adequate for *Didymo* growth. Hence, the substrates used in the troughs were suitable for *Didymo* colonization.

Nutrient requirements for diatoms were met by using a growth media supplemented by silica. Different growth media were used to fit the specific needs of the algae and for the purposes of this study it was decided to use the F/2 Medium™ as positive results were shown when used for colonizing *Chlodophra* (Brinkman, Personal Communication, 2009). Since *Didymo* is found in nutrient-poor waters finding a balance that gives *Didymo* its desired nutrient levels while maintaining a concentration that allows for *Didymo* not to be in direct competition from other algae proved the trickiest part of attempting *Didymo* colonization. In a nutrient-poor river system *Didymo* is able to outcompete other algae by its ability to hydrolyze inorganic phosphorus within its stalk material (Maurer 2008). Despite efforts to replicate a nutrient-poor system in the troughs, by slowly adding the media over time and eventually decreasing the concentration of the media used, the *Didymo* in the troughs never colonized new substrate and were overgrown by other algae. This in turn was attributed to the possibility that levels of inorganic phosphorus were high enough to where *Didymo* lost its competitive edge of being able to hydrolyze organic phosphorus within its stalks to survive in nutrient-poor waters. In natural

systems, competing algae have been shown to go through different successions of dominant algae (Holm and Armstrong 1981).

Iron availability and reactivity has recently been shown to be a key factor in *Didymo's* ability to sequester phosphorus that is bound within the stalk material making it readily available for use (Sundareshwar *et al.* 2011). The F/2 Media™ contained levels of iron appropriate for algae growth as evidenced by the growth of other algae within the troughs. Overall, *Didymo* was supplied with everything it needed to grow within the troughs. What did not allow for *Didymo* growth was an environment too nutrient-rich where other algae were no longer at a disadvantage to *Didymo* but instead could easily outcompete it.

4.2 Water quality parameters and their affect on *Didymo* survivability

Results from testing different water quality parameters on *Didymo* showed that after 60 minute treatments only pH, copper, zinc, and chlorine affected *Didymo* survivability. The main mechanism of death in the *Didymo* cells is cell lysis, which is the rupturing of the cellular wall and leaking of the cytoplasm (Kilroy *et al.* 2006c). All other parameters tested proved to have no effect or were inconclusive and included ammonia, nitrate, nitrite, phosphate, chloride, calcium, and magnesium.

4.2.a pH

Although the percent of live *Didymo* cells decreased only for the lowest pH at 4.3, this does not necessarily mean that *Didymo* can survive at the other pH levels tested. The lowest pH water that *Didymo* has been found to colonize had a pH of 6.4 (Kilroy *et al.* 2006b). Results from the tests at pH 5.9 showed to have no effect on *Didymo*. Therefore, it is possible that *Didymo* could live in water with a pH lower than 6.4.

Despite that none of the high level pH treatments affected *Didymo* cell viability, in that the Neutral Red stain was always taken up, cell lysis was occurring in the treatments at pH 10.7. With the maximum pH level where *Didymo* is found at 9.0, it is understandable to see cell lysis occurring at a pH of 10.7 (Kilroy *et al.* 2006b). However, the treatment of a pH of 9.9 showed no sign of cell lysis, so again longer exposure times should be performed to verify whether *Didymo* can survive in higher pH waters.

4.2.b Copper

Overall, results from the copper toxicity tests agreed with findings from previous studies in that copper does affect *Didymo* cell viability. Although experimental setups differed, previous studies found that a 60 minute exposure to a concentration of 5 mg/L of chelated copper at 12.1°C resulted in 94% of *Didymo* cells dying (Jellyman *et al.* 2006). Only 50% of the *Didymo* cells were killed for the same concentration and exposure time at 13°C in this study. Differences in the use of chelated copper and copper sulfate could explain why the mortality rates were different. Water chemistry also could have played a role in the differences in mortality rates. Water hardness and pH have been shown to affect the bioavailability and proportion of toxic copper species which in turn affects the toxicity of it on aquatic organisms (Jellyman *et al.* 2006). Although results testing how pH affects copper toxicity are variable, higher water hardness has been found to decrease copper toxicity (Howarth and Sprague 1978; Perschbacher and Wurts 1999). Unfortunately the water hardness was not reported in the previous study so a direct comparison cannot be made.

A significant difference between nominal and measured concentrations of copper occurred, with most measured concentrations being much lower than nominal concentrations. Differences between nominal and measured concentrations of copper also occurred in survivability tests in New Zealand (Kilroy *et al.* 2006c). This can be explained by adsorption and absorption of copper by *Didymo*, and adsorption to the walls of the exposure chambers themselves. It has been shown that algae has a

high affinity to Cu(II) ions (Xue *et al.* 1988). How much copper is adsorbed by the algae is a factor of mucilage structure or extracellular polymeric substances, pH, and the ratio of surface area to dry weight, and competition from other ions (Tien *et al.* 2005). Exposure time for maximum adsorption of copper in four different algae species were all between 60 and 75 minutes, which ensures that there was enough time for the adsorption of copper to occur (Tien *et al.* 2005). Another study found that nearly all of the copper bound itself to blue-green algae within 10 minutes, which again suggests copper concentrations appear lower due to absorption and adsorption (Les and Walker 1984).

Results from the three different temperature tests showed that copper was more toxic to *Didymo* at colder temperatures. Since temperatures affect on the toxicity of copper is species dependent, some previous studies have found the opposite to be true for other algae (Cairns *et al.* 1975). As discussed previously the toxicity of copper can depend on many other parameters such as water hardness and pH, but it appears that temperature affects the toxicity as well.

An initial investigation, previous to this research, into the effect of copper on *Didymo* was conducted with a 60 minute exposure to 0.63, 1.3, 2.5, 5.0, 10. mg/L of copper. Since 100% mortality of *Didymo* was never reached during the 60 minute exposure to copper, the test was continued for a total of 360 minutes to determine if the longer exposure would achieve this result. After 60 minutes the amount of live cells were counted and the highest concentration of 10 mg/L of copper resulted in an average of 31% of *Didymo* cells surviving (Figure 4.1). After 360 minutes the highest concentration of 10 mg/L of copper had an average of 10% of *Didymo* cells surviving. The longer duration did achieve greater copper toxicity but 100% mortality was still not reached.

4.2.c Influence of pH on copper toxicity

In the low pH copper tests the water sample analysis had a higher copper concentration of 82.3 mg/L than the nominal concentration of 40 mg/L. This can possibly be attributed to measurement error,

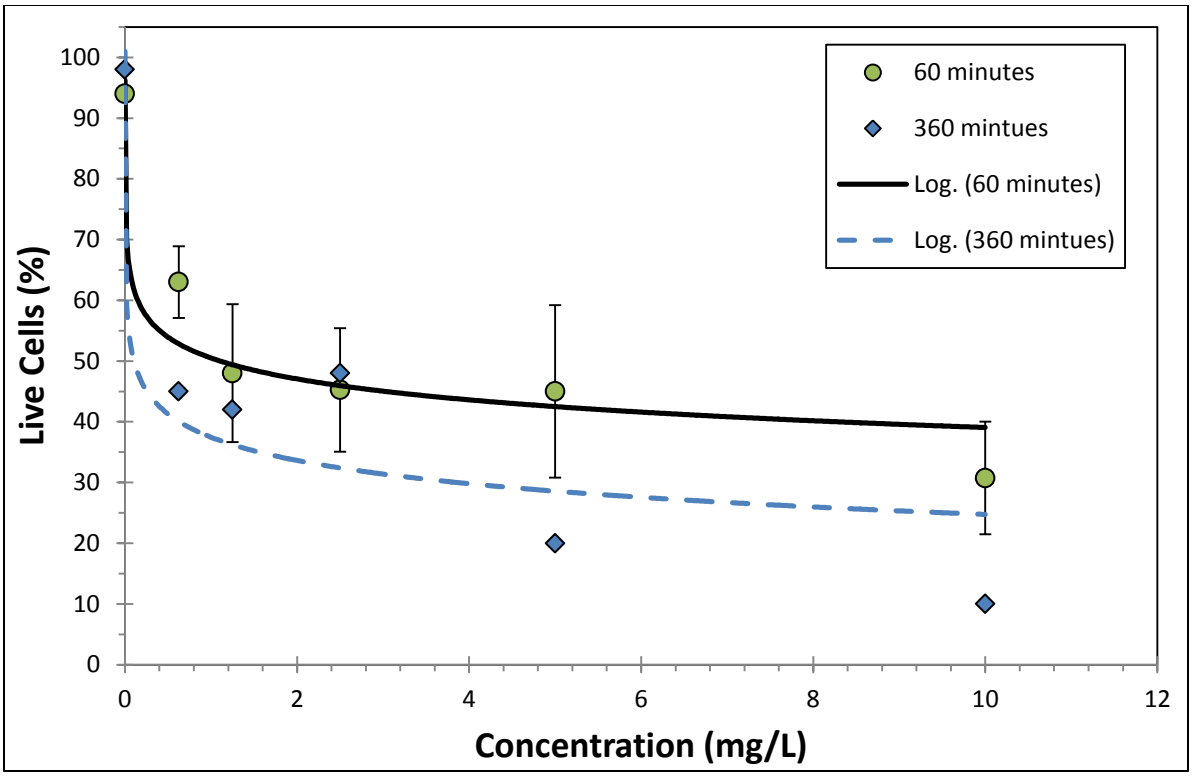

Figure 4.1 - *Didymo* survivability after a 60 and 360 minute exposure to 0.63, 1.3, 2.5, 5.0, 10. mg/L of copper. Error bars represent the standard error for the percent of live cells at each concentration.

since 36% of cells survived the treatment which is higher than the control pH test that had only 24% of the cells survive. The measurement error may have been forgetting to filter the water sample that was used to measure the total dissolved copper concentration. The result would be a higher copper concentration due to the undissolved copper not being filtered out and included in the analysis to determine the copper concentration.

Although copper toxicity generally decreased with decreasing pH the opposite occurred in the highest copper concentration (40 mg/L) test not allowing for any conclusive statements (Figure 4.2). Other studies have also reported mixed results with some showing copper toxicity increasing with a

decrease in pH due to the predominance of the free metal ion; while other show the opposite due to competition with H^+ at the cell membrane surface (Franklin *et al.* 2000). Copper toxicity and pH is complex and conclusive affects of pH on metal toxicity have not been established (Peterson *et al.* 1984; Starodub *et al.* 1987). Three factors have been identified as controlling how pH effects copper toxicity: complexation, precipitation, and adsorption (Flemming and Trevors 1989). These three factors directly control copper speciation and bioavailability. Complexation is controlled by pH, hardness, and the amount of dissolved organic carbon present in the water. Precipitation of copper occurs in waters with high pH and hardness. Adsorption of copper by algae can happen quickly and in substantial amounts as discussed in the previous section. amount of dissolved organic carbon present in the water. Precipitation of copper occurs in waters with high pH and hardness. Adsorption of copper by algae can happen quickly and in substantial amounts as discussed in the previous section.

4.2.d Zinc

Zinc did affect *Didymo* cell viability but not as much as copper did, similar to results from a previous study (Jellyman *et al.* 2006). It is unclear how temperature influences zinc toxicity (Figure 3.4). For all the concentrations tested, except for the highest (40 mg/L), the coldest water temperature (9.6°C) was the most toxic, then the warmest (15.7°C), and finally the middle temperature (14.1°C). However, for the highest zinc concentration tested (40 mg/L), the coldest water temperature (9.6°C) was the least toxic, while the other two temperature tests had increased toxicity. These mixed results can be seen in previous studies that found temperature's effect on zinc toxicity to be very species dependent and in some cases found temperature to have very little influence (Cairns *et al.* 1975; Rand and Petrocelli 1985). Other studies have come to similar conclusions but have found that warmer water temperatures can decrease the survival time of aquatic species exposed to zinc and other metals due to elevated respiration and metabolism (Wang 1987). Therefore, it is possible *Didymo* in warmer water

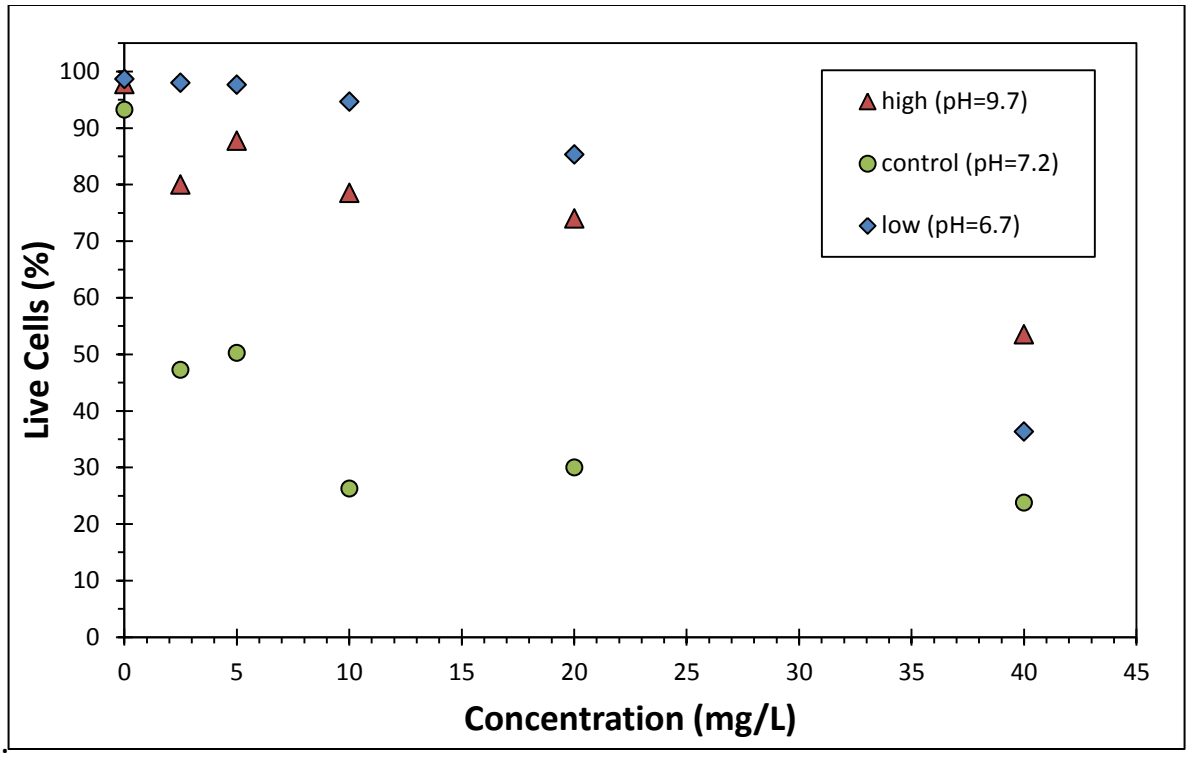

Figure 4.2 - *Didymo* survivability after a 60 minute exposure to 2.5, 5, 10, 20, 40 mg/L of copper at different pH levels.

could die sooner than in cold water if exposed to zinc, however this would need to be tested with longer duration tests.

4.2.e Ammonia

Ammonia is a known algaecide, yet even at the relatively high concentrations tested, the cells survived. Shrunken chloroplasts were an indication that cell lysis was beginning to occur so there were signs that the ammonia was negatively affecting *Didymo*. Due to the visual clues that cell lysis may be occurring, the highest concentration test was continued and rechecked after three and half hours to

determine if the cells would rupture with a longer exposure time. The cell lysis had progressed since 60 minutes, but the *Didymo* cells still took up the stain. How much of the unionized toxic form of ammonia is present is dependent on pH level and temperature, so it is possible that at different temperatures and pH levels *Didymo* cell viability could have been more greatly affected. Studies with other algal species have shown that elevated levels of ammonia can inhibit nitrogen uptake by the algae which can disrupt normal function of the chloroplasts (Abeliovich and Azov 1976; Ohmori *et al.* 1977). The blocking of nitrate uptake by ammonia was to found to last only as long as the ammonia was present so it is possible that the cell lysis shown in *Didymo* may be reversible for the 60 minute exposure time. This would have to be determined *in situ* with longer duration tests.

4.2.f Chlorine

Results from the chlorine tests were in agreement with previous *Didymo* control tests performed in New Zealand in that chlorine did affect *Didymo* cell viability (Jellyman *et al.* 2006). Due to the onset of snowmelt runoff, the flows on the Cache la Poudre River were too high to allow for sampling of *Didymo* in order to conduct a third temperature test. Results from the two previous tests show that colder temperatures increased the toxicity of chlorine. However, a third test at a different temperature should be performed to validate the results. Previous results have shown temperatures affect on chlorines toxicity to algae are mixed and that temperature main affect on toxicity to algae may be the rate at which toxic chlorine changes into non-toxic compounds (Cairns *et al.* 1975).

Overall, the measured concentrations of chlorine were on average 85% different from nominal concentrations in the two temperature tests. This discrepancy may be due to adsorption/absorption of chlorine by *Didymo* and the exposure chamber itself. Additional research is needed since there is little to no literature on the subject.

4.2.g Nitrate, nitrite, phosphate, chloride, calcium, and magnesium

Tested concentrations of nitrate, nitrite, chloride, calcium, and magnesium tests did not affect *Didymo* cell viability. Nitrite is the only parameter that is known to be toxic to aquatic life, but since it is rapidly oxidized to non-toxic nitrate, it is not often found in large concentrations in natural waters (Rand and Petrocelli 1985). Having tested nitrite concentrations up to 10 mg/L and not seeing any effect on *Didymo* cell viability it can be inferred that nitrite does not affect *Didymo* at concentrations up to 10 mg/L. Therefore in the case of the Spring Creek Study, it is possible that they can rule out nitrite as leading to *Didymo's* mortality if nitrite levels in the spring-fed tributaries are up to 10 mg/L (Sutherland *et al.* 2007).

Nitrate, chloride, calcium, and magnesium are not considered to be toxic parameters (Environmental Protection Agency 1986) and results from this study are in agreement. Large differences between the nominal and measured concentrations of calcium can be explained by the solubility of calcium chloride in water. At 10°C the solubility of calcium chloride is 64.7 mg/L. For the test with a nominal concentration of 100 mg/L the measured concentration was 65.9 mg/L. This agrees with observations made during the test that the 100 mg/L exposure chambers had precipitate. Minor discrepancies between nominal and measured concentrations for other parameters were insignificant and probably resulted from the imprecision of the calibration of the diluter system.

5. CONCLUSION

The goal of this study was to determine if *Didymo* could be grown in an artificial stream setting within a laboratory, and to identify water quality parameters that affect *Didymo* survivability. Colonization of *Didymo* was unsuccessful despite water source, water temperature, flow velocity, and light intensity and duration being all within parameters observed in the field where *Didymo* is present.

Overall, *Didymo* survivability as affected by a 60 minute exposure to different water quality parameters followed previously determined results in that known algaecides affected cell survivability, while other non-toxic parameters showed no effect on *Didymo*. Nitrate, nitrite, phosphate, chloride, calcium, and magnesium did not affect *Didymo* survivability (Table 5.1). Ammonia also did not affect *Didymo* but signs of cells lysis were observed and possible mortality may occur at longer exposure times. Copper, zinc, chlorine, and pH affected *Didymo* survivability (Table 5.1). Copper showed the greatest affect on *Didymo* survivability with the LC_{50}s for copper at 9.3°C and 13.0°C being 3.3 mg/L and 5.4 mg/L respectively at pH 7.7. For copper toxicity in waters with a lower pH (6.7) the resulting LC_{50} was 33 mg/L. Median lethal concentrations could not be calculated for the copper tests at 16.3°C and at a higher pH (9.7) because more than 50% of the data needed to be trimmed not allowing for a calculation. Generally, both colder water temperature and higher pH increased copper toxicity on *Didymo*. The affect of temperature on copper toxicity was shown to be statistically significant with a resulting p-value of 0.02. However, there was no statistically significant affect of pH on copper toxicity with a p-value of 0.07.

The LC_{50} could also not be determined for all three zinc tests but the highest zinc concentration of 40 mg/L had on average 56% of *Didymo* cells surviving which was higher than copper which averaged 20% of cells surviving. No apparent trend on the affect of temperature to zinc toxicity on *Didymo* could

Table 5.1 – Results summary table for different water quality parameters and their affect on *Didymo*. Concentrations and live cells are presented as the range of resulting values.

Parameter	Concentration (mg/L)	Temperature (°C)	pH	Live Cells (%)
Copper	2.5-40	9.3	7.7	4-62
		13	7.7	24-50
		16.3	7.7	33-84
		11.1	6.2	36-98
		11.4	9.7	54-80
Zinc	2.5-40	9.6		53-87
		14.1		58-97
		15.7		42-91
Chlorine	0.63-10	11.5		9-98
		17.3		43-97
pH		11.1	4.3-7.5	84-100
		11.3	7.6-10.8	84-100
Ammonia	1.6-25	9.9	7.6	95-99
Calcium Chloride	6.3-100	9.9		92-99
Magnesium	0.63-10	10.0		93-98
Nitrate	0.63-10	9.4		95-100
Nitrite	0.63-10	9.5		94-100
Phosphate	0.19-2.5	10.4		94-98

be determined; however, the interaction of temperature on zinc toxicity was statistically significant with a p-value of 0.02.

Chlorine at temperatures of 11.5°C and 17.3°C had LC_{50}s of 5.67 and 8.46 mg/L respectively. A third temperature test could not be conducted due to peak flows not allowing for collection of *Didymo* samples. The highest chlorine concentration of 10 mg/L had on average 26% of *Didymo* cells surviving. The affect of temperature on chlorine toxicity was statistically significant with a p-value <0.001.

Didymo survivability was affected by water with pH 4.3 but not by water with pH 5.9 and 6.9. Cell lysis was occurring in water with pH 10.7 but no sign of any affect on *Didymo* survivability was found in water with pH 9.9. The survivable range of pH values for *Didymo* may be greater than thought but longer duration tests would be needed to confirm.

6. Recommendations

Didymo's success in the natural environment is attributed to its ability to sequester nutrients it needs for growth in an oligotrophic environment essentially allowing it to outcompete other algae. In order to better understand this, an artificial stream setting could be constructed streamside with river water diverted through it continuously to ensure water chemistry and nutrient levels are suitable for *Didymo* growth. Once the artificial stream setting was colonized certain parameters could then be manipulated to determine the effect on *Didymo*. Such manipulations of interest could be light intensity and duration, water clarity, water velocity and depth, and manipulating nutrient levels to see if there is any shift in the periphyton community away from *Didymo*.

The use of an artificial stream would not only help in the pursuit of colonizing *Didymo* but also allow for greater control in toxicity tests on *Didymo* that would help with future management decisions. Having toxicity test in an artificial stream environment would allow for a more realistic application of toxic parameters over varying concentrations and exposure times. Determining dose concentrations and number of applications to reach 100% mortality would give managers a better idea of the practicality of dosing natural systems with toxic materials. Also studying *Didymo's* ability and the speed with which it can recover from doses that do not cause 100% mortality will allow managers a better decision on using algaecides in rivers. Managers should also consider the effect of water temperature and pH on toxicity of certain water quality parameters before making decisions on what concentrations to apply.

Since the use of toxic water quality parameters in natural environments can have negative side effects on the surrounding ecosystem more emphasis should be placed on two things. First, the resurgence of *Didymo* in its native areas may be linked to the altered flow regimes that have been created by water dams and diversions (Kirkwood *et al.* 2007). With this reduction in peak flows and their durations, scouring of the stream bed which reduces *Didymo* biomass is no longer occurring with

enough frequency. Managers in charge of dam releases and water diversions should try and restore a more natural flow regime where higher flows of longer durations occur annually to help to reduce *Didymo* biomass. Secondly, stopping the spread of *Didymo* within or between watersheds can only be done by educating the people using the waterways on how to clean and disinfect any gear that may transport *Didymo* cells. This will help slow *Didymo* spread and growth until future ways to control *Didymo* are discovered.

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APPENDIX A

Test data for non-toxic parameters on *Didymo*

Table A.1 – Results from tests of non-toxic parameters on *Didymo*. Lives cells (%) is an average value based upon 3 replicates.

	Temperature (°C)	Nominal Concentration (mg/L)	Measured Concentration (mg/L)	Live Cells (%)
Ammonia	Temperature 9.9	29	31	96
		15	17	99
		7.3	10	96
	pH 7.5	3.6	7.0	97
		1.8	5.0	97
		Control	3.7	97
Calcium	9.9	100	86	96
		50	54	96
		25	39	97
		13	30	97
		6.3	24	97
		Control	20	94
Chloride	9.9	178	450	96
		89	260	96
		45	113	97
		22	55	97
		11	28	97
		Control	7.8	94
Magnesium	10.0	10	12	95
		5.0	6.9	97
		2.5	4.3	93
		1.1	3.2	96
		0.63	2.6	94
		Control	1.9	96
Nitrate	9.5	10	17	95
		5.0	11	99
		2.5	9.1	98
		1.3	7.1	97
		0.63	6.4	97
		Control	5.3	98
Nitrite	9.5	10	9.5	97
		5.0	5.0	98
		2.5	3.0	95
		1.3	2.4	97
		0.63	0.7	96
		Control	0.02	98
Phosphate	9.5	2.5	2.5	96
		1.3	1.3	97
		0.63	0.66	97
		0.31	0.35	96
		0.16	0.16	96
		Control	0.03	97

Table A.2 – Raw results data for different temperature copper tests on *Didymo*.

Concentration (mg/L)	Temperature (°C)	Lives Cells (%)	Temperature (°C)	Lives Cells (%)	Temperature (°C)	Lives Cells (%)
Control	9.4	91	13.2	99	16.0	95
	9.4	97	13.2	91	16.0	97
	9.3	98	13.0	92	16.0	98
	9.3	96	13.0	91	16.0	90
	9.4	92	13.2	91	16.4	99
	9.4	94	13.2	86	16.4	97
	9.3	94	12.8	100	16.4	99
	9.3	93	12.8	96	16.4	98
2.5	9.2	52	13.0	54	16.3	24
	9.1	60	12.8	36	16.1	52
	9.2	68	13.0	33	16.3	54
	9.1	67	12.8	66	16.0	62
5.0	9.2	37	12.8	53	16.4	70
	9.2	15	13.1	36	16.1	30
	9.2	19	12.9	64	16.3	41
	9.2	21	12.5	48	16.4	31
10	9.2	39	13.0	18	16.4	20
	9.6	5	13.6	12	16.4	10
	9.2	31	12.9	43	16.4	8
	9.2	27	12.8	32	16.4	46
20	9.2	49	13.0	40	16.4	55
	9.2	17	12.9	19	16.1	52
	10.0	9	13.7	49	16.4	15
	9.3	15	12.7	12	16.4	32
40	9.8	4	13.3	45	16.2	35
	9.5	6	13.4	2	16.1	23
	9.5	2	13.2	28	16.2	39
	9.5	2	12.8	20	16.4	36

Table A.3 – Raw results data for different temperature zinc tests on *Didymo*.

Concentration (mg/L)	Temperature (°C)	Lives Cells (%)	Temperature (°C)	Lives Cells (%)	Temperature (°C)	Lives Cells (%)
Control	9.5	98	13.8	98	15.5	99
	9.5	93	13.8	98	15.5	90
	9.8	96	14.1	99	15.5	95
	9.8	92	14.1	98	15.5	95
	9.9	93	14.3	96	15.9	97
	9.9	94	14.3	97	15.9	99
	9.6	98	14.2	99	15.8	98
	9.6	96	14.2	95	15.8	96
2.5	9.5	87	14.0	98	15.6	84
	9.7	81	14.2	99	15.6	90
	9.5	90	14.2	95	15.6	90
	9.7	89	14.0	95	15.8	99
5.0	9.6	82	13.7	94	15.7	93
	9.6	97	13.9	94	15.6	81
	9.7	96	14.1	98	15.9	98
	9.5	87	14.0	95	15.8	95
10	9.3	93	13.7	91	15.6	89
	10.0	73	14.2	86	15.8	97
	9.7	81	14.3	99	15.9	94
	9.4	82	14.1	99	15.7	91
20	9.5	27	13.9	78	15.6	73
	9.7	66	14.2	76	15.9	52
	9.8	59	14.3	84	15.9	54
	9.5	60	14.1	76	15.9	79
40	9.6	40	14.1	72	15.6	48
	9.8	85	14.3	43	15.8	41
	9.8	63	14.2	42	15.9	42
	9.5	81	14.2	73	15.8	36

Table A.4 – Raw results data for different temperature chlorine tests on *Didymo.*

Concentration (mg/L)	Temperature (°C)	Lives Cells (%)	Temperature (°C)	Lives Cells (%)
Control	11.5	100	17.3	100
	11.4	98	17.3	98
	11.4	100	17.3	100
	11.6	97	17.3	98
0.63	11.4	98	17.0	99
	11.5	95	17.2	98
	11.5	99	17.2	98
	11.4	99	17.2	94
1.3	11.5	96	17.1	99
	11.2	93	17.3	93
	11.7	98	17.3	93
	11.6	96	17.3	98
2.5	11.6	95	17.2	97
	11.6	97	17.4	97
	11.4	92	17.3	96
	11.6	97	17.3	96
5.0	11.4	70	17.3	49
	11.3	51	17.4	90
	11.8	68	17.3	60
	11.5	59	17.3	82
10	11.7	4	17.3	63
	11.4	8	17.4	42
	11.4	5	17.4	44
	11.8	19	17.3	21

Table A.5 – Raw results data for different pH copper tests on *Didymo.* Values of pH for the low and control tests were averaged for each concentration.

Concentration (mg/L)	pH	Temperature (°C)	Lives Cells (%)	pH	Temperature (°C)	Lives Cells (%)	pH	Temperature (°C)	Lives Cells (%)
Control	6.7	11.2	99	7.7	13.2	99	10	11.4	98
		11.1	99		13.2	91	10	11.4	95
		11.2	98		13.0	92	10.1	11.5	99
		11.2	98		13.0	91	10.1	11.4	99
					13.2	91			
					13.2	86			
					12.8	100			
					12.8	96			
2.5	6.7	11.0	98	7.2	13.0	54	9.7	11.4	68
		11.0	97		12.8	36	9.7	11.4	79
		11.0	99		13.0	33	9.7	11.4	84
		11.0	98		12.8	66	9.8	11.4	89
5.0	6.7	11.2	97	7.3	12.8	53	9.4	11.4	85
		10.9	97		13.1	36	9.5	11.4	86
		11.0	99		12.9	64	9.5	11.4	92
		11.0	98		12.5	48	9.5	11.4	88
10	6.7	11.2	87	7	13.0	18	8.3	11.4	90
		11.4	99		13.6	12	8.4	11.4	72
		11.0	98		12.9	43	8.6	11.4	58
		11.2	95		12.8	32	9.2	11.4	94
20	6.5	11.0	82	6.6	13.0	40	8.2	11.4	93
		10.9	85		12.9	19	8.2	11.4	56
		11.3	89		13.7	49	8.2	11.4	62
		11.1	85		12.7	12	8.2	11.4	85
40	6.0	11.1	36	6.2	13.3	45	6.7	11.4	52
		11.2	39		13.4	2	6.7	11.4	51
		11.1	34		13.2	28	6.7	11.4	52
		11.2	36		12.8	20	6.7	11.4	59

Table A.6 – Raw results data for different water quality parameter tests on *Didymo.*

Calcium Chloride			Phosphate			Magnesium		
Concentration (mg/L)	Temperature (°C)	Lives Cells (%)	Concentration (mg/L)	Temperature (°C)	Lives Cells (%)	Concentration (mg/L)	Temperature (°C)	Lives Cells (%)
Control	9.7	90	Control	10.3	99	Control	9.9	94
	10.3	90		10.5	96		10.2	98
	9.7	95		10.2	98		9.8	98
	8.6	95		10.4	98		10.0	92
	9.8	97		10.2	96		9.7	94
	9.5	94		10.5	92		9.8	98
6.3	9.7	94	0.19	10.4	95	0.63	9.7	95
	10.2	98		10.5	96		10.0	94
	10.0	98		10.5	96		9.9	94
13	10.3	98	0.4	10.9	94	1.3	10.5	97
	9.9	98		10.3	97		10.0	95
	9.7	96		10.4	97		10.0	97
25	10.0	94	0.75	10.4	96	2.5	10.0	94
	10.1	97		10.3	98		10.2	93
	10.5	99		10.4	96		10.5	93
50	9.8	98	1.3	10.4	97	5.0	10.0	96
	9.8	95		10.2	96		10.0	98
	9.9	95		10.4	97		10.2	97
100		92	2.5		96	10		97
		98			97			94
		98			95			93

Table A.7 – Raw results data for different water quality parameter tests on *Didymo.*

Nitrate			Nitrite			Ammonia			
Concentration (mg/L)	Temperature (°C)	Lives Cells (%)	Concentration (mg/L)	Temperature (°C)	Lives Cells (%)	Concentration (mg/L)	pH	Temperature (°C)	Lives Cells (%)
Control	9.2	96	Control	9.5	98	Control	7.5	10.2	98
	9.7	93		9.5	97		7.5	10.2	98
	9.4	97		9.5	99		7.5	9.8	89
	9.2	97		9.5	97		7.5	9.8	99
	9.4	98		9.6	96		7.5	10.0	98
	9.2	93		9.6	98		7.5	10.0	97
0.63	9.2	95	0.63	9.2	97	1.6	7.5	9.9	96
	9.2	99		9.4	97		7.5	9.8	99
	9.7	99		9.4	94		7.6	9.9	97
1.3	9.5	96	1.3	9.4	97	3.1	7.5	9.7	98
	10.0	99		9.2	98		7.5	9.7	98
	9.4	99		9.4	96		7.5	9.8	96
2.5	9.4	95	2.5	9.3	96	6.3	7.5	9.7	95
	9.2	100		9.8	95		7.5	10.1	98
	9.4	98		9.4	95		7.5	9.7	96
5.0	9.7	98	5.0	9.3	97	13.0	7.6	9.8	98
	10.0	99		9.5	98		7.5	9.9	100
	9.2	97		9.9	99		7.5	10.2	98
10	9.2	99	10	9.4	97	25	7.6	10.1	95
	9.5	97		9.6	100		7.5	9.9	99
	9.4	98		9.4	94		7.5	10.0	95

Table A.7 – Raw results data for pH tests on *Didymo.*

pH	Temperature (°C)	Lives Cells (%)	pH	Temperature (°C)	Lives Cells (%)
4.3	11.5	91	7.6	11.3	99
4.3	11.1	84	7.7		98
4.3	11.0	91	7.7	11.3	99
5.9	10.9	98	9.8	11.4	99
5.9	11.0	99	9.8	10.9	99
5.9	10.9	99	10.0	11.2	99
6.9	11.4	99	10.6	11.1	84
6.9	11.4	100	10.7	11.1	100
6.9	11.0	99	10.8	11.7	91
7.3	11.1	98			
7.4	10.9	100			
7.5	10.7	99			
7.5	10.9	99			
7.5	11.2	99			
7.5	11.0	99			

APPENDIX B

Observations on *Didymo* in the Cache la Poudre River, Fort Collins, CO

Didymo is found in large abundance throughout most of the Cache la Poudre River upstream of Fort Collins, CO. After observing *Didymo* in the river for over a couple years, the peak blooming appears to occur in early Spring and Fall. It occurs just after the ice is off the river, usually around February, until runoff due to snowmelt begins around June. The higher flows from runoff mobilize the streambed and scour the *Didymo* which reduces biomass along the stream bottom. Blooms begin to form again under low flow conditions of late Summer and early Autumn and seem to peak again in Fall where entire stretches of river bottom can be covered. This lasts until the river becomes iced over and then observations could not be made again until the ice begins to melt. *Didymo* is present as soon as the ice melts so it may be assumed that it can live through an ice-covered winter and then form peak blooms again immediately after the ice is off the river again in early Spring.

Of note, peak flows in 2010 were higher than 2011, but the duration of the peak flows were much longer in 2011 which resulted in more removal of *Didymo* from the stream bottom. This emphasizes the fact that the size of the peak flow is only as important as the duration of the flows that are mobilizing the bed and scouring the *Didymo*.

Figure B.1 – *Didymo* covering entire streambed of the Cache la Poudre River upstream of Picnic Rock, Fort Collins, CO. Photo taken 4/7/09.

Figure B.2 - *Didymo* covering entire streambed of the Cache la Poudre River upstream of Picnic Rock, Fort Collins, CO. Photo taken 4/7/09. Notice healthy white tufts of *Didymo*.

Figure B.3 – Underwater photo of *Didymo* in the Cache la Poudre River upstream of Picnic Rock, Fort Collins, CO. Photo taken 4/7/09.

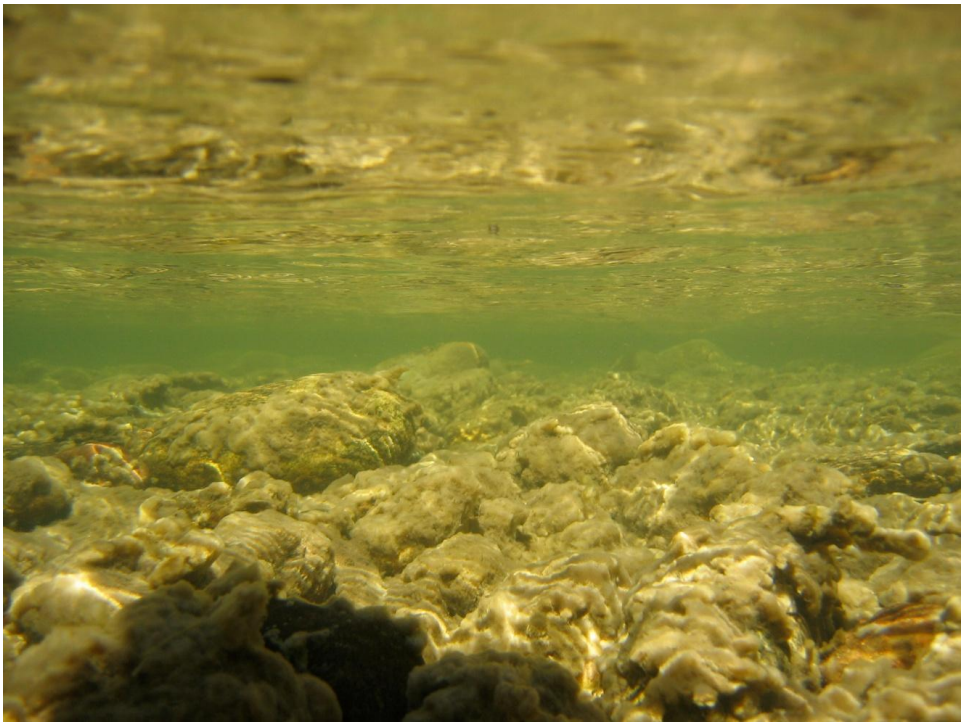

Figure B.4 – Underwater photo of *Didymo* covering entire streambed of the Cache la Poudre River upstream of Picnic Rock, Fort Collins, CO. Photo taken 4/7/09.

Figure B.5 – *Didymo* covered rock from the Cache la Poudre River upstream of Picnic Rock, Fort Collins, CO. Photo taken 4/19/09.

APPENDIX C

Didymo colonization

C.1 Shallow troughs

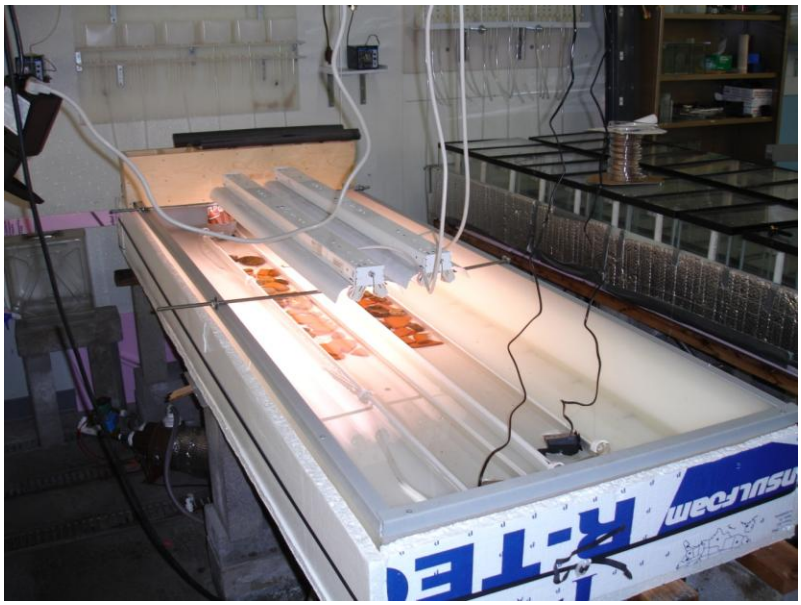

Figure C.1 – Artificial stream setting at the Colorado Division of Wildlife Aquatic Toxicology Laboratory, Fort Collins, CO. Two shallow trough treatments are pictured.

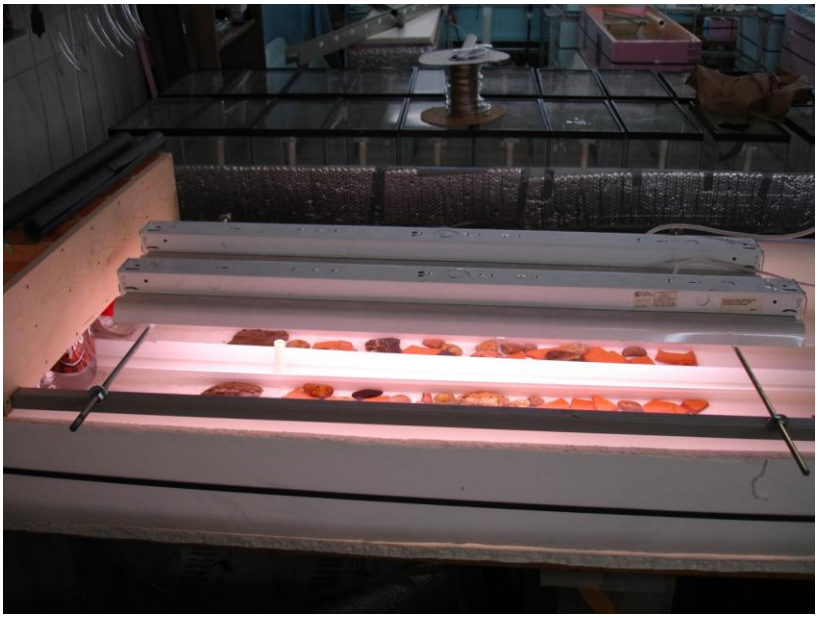

Figure C.2 – Side view of artificial stream setting at the Colorado Division of Wildlife Aquatic Toxicology Laboratory, Fort Collins, CO. Two shallow trough treatments are pictured.

C.2 Deep Troughs

Figure C.3 – Close up of deep trough setup looking upstream. Rocks colonized by *Didymo* can be seen.

Figure C.4 - Close up of deep trough setup looking downstream. Rocks colonized by *Didymo* can be seen.

Figure C.5 – Close up of rock colonized with *Didymo* in deep trough.

C.3 Didymo comparison photos

Figure C.6 – Rock with *Didymo* immediately after placement into the shallow trough setup on 10/23/09.

Figure C.7 – Same rock as in Figure C.6, twenty six days later. Notice the deterioration and the color of the *Didymo* is now green as this algae has become dominant.

Figure C.8 – Rock with *Didymo* immediately after placement into the shallow trough on 10/23/09.

Figure C.9 – Same rock as in Figure C.8, twenty six days later. Notice the deterioration and the color of the *Didymo* is now green as this algae has become dominant.

C.4 Brick Application

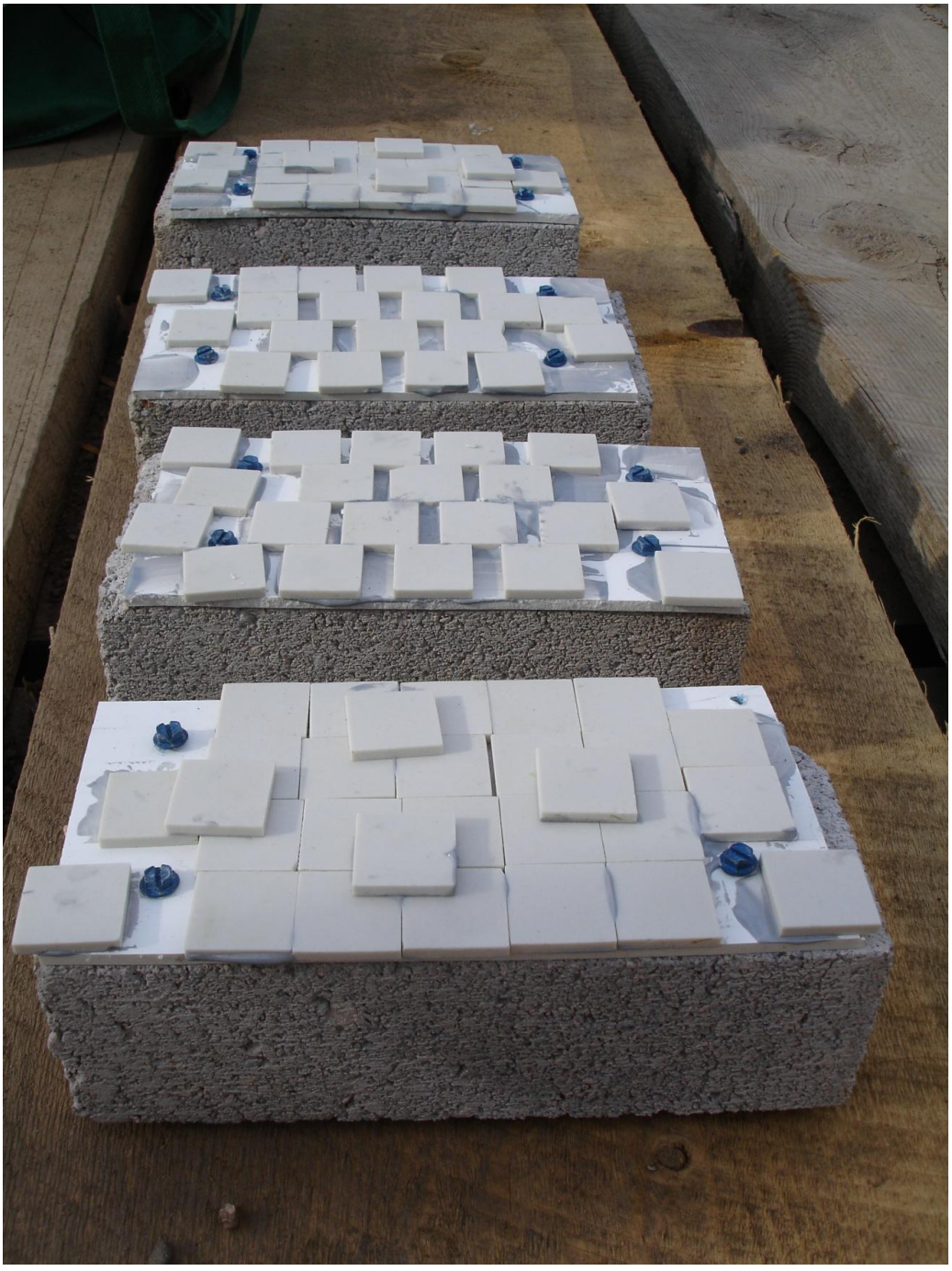

Figure C.10 – Bricks before being placed into sampling site on Cache la Poudre River, Fort Collins, CO.

Figure C.11 – Bricks placed into Cache la Poudre River, Fort Collins, CO.

Figure C.12 - Bricks placed at the *Didymo* sampling site on Cache la Poudre River , Fort Collins, CO.

Figure C.13 – Twelve days after placing the bricks into the Cache la Poudre River, Fort Collins, Co. Colonization of tiles has begun.

Figure C.14 – Overhead shot of artificial stream setup with colonized and new tiles being shown.

APPENDIX D

Water quality and its affect on *Didymo* survivability

Figure D.1 - Sampling site on the Cache la Poudre River at Gateway Park, Fort Collins, Co. Photo taken on 1/27/10.

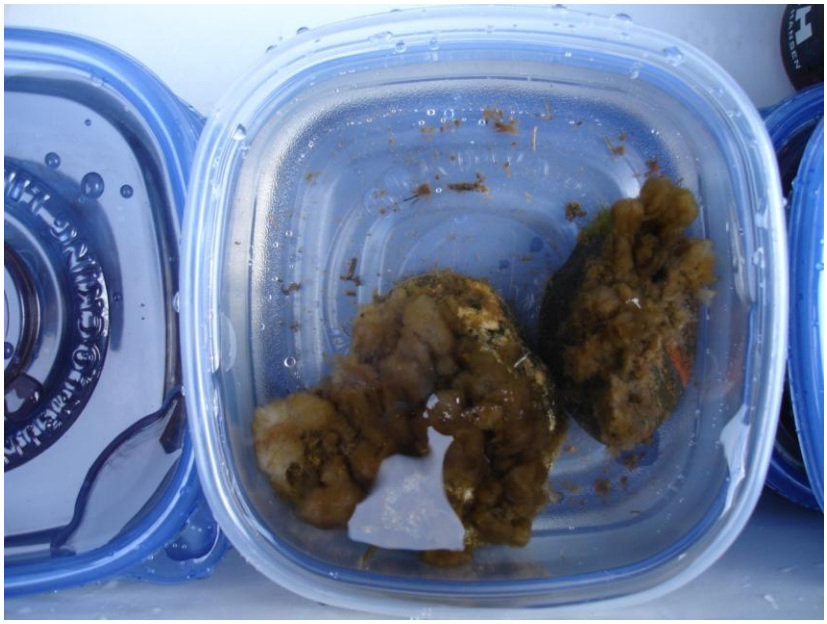

Figure D.2 – *Didymo* samples in one liter containers stored in cooler, taken from the Cache la Poudre River at Gateway Park, Fort Collins, Co. Photo taken on 1/27/10

Figure D.3 – Close-up of an exposure chamber setup for testing different water quality parameters on *Didymo* at the Colorado Division of Wildlife Aquatic Toxicology Laboratory, Fort Collins, CO.

APPENDIX E

***Didymo* cell viability**

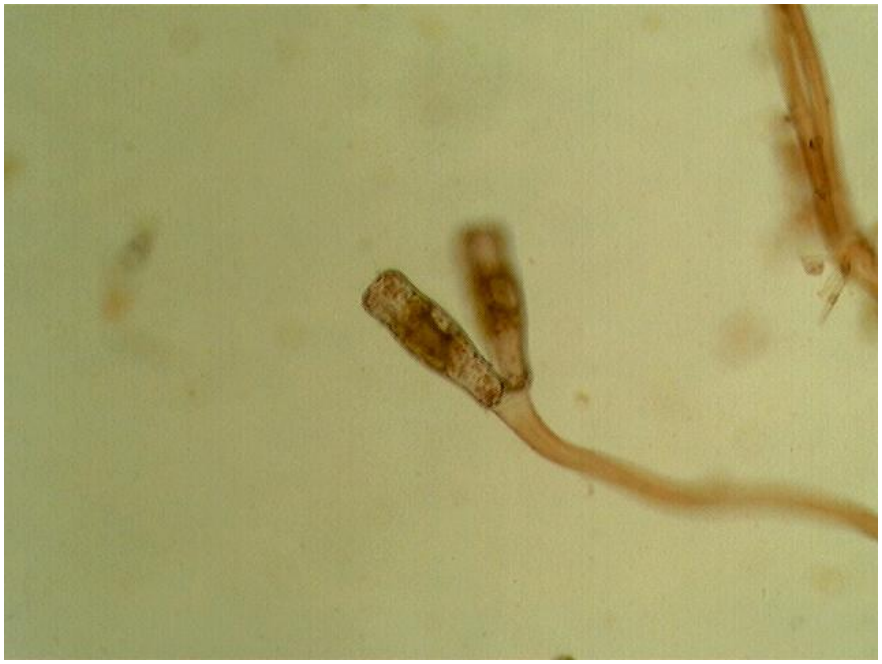

Figure E.1 – Neutral Red stain to indicate live and dead cells. The red granules within the cell indicate the cell has taken up the stain and is counted as a live *Didymo* cell. Notice the chloroplast's healthy shape.

Figure E.2 - Red granules within the cell indicate the cell has taken up the stain and is counted as a live *Didymo* cell. Notice the chloroplasts healthy X shape.

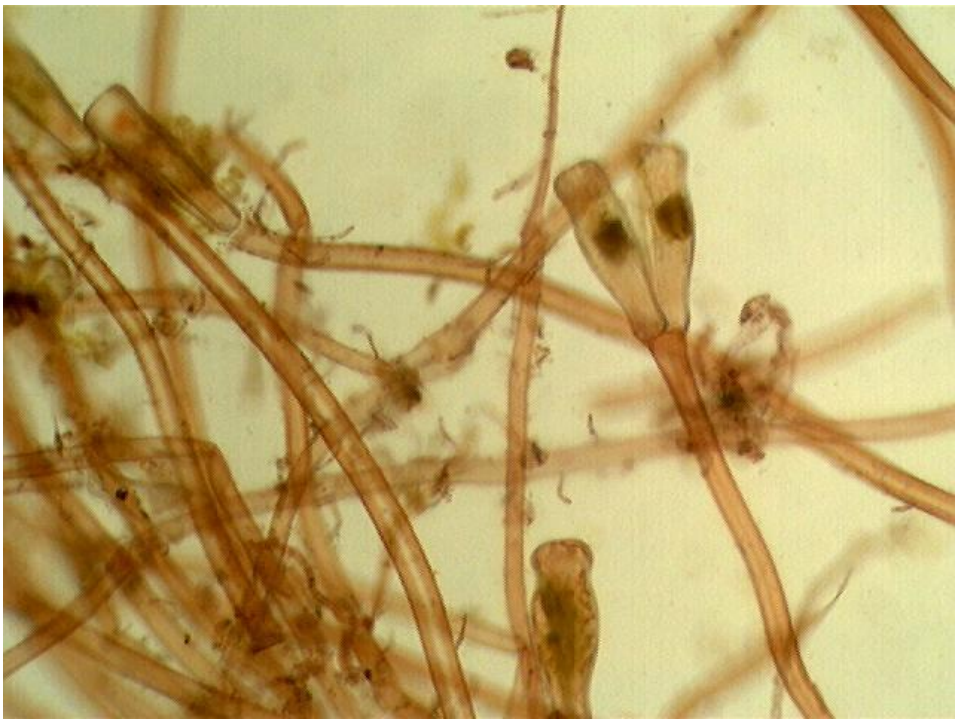

Figure E.3 – After a 60 minute exposure to copper sulfate. The two cells in the upper right of the photo do not have red granules within the cell and the chloroplasts have changed from an X shape to circular shapes indicating the cells are not alive.

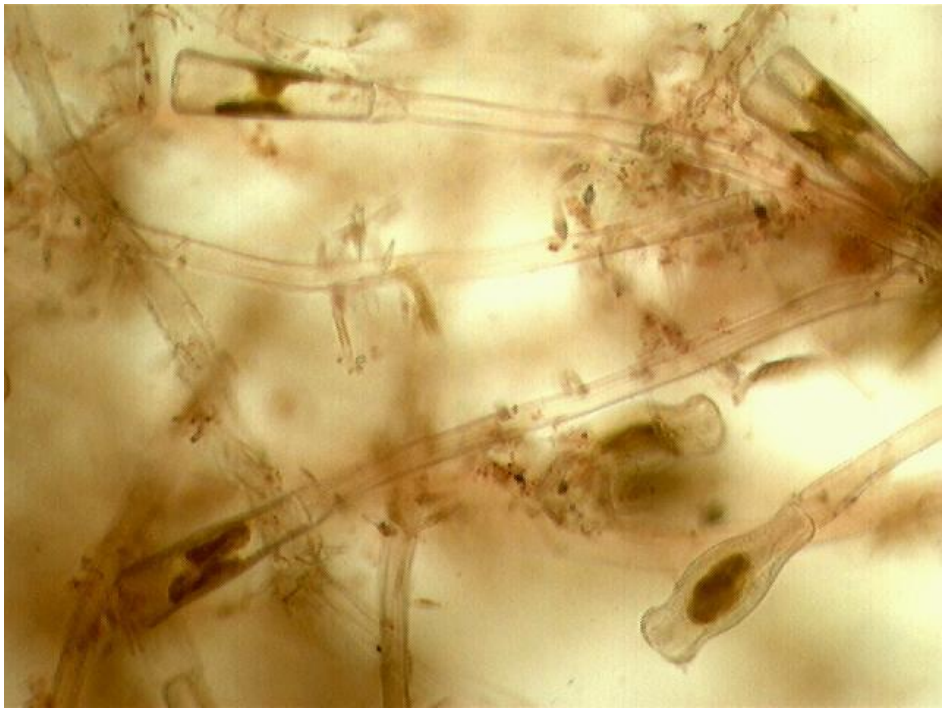

Figure E.4 - After a 60 minute exposure to copper sulfate. All the cells do not have red granules so they did not take up the Neutral red stain. Also note the chloroplasts have changed from an X shape to circular and deformed shapes indicating the cells are not alive.

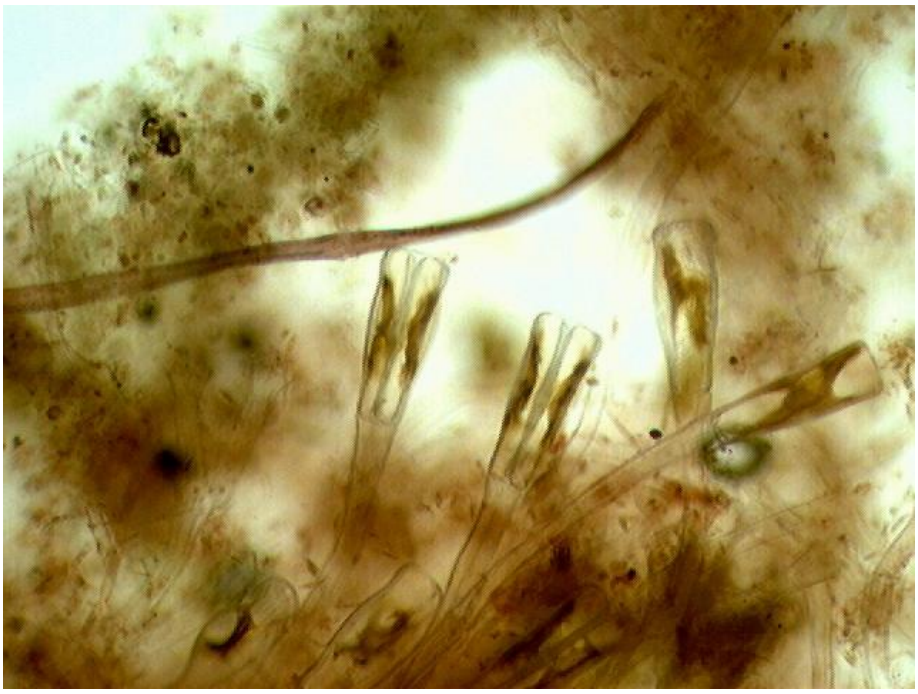

Figure E.5 - After a 60 minute exposure to copper sulfate. All the cells do not have red granules so they did not take up the Neutral red stain. Also note the chloroplasts have a deformed shape indicating the cells are not alive.

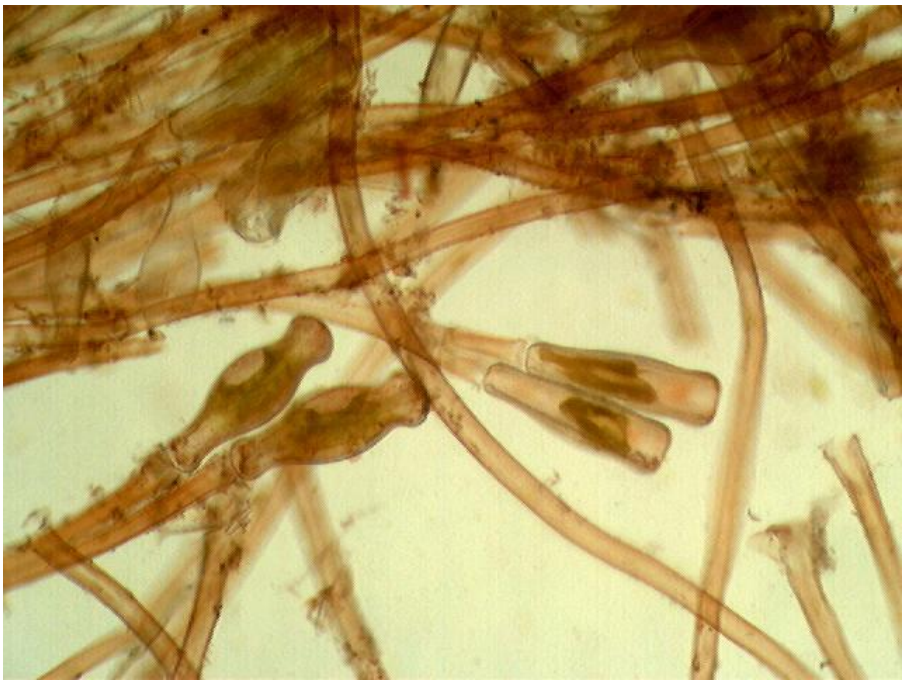

Figure E.6 - After a 60 minute exposure to copper sulfate. The two cells on the right do not have red granules within the cell so they did not take up the Neutral red stain. Also note the chloroplasts have changed from an X shape to a deformed shape indicating the cells are not alive. The two cells on the left have taken up the stain and the chloroplasts are still healthy indicating they are alive.

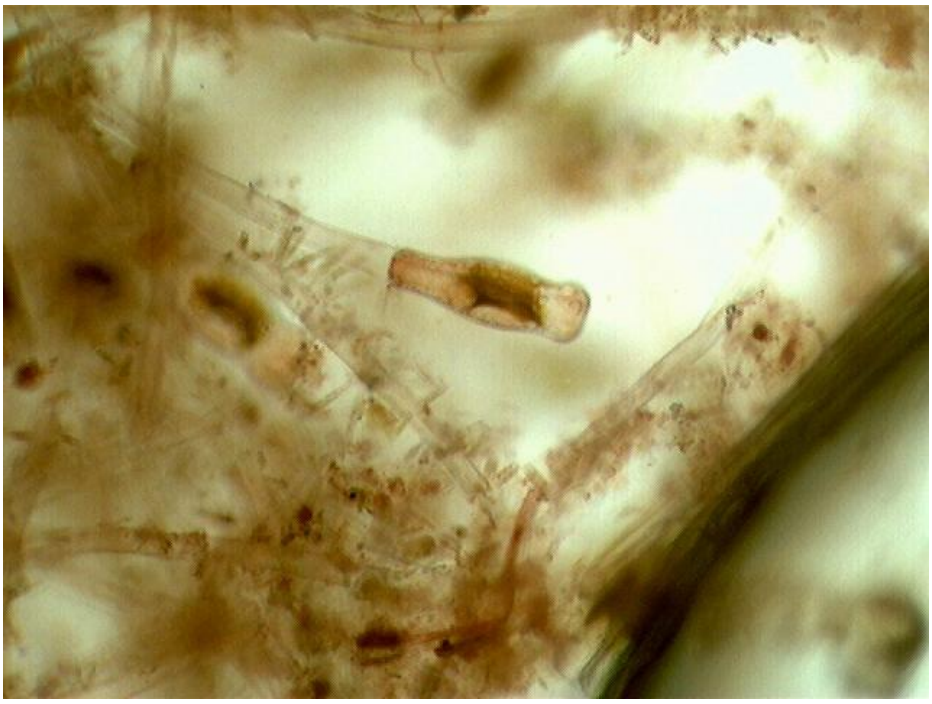

Figure E.7 - After a 60 minute exposure to copper sulfate. The cell has taken up the stain and the chloroplasts looks somewhat healthy so the cell is counted as alive. However, notice the bubbles forming inside the cell which can indicate cell lysis may be occurring.

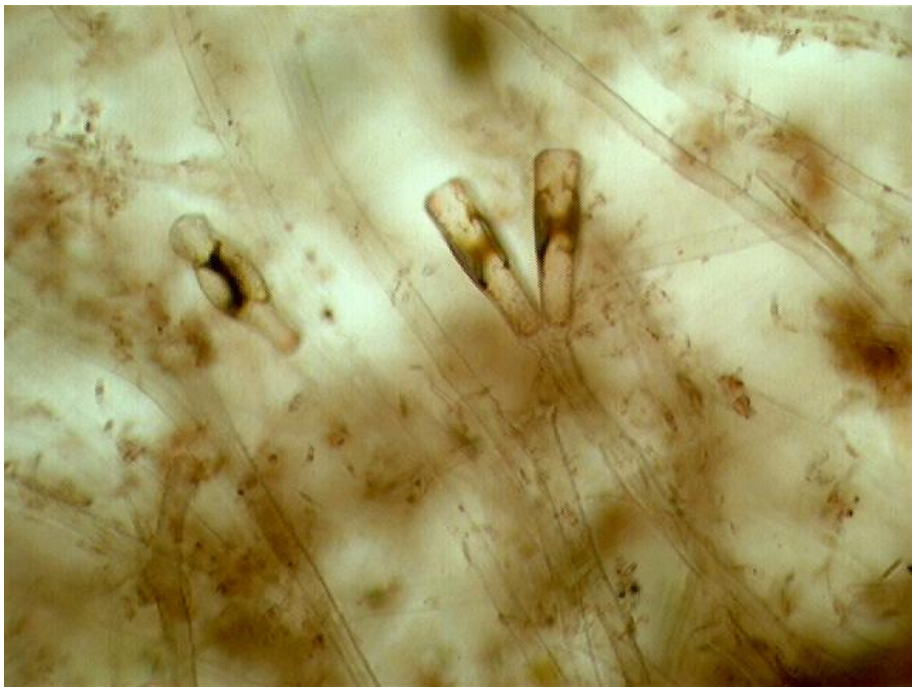

Figure E.8 - After a 60 minute exposure to copper sulfate. The two cells on the right have red granules within the cell so they did take up the Neutral red stain and are counted as alive. However, notice the chloroplasts looked deformed compared to a healthy live cell. Cell lysis may be occurring.

Figure E.9 – Ruptured *Didymo* cell that may have occurred from separating *Didymo* tufts. This type of cell was not counted as alive or dead and therefore not included in the first 100 *Didymo* cells viewed. Notice the chloroplast is leaking out from the cell.

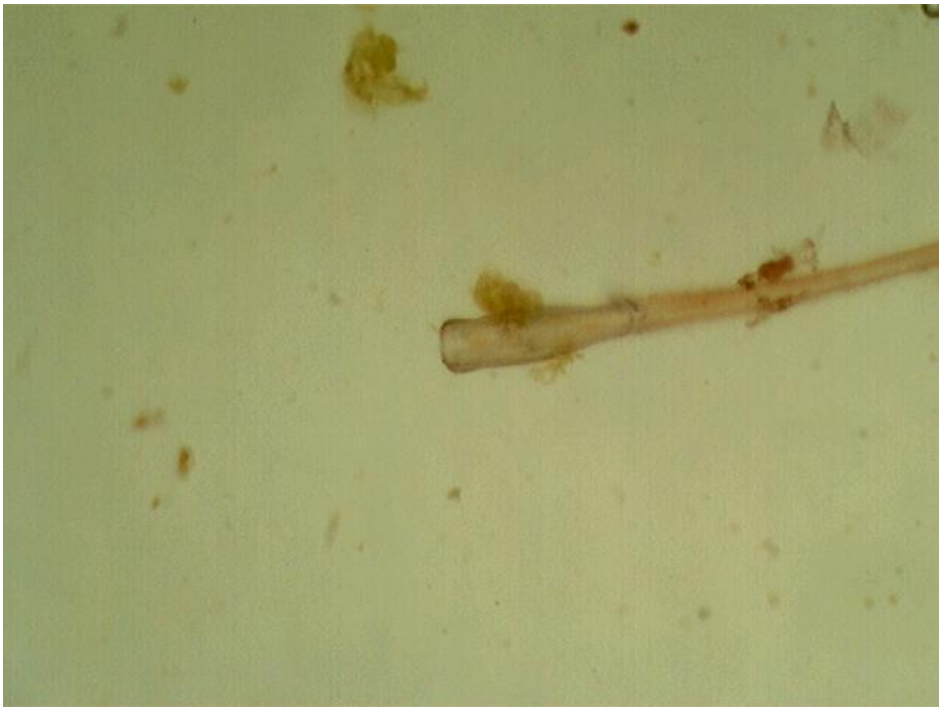

Figure E.10 - Ruptured *Didymo* cell that may have occurred from separating *Didymo* tufts. This type of cell was not counted as alive or dead and therefore not included in the first 100 *Didymo* cells viewed. Notice the chloroplast is leaking from the cell.