

DEVELOPMENT OF A LIFE CYCLE BIOASSAY WITH
CHIRONOMUS TENTANS IN ARTIFICIAL STREAMS

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ABSTRACT

The objective of the work described in this thesis was to develop a novel life cycle bioassay for evaluating metal mine effluent effects using the midge *Chironomus tentans* in artificial streams. The bioassay was tested *in situ* using an environmentally relevant concentration (45%) of effluent from the Copper Cliff Wastewater Treatment Plant at INCO Ltd. in Sudbury, ON, Canada. There was reduced survival ($p=0.001$), reduced total emergence ($p<0.001$), increased time to emergence ($p<0.001$), and reduced hatching success ($p=0.001$) of *C. tentans* exposed to 45% Copper Cliff effluent relative to a reference water treatment. This research showed that the life cycle bioassay in artificial streams could be used *in situ* to assess metal mine effluent effects on life cycle endpoints in a benthic invertebrate.

The artificial stream bioassay was also evaluated in the laboratory where sensitivity of the bioassay was compared to an existing beaker life cycle bioassay in a side-by-side exposure. Response patterns were consistent between the two bioassays for hatching success and time to emergence. However, effluent effects were observed for growth, survival, numbers emerged, and number of eggs per egg case in the artificial stream bioassay, but not in the beaker bioassay. Conversely, significant responses in sex ratios and number of egg cases per female were observed in the beaker bioassay, but not in the artificial stream bioassay. The inconsistencies in response between the two bioassays may be explained by the larger number of organisms per replicate in the artificial stream bioassay which may have resulted in decreased variability in endpoint measurement and smaller effect sizes. Results were compared between the laboratory

and field artificial stream bioassay studies. Measures of survival, emergence, and hatching success were consistent between experiments, although the magnitudes of effect were generally greater in the field than in the laboratory.

This research was significant because it developed a more environmentally-relevant bioassay for use in the laboratory and *in situ*. The results from this research provided fundamental information on effects of a metal mine effluent on a benthic invertebrate. This bioassay also has potential for technology transfer to the federally legislated environmental effects monitoring program.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ASB	Artificial Stream Bioassay
BB	Beaker Bioassay
CCE	Copper Cliff Effluent
CV	Coefficient of Variation
EEM	Canadian Environmental Effects Monitoring Program
ES	Effect Size
INCO	International Nickel Company
LC ₅₀	Median Lethal Concentration
MME	Metal Mine Effluent
n	Number of replicates
SD	Standard Deviation
SE	Standard Error
t _α	Critical t value for α level of significance
t _β	Critical t value for β level of significance
WQG	Canadian Water Quality Guidelines for the Protection of Aquatic Life
WWTP	Wastewater Treatment Plant

Chapter 1

GENERAL INTRODUCTION

1.1 Introduction

The objective of the work described in this thesis was to develop an environmentally relevant bioassay for a model test species that could be used to measure effects of metal mine effluent (MME) discharged into aquatic receiving environments. To achieve this objective, a life cycle bioassay using *Chironomus tentans* (order Diptera, family Chironomidae) was developed and tested both *in situ* and in the laboratory using artificial streams and treated MME. A direct comparison was made between this bioassay and a standard *C. tentans* life cycle bioassay (Benoit et al. 1997) commonly used to measure the toxicity of contaminated sediments. This introduction summarizes the advantages and disadvantages of existing laboratory and in-field or *in situ* bioassays and provides background on the model species selected for bioassay development (*C. tentans*). This introduction also provides a basis for selecting bioassay endpoints and for selecting MME as the contaminant for bioassay testing and evaluation.

1.2 Laboratory bioassays

1.2.1 Acute toxicity tests

An acute toxicity test generally measures the effects of a chemical on an organism following an exposure of hours to days (Rand et al. 1995). The endpoint typically measured is mortality, which is reported as a median lethal concentration (LC₅₀). The LC₅₀ is the chemical concentration at which there is 50% mortality among test organisms over the duration of the test (Rand et al. 1995). Acute toxicity tests are commonly used to screen new chemicals (Parrish 1995) or to assess the quality of industrial effluents discharged to surface waters (USEPA 2002). In Canada, for example,

acute tests are conducted on treated pulp mill (*Pulp and Paper Effluent Regulations* 1992) and metal mine (*Metal Mining Effluent Regulations* 2002) effluents on a frequent basis (e.g., once per month) as a condition of a regulated approval or permit for discharge. Commonly used acute toxicity tests include the 48-hour *Daphnia magna* test (Environment Canada 2000a) and the 96-hour rainbow trout (*Oncorhynchus mykiss*) test (Environment Canada 2000b).

Acute toxicity tests are advantageous because they are quick, relatively easy to use, cost-effective (Cairns 1995, Kimball and Levin 1985), and many of the methods are standardized (Environment Canada 2000a, Environment Canada 2000b). However, these short-term toxicity tests generally lack environmental relevance because they typically measure mortality as the only endpoint, use concentrations of a chemical that are often much higher than is seen in the environment, and are conducted in the laboratory under tightly controlled conditions (Cairns 1995, Kimball and Levin 1985).

The advantage of running toxicity tests under controlled conditions in the laboratory is that most conditions can be regulated to reduce variability within the data to focus on the single stressor being evaluated (Cairns 1995). However, the constant and artificial conditions in the laboratory make it difficult to extrapolate results to the more complex conditions found in the field (Kovacs and Megraw 1996, Sibley et al. 1999, Tucker and Burton 1999). Several studies have reported effects in the field that cannot be predicted by observations made in the laboratory (Robinson et al. 1994, Tucker and Burton 1999). For example, *C. tentans* larvae were exposed in the laboratory and *in situ* to sediments contaminated with agricultural and urban runoff. In most exposure periods, larvae exposed *in situ* exhibited lower survival than larvae exposed to the same

sediments in the laboratory (Tucker and Burton 1999). Complex interactions between abiotic and biotic factors, which are difficult to replicate in the laboratory, influence the response of an organism to stress. For example, many controlled studies have shown increased susceptibility of aquatic organisms to metals at suboptimal ranges of temperature, salinity, or food availability (Chapman et al. 1982, Cotter et al. 1982). Thus, laboratory tests carried out under artificial conditions might not correctly estimate contaminant effects on organisms in the field.

1.2.2 Chronic toxicity tests

Chronic toxicity tests expose organisms to the contaminant of concern for longer periods of time (e.g., weeks, months, years) (Rand et al. 1995). These tests typically include the measurement of sublethal endpoints, such as growth and reproduction. Some examples of chronic toxicity tests include the 10-day sediment toxicity test with *C. tentans* or *C. riparius* (Environment Canada 1997), the *Ceriodaphnia dubia* reproduction and survival test (Environment Canada 1992c), and 7-day toxicity tests with fathead minnows (*Pimephales promelas*) (Environment Canada 1992b) and inland silverside (*Menidia beryllina*) (USEPA 1994). The *C. tentans/C. riparius* test method evaluates the growth and survival of larvae after 10 days of exposure. Effects on chironomid growth and survival have been observed after 10 days of exposure to sediments contaminated with metals and organic compounds (Burton et al. 1996, Kemble et al. 1994, Watts and Pascoe 2000, West et al. 1993).

Due to the longer exposure duration, effects are typically detected at lower contaminant concentrations than in acute toxicity tests. In Canada, sublethal toxicity

testing is performed on pulp mill (Environment Canada 1998) and metal mine (Environment Canada 2002) effluent once or twice per year as part of the industry's environmental effects monitoring (EEM) program. The data from these toxicity tests are used to estimate the extent of potential effluent-related effects on biological components in the receiving environment (Environment Canada 2002).

Chronic toxicity tests are typically run in the laboratory, so they have the same advantages and disadvantages of being run in the controlled, laboratory environment as discussed in the section on acute toxicity testing (section 1.2.1). In addition, chronic toxicity tests do not always evaluate effects on the most sensitive stages of an organism's life cycle. For example, Nebeker et al. (1984) found that first instar *C. tentans* larvae were the most sensitive larval stage to copper exposure. However, 10 day old third instar larvae are used for the 10-day toxicity test because they are easier to handle than first instar larvae (Environment Canada 1997, Nebeker et al. 1988, USEPA 2000). In addition, while some chronic toxicity tests measure effects on reproductive endpoints (Environment Canada 1992a, Environment Canada 1992c), other chronic toxicity tests only measure effects on survival and/or growth (Environment Canada 1992b, Environment Canada 1997, USEPA 1994). The measurement of reproductive endpoints is important because impairments in reproduction may lead to a decrease in population density in future generations (Allan and Daniels 1982, Barnthouse 1993, Moriarty 1999).

1.2.3 Life cycle bioassays

A life cycle bioassay measures the effects of contaminants throughout the life cycle of an organism. Therefore, effects on sensitive life stages and reproductive endpoints can be assessed in the same bioassay. Examples of life cycle bioassays include the fathead minnow (*Pimephales promelas*) life cycle bioassay (Borton et al. 2000, Mount and Stephan 1967) and the *Chironomus tentans* life cycle bioassay (ASTM 2000, Benoit et al. 1997, USEPA 2000). However, life cycle bioassays are typically carried out in the laboratory, so they also have the same advantages and disadvantages as acute and chronic tests with respect to running tests under controlled laboratory conditions (section 1.2.1)

1.3 *In situ* or outdoor exposures

Existing laboratory bioassays continue to serve important roles in assessing effluent quality and for screening contaminants over short periods of time. However, with the continuing discharge of chemicals and complex mixtures into aquatic receiving environments, there is a need for improved in-field or *in situ* methods to evaluate contaminant effects over the life cycle of an organism and under realistic environmental conditions of photoperiod, temperature, water quality, and effluent quality and quantity. Effects can be studied under more realistic exposure scenarios through the use of field experiments. A field-based bioassay could be an important tool for studying effects because, like laboratory-based bioassays, a bioassay performed in the field would be replicable (Guckert 1993), but it could be carried out under more realistic physicochemical exposure conditions.

1.3.1 In-stream or in-lake systems

There are many different types of in-stream or in-lake systems that have been used to test the effects of chemicals on organisms in the field. The use of these systems allows direct exposure to contaminants within the dynamic aquatic environment because organisms are transplanted and contained within the water body of interest (Chappie and Burton 1997). One type of system requires organisms to be transplanted from the laboratory into small cages or enclosures that are placed directly in the aquatic system. These *in situ* exposure systems have been used to evaluate the effects of contaminants on the feeding rate of *Gammarus pulex* (Forrow and Maltby 2000), survival and growth in *C. tentans* (Sibley et al. 1999, Tucker and Burton 1999), survival and bioaccumulation in *Lumbriculus variegatus* (Sibley et al. 1999), survival of *Hyaella azteca* (Tucker and Burton 1999), and survival in *Ceriodaphnia dubia* (Sasson-Brickson and Burton 1991). Other types of larger scale, in-field exposure systems have been used to test the effects of single contaminants and complex effluents on more complex assemblages of organisms. Examples include in-stream troughs (Perrin et al. 1992) and in-lake enclosures (Brazner et al. 1989, Liber et al. 1997, Liber et al. 1999).

1.3.2 Stream-side systems

There are various types of stream-side systems that have been used to test the effects of contaminants on single species, species assemblages, or communities (Dubé et al. 2002a, Perrin et al. 1992). Field-based artificial stream systems have been developed and tested at the National Water Research Institute of Environment Canada since 1991 (Dubé et al. 2002a). These systems have been used to evaluate the effects of complex

effluents, such as pulp mill effluent and metal mining effluent on benthic invertebrate communities (Culp et al. 1996, Culp et al. 2000a, Culp et al. 2003) and fish populations (Dubé et al. 2002b, Dubé et al. 2004b, Dubé et al. 2004c, Dubé and MacLatchy 2000).

One advantage these systems have over other *in situ* systems is that the concentration of an effluent can be tightly controlled (Dubé and Culp 1996). Thus, it is possible to evaluate the effects of a single effluent at a variety of concentrations, such as Dubé and Culp (1996) demonstrated using pulp mill effluent (concentrations ranging between 0.25% and 10%) and chironomids in the Thompson River, BC, Canada. It is also possible to identify the causative stressor in a pulp mill effluent using these systems. For example, Culp et al. (1996) and Podemski and Culp (1996) exposed a benthic food web from the Athabasca River, AB, Canada, to three treatments (1% treated pulp mill effluent, 1% nutrients (nitrogen and phosphorus), and reference river water) to show that effects were due to nutrient enrichment rather than contaminants. Finally, the flexibility of these systems allows for assessment of several effluents individually and in combination in rivers where the simultaneous discharge of multiple effluents confounds interpretations of impacts on aquatic biota (Dubé et al. 2002a, Dubé et al. 2002b). In one study, the effects of a pulp mill effluent and a municipal sewage effluent were evaluated separately and together for both fish (Dubé et al. 2004a) and benthic invertebrates (Culp et al. 2004) in the Wapiti River, AB, Canada.

To date, when these artificial stream systems have been used with benthic invertebrates, they have measured the effects of point source effluents on community-level endpoints, such as total invertebrate density, taxon richness, Ephemeroptera-Plecoptera-Trichoptera richness, Simpson's diversity index, Bray-Curtis index, biomass,

and emergence in benthic invertebrates (Cash et al. 2003, Culp et al. 2003, Culp et al. 2004). Life cycle endpoints in benthic invertebrates, such as reproduction, have not been directly measured in these systems. The measurement of reproductive endpoints may provide additional information about how an effluent affects a species. If community-level changes are documented in an effluent-exposed system, then a potential next step would be to look at effects at the level of the individual or population to help identify mechanisms underlying community-level changes. In order to understand effluent-related, adverse ecological effects, it is important to understand the effects of the effluent at each level of biological organization (e.g., individual, population, community) and on the interrelationships between these levels of organization (Rand et al. 1995). Research using these stream-side artificial stream systems also typically used substrates common to riffle habitats, such as rocks and pebbles (Culp et al. 2003). It is important to expand their applicability by using other substrates (e.g., sand).

1.4 Selected test species (*Chironomus tentans*)

Chironomus tentans is a species of non-biting midge that is widely distributed in freshwater environments. It was chosen as the test species for this thesis research for a number of reasons. First, it is a commonly used test species for freshwater toxicity testing. Sediment toxicity tests and laboratory-based life cycle bioassay techniques are well-established for *C. tentans* (ASTM 2000, Environment Canada 1997, USEPA 2000), and there is good inter-laboratory agreement from sediment toxicity tests carried out with *C. tentans* (Burton et al. 1996). Second, *C. tentans* is quite easy to culture and handle in the laboratory (Environment Canada 1997, USEPA 1993). Third, *C. tentans*

larvae have been successfully used *in situ* (Chappie and Burton 1997, Sibley et al. 1999). Finally, chironomids are an ecologically important component of many freshwater food webs, where the larvae comprise a large proportion of the benthic biomass (Armitage et al. 1995).

Chironomus tentans has four distinct life stages: egg, larva, pupa, and adult. Individuals typically undergo a complete life cycle within 23 to 30 days under laboratory conditions at 23°C (Benoit et al. 1997). At this temperature, eggs will hatch within two to six days of oviposition and will then remain with the gelatinous egg case, possibly using it as an initial food source, for up to 24 hours. The larvae go through four instars, during which time they live in the sediments in tubes constructed of sedimentary materials connected by mucous secretions. Towards the end of the fourth instar, the larvae cease feeding and pupation commences. Emergence tends to exhibit a bimodal distribution with the males emerging on average five days earlier than the females (Benoit et al. 1997). Females can be distinguished from the males by the absence of plumose antennae and by a much larger and darker abdomen (USEPA 1993). Females will generally produce one egg case about 24 hours after emergence. The adults typically die within seven days of emergence (Benoit et al. 1997).

1.5 Bioassay endpoint selection for test species (*C. tentans*)

1.5.1 Growth

Growth will be evaluated in the bioassay developed under this research by measuring weight, length and head capsule length several times during the larval stages. This endpoint may provide important information because growth of *C. tentans* has been

shown to be quite sensitive to contaminant exposure (Giesy et al. 1988, Timmermans et al. 1992). Growth was inhibited when *C. tentans* larvae were placed in sediments contaminated with metals (Besser et al. 1995). Growth of chironomid larvae was stimulated during exposure to low levels of a bleached kraft pulp mill effluent, and inhibited during exposure to high levels of the same effluent (Dubé and Culp 1996). In addition, alterations in growth may have serious consequences on a population of *C. tentans*. Growth affects survival and reproduction of *C. tentans*, so alterations in growth rate may have an effect on the population growth rate (Sibley et al. 1997). Larvae have to reach a certain size in order to be able to emerge (Liber et al. 1996, Sibley et al. 1997) and reproduce (Sibley et al. 1997, Sibley et al. 2001). In fact, changes in growth have been used to predict effects on reproduction and population-level endpoints (Sibley et al. 1997). In addition, reductions in growth have been correlated with effects seen in the field. A 30% inhibition in growth in a laboratory 10-day sediment toxicity test with *C. tentans* was correlated with a lack of chironomids in those sediments in the field (Giesy et al. 1988).

1.5.2 Survival

Survival will be measured in this bioassay because it is relatively easy to measure and it is a common endpoint across different bioassays (Harrahy and Clements 1997, Nebeker et al. 1988, Watts and Pascoe 2000). Percent survival will be calculated by dividing the number of animals that survived to the end of the bioassay by the number expected. Changes in survival can have important effects on population-level parameters (Barnthouse 1993). Almost all chemicals will have an effect on survival at

some concentration (Rand et al. 1995). The lethal toxicity of different chemicals varies widely, depending on factors such as species, life history, and water quality. Although survival is less sensitive than sublethal endpoints, such as reproduction and behaviour, results of a multi-generational study exposing *C. riparius* to cadmium suggested that effects on survival were more consistent than other life cycle endpoints, such as reproduction (Postma and Davids 1995).

1.5.3 Emergence

Emergence will be measured in this bioassay because it has been shown to be a good indicator of contaminant stress in *Chironomus* spp. (Gerhardt and Janssens de Bisthoven 1995, Ingersoll et al. 1990, Pascoe et al. 1989, Timmermans et al. 1992, Wentsel et al. 1978). Wentsel et al. (1978) observed that emergence of *C. tentans* was delayed by two days and the number of adults emerging was reduced by over three times after exposure to sediments containing high levels of cadmium, zinc, and chromium. The emergence of *C. riparius* was delayed after exposure to waterborne selenium at concentrations greater than 837 µg/L and percent emergence was reduced at 6050 µg/L of selenium in the water (Ingersoll et al. 1990). Emergence in benthic invertebrate communities has also been studied in artificial streams. Culp et al. (2003, 2004) and Cash et al. (2003) have observed reductions in numbers and delays in emergence after exposure to pulp mill effluent.

Alterations in emergence patterns and reductions in the number of adults emerging can have serious population-level consequences by reducing the number of individuals in a mating swarm, which may adversely affect mating success (Pascoe et al.

1989). Previous studies have demonstrated a correlation between emergence and larval growth rates (Liber et al. 1996). Therefore, factors that affect the growth rate of the developing larvae may also affect adult emergence patterns.

1.5.4 Reproduction

Reproduction will also be measured in the developed bioassay because reproductive processes are often quite sensitive to metal exposure (Langston 1990). Effects on reproduction may also result in population-level consequences (Luoma and Carter 1991). The reproductive endpoints measured in this bioassay include sex ratios, fecundity, and hatching success.

Sex ratios are a measure of the relative number of males to females in the population. Chironomid species typically have sex ratios close to 1:1 and there is little evidence that deviations from this typical sex ratio would be beneficial to aquatic insects (Armitage et al. 1995). In a clean sediment study, food quantity was used to manipulate the growth rate of *C. tentans*. At the lower feeding rates, sex ratios were skewed with a higher proportion of males, likely due to higher energy requirements for female emergence (Liber et al. 1996). However, some studies have found no significant effects on sex ratios after exposure to various environmental contaminants (Kahl et al. 1997, Pascoe et al. 1989, Sibley et al. 1996).

Fecundity is a measure of the number of offspring produced by each female. Fecundity in *C. tentans* is measured by a combination of two factors: number of egg cases per female and number of eggs per egg case. A reduction in either of these factors has the potential to reduce recruitment in the next generation. In a study where growth of

C. tentans larvae was manipulated by feeding rate, it was shown that the mean number of eggs per female decreased below a larval dry weight of 1.5 mg per individual (Sibley et al. 1997). The number of egg cases per female was not affected in *C. tentans* after life cycle exposure to 91 µg/L of 4-nonylphenol (Kahl et al. 1997), or to sediments contaminated with low levels of organic compounds (Benoit et al. 1997). However, Sibley et al. (1996) observed fewer eggs per female in treatments where zinc was available for uptake from the sediments.

A decrease in hatching success may have consequences on recruitment in the next generation. Some studies have concluded that hatching success is a relatively insensitive endpoint (Benoit et al. 1997, Gauss et al. 1985, Sibley et al. 1996). For example, there was no significant effect on hatching success in *C. tentans* after life cycle exposure to zinc in contaminated sediments (Sibley et al. 1996), or after growth was manipulated throughout the life cycle by feeding rate (Sibley et al. 1997). However, in the above studies, the egg cases were not oviposited directly into the treatment solution but, instead, were transferred to the treatment solution after being laid. In a different study where females oviposited egg cases directly into cadmium solutions of 30 and 300 mg/L, hatching success was 50% and 0%, respectively (Williams et al. 1987). When the egg cases are laid directly into the treatment solution, contaminants may be incorporated into the egg case as the process of water-hardening occurs, which may increase contaminant exposure to the embryos (Benoit et al. 1997).

1.6 Selection of test effluent

1.6.1 Metal mining effluent

Metal mines can have effects on aquatic systems in a variety of ways (Farak et al. 1998, Gunn et al. 1995, Havas et al. 1995). One impact of a metal mine on the aquatic environment is the increased metal loading that occurs downstream through the discharge of treated effluents with elevated levels of metals (Farak et al. 1998, Jaagumagi and Bedard 2002). The release of metals into the environment is an issue because metals do not biodegrade, so they can persist in the environment for extended periods of time. Metals are also known to be toxic to invertebrates (Luoma and Carter 1991). Heavy metals have been shown to decrease benthic invertebrate community density and diversity (Clements et al. 1988, Clements et al. 1992, Roline 1988). In invertebrates, metals have been shown to have effects on growth (Besser et al. 1995), survival (Postma and Davids 1995), emergence (Ingersoll et al. 1990, Pascoe et al. 1989, Timmermans et al. 1992, Wentzel et al. 1978), and reproduction (Luoma and Carter 1991, Sibley et al. 1996, Williams et al. 1987).

Metal mines in Canada are now required to perform environmental effects monitoring (EEM) in accordance with the *Metal Mining Effluent Regulations* (2002). The standard procedure for a typical EEM program is to conduct a field survey comparing fish and benthic invertebrate communities downstream of the mine to a reference site. An effect is defined as a significant difference in fish population-level endpoints (i.e., age, size-at-age, gonadosomatic index, condition, and liversomatic index) or benthic invertebrate community-level endpoints (i.e., total invertebrate density, taxon richness, Simpson's diversity index, and Bray-Curtis index) between the exposure

and reference sites (Environment Canada 2002). However, there are situations where standard field surveys are not possible because the receiving environment is too unsafe for a field survey or too confounded to determine the effects of a single effluent. In these situations, other tools, such as caged bivalve studies and artificial streams, have been approved as alternative methods to measure effluent effects (Environment Canada 2002). The *C. tentans* life cycle bioassay developed in this research may provide another tool for assessing the effects of MME on a benthic invertebrate.

1.6.2 Description of the study site

The project study site is located in Sudbury, ON, Canada. Metal mining has been occurring in the Sudbury region since 1883 (Jaagumagi and Bedard 2002), and the local environment has been severely impacted by these mining activities (Griffiths 1992, Matuszek et al. 1992, Nriagu et al. 1998). As a result of these impacts, there is decreased abundance and diversity in benthic invertebrate communities and fish populations in nearby watersheds (Conlon et al. 1992, Griffiths 1992, Griffiths and Keller 1992, Matuszek et al. 1992). Reductions in aerial emissions and remediation efforts have reduced the metal and sulphur dioxide inputs into the environment and have resulted in improved biological status of many of the surrounding water bodies (Gunn et al. 1995, Havas et al. 1995, Nriagu et al. 1998). Despite these improvements, historical effects due to acidification and metal loading remain (Havas et al. 1995, Keller 1992). The effects of mining and remediation efforts in the Sudbury region have been documented and provide an understanding of the effects of metal mining and acidification on terrestrial and aquatic ecosystems (Keller 1992). However, there has been less research

on the impacts of the current discharge of treated MMEs into receiving waters. Several metal mines operate in the area and continue to release contaminants into the aquatic receiving environment (Dubé et al. 2004b).

Junction Creek flows southwest through the city of Sudbury. Downstream of Sudbury, Junction Creek enters Kelly Lake. Further downstream, Junction Creek ends in McCharles Lake where the creek system joins the Vermillion River. Junction Creek is an example of a confounded receiving environment because it receives effluents from multiple point sources. In addition, Junction Creek has a long history of impact from metal mining in the region and various non-point sources of contamination (Jaagumagi and Bedard 2002). Treated MME is released into the creek from three wastewater treatment plants (WWTP) or mines that are currently in operation. Garson mine, which is located near the head of the creek, discharged 7.79×10^5 m³/year of treated MME into the creek in 2001, which comprised 20% of the creek's volume at the point of discharge (Dubé et al. 2004b). Nolin WWTP released 1.87×10^6 m³/year of treated MME in 2001, which comprised 30% of the creek's volume at the point of discharge. The Copper Cliff WWTP released 8.31×10^6 m³/year of treated MME in 2001, which comprised 45% of Junction Creek's volume at the discharge point (Dubé et al. 2004b). Other sources of contamination in Junction Creek include the following: historical metal deposition in sediments from previous mining activities, storm sewers and treated sewage discharge from the city of Sudbury, high levels of creosote from a former wood creosoting plant, and atmospheric deposition of metals from smelting activities. The presence of other factors confounds the assessment of current MME discharges on aquatic organisms in

Junction Creek. Thus, this study site provides a good opportunity to develop and test a new artificial stream bioassay.

Treated MME from the Copper Cliff WWTP was the effluent used in the study. Of the three MMEs released into Junction Creek, Copper Cliff WWTP releases the largest volume of effluent and comprises the largest portion of the creek flow at complete mix (Dube et al., 2004b). The Copper Cliff WWTP, which is located at Latitude N46° 28' 13.3" Longitude W81° 03' 37.3", has the capacity to treat 2.27×10^5 m³ of wastewater per day and can remove heavy metals and suspended solids. Dissolved metals are precipitated using pH adjustment with lime and settling. Suspended solids are removed by flocculent addition. After treatment, sulphuric acid is added to the effluent in order to return the treated effluent to a neutral pH (G. Watson, INCO Limited, Sudbury, ON, Canada, personal communication).

1.7 Specific research objectives

The overall objective of this research was to develop a more environmentally relevant toxicity bioassay. This research was undertaken in three phases. In phase I, the existing laboratory-based *C. tentans* life cycle bioassay (Benoit et al. 1997) was transferred to the artificial stream systems. During this phase, methodology for the *C. tentans* life cycle bioassay in artificial streams was developed under controlled conditions in the laboratory. In phase II (Chapter 2), the artificial stream life cycle bioassay was evaluated in the field using an environmentally relevant concentration of a MME from Copper Cliff WWTP. In phase III (Chapter 3), the artificial stream bioassay was tested in the laboratory using the same concentration of MME from Copper Cliff

WWTP. The life cycle bioassay developed by Benoit et al. (1997) was also run simultaneously in the laboratory using the same effluent in order to compare the sensitivity of the two bioassays. A comparison of responses in the field and laboratory artificial stream bioassays was also made. The specific objectives and a description of each chapter are outlined in Table 1.1. This thesis is written in articles format.

Table 1.1: Research objectives by chapter

Chapter	Objectives	Description of Chapter
1	Introduction	<ul style="list-style-type: none"> ➤ Background information including existing laboratory bioassays, existing outdoor exposure systems, biology of <i>Chironomus tentans</i>, biological endpoints, environmental effects of metals and metal mine effluents, and metal mine effluent treatment process
2	<ul style="list-style-type: none"> ➤ To develop a life cycle bioassay using <i>C. tentans</i> in artificial streams under field conditions ➤ To use the bioassay to test the effects of a metal mine effluent on the life cycle of <i>C. tentans</i> 	<ul style="list-style-type: none"> ➤ First-time implementation of the <i>C. tentans</i> life cycle bioassay in a field-based artificial stream system
3	<ul style="list-style-type: none"> ➤ To compare the life cycle bioassay in artificial streams to the beaker life cycle bioassay in comparative laboratory exposures ➤ To test the effects of a metal mine effluent on the life cycle of <i>C. tentans</i> in a laboratory setting ➤ To compare artificial stream bioassay responses in the laboratory and field 	<ul style="list-style-type: none"> ➤ Artificial stream bioassay evaluated under laboratory conditions to compare the utility and sensitivity to a standardized beaker bioassay
4	General discussion	<ul style="list-style-type: none"> ➤ Summary of project, synthesis of results, discussion of bioassay relevance, and future research directions

Chapter 2^a

**USING ARTIFICIAL STREAMS TO ASSESS THE EFFECTS OF METAL
MINING EFFLUENT ON THE LIFE CYCLE OF THE FRESHWATER MIDGE
(*CHIRONOMUS TENTANS*) *IN SITU***

^a This chapter has been accepted for publication in the journal of Environmental Toxicology and Chemistry under joint authorship with Monique G. Dubé (National Water Research Institute)

2.1 Abstract

In 2002, an *in situ* life cycle bioassay with *Chironomus tentans* in artificial streams was developed to evaluate the effects of a complex metal mine effluent (MME) under ambient environmental conditions. The bioassay was tested in the field using effluent from the Copper Cliff WWTP at INCO in Sudbury, ON, Canada. *Chironomus tentans* were exposed throughout the life cycle to 45% Copper Cliff effluent, which is the average effluent concentration measured in Junction Creek, ON, Canada, the natural receiving environment. *Chironomus tentans* in the effluent treatment had reduced survival ($p=0.001$), reduced total emergence ($p<0.001$), increased time to emergence ($p<0.001$), and reduced hatching success ($p=0.001$) relative to animals in the reference water treatment. *Chironomus tentans* in the effluent treatment were not significantly different from those in the reference in terms of growth, sex ratio, number of egg cases per female and number of eggs per egg case. This research showed how a life cycle bioassay could be used *in situ* to assess MME effects on a benthic invertebrate.

2.2 Introduction

There is a large degree of uncertainty when extrapolating from the laboratory to the field. Certain conditions, such as temperature, photoperiod, and water quality, which are held at optimal levels in laboratory bioassays, tend to fluctuate in the field. These fluctuations may affect the degree or nature of effect that a contaminant may have on a study organism (Bervoets et al. 1996, Bervoets and Blust 2000, Boone and Bridges 1999, Chappie and Burton 1997, Gauss et al. 1985, Lohner and Fisher 1990). As a result, it is not always possible to predict effects observed in the field based on results of

laboratory studies (Robinson et al. 1994, Tucker and Burton 1999). Thus, it is important to test the effects of complex effluent under field conditions in order to determine the nature and degree of response that an organism has to the effluent in the environment.

One method of evaluating the effects of contaminants under field conditions is through the use of artificial streams. In artificial streams, organisms are exposed to ambient temperature, water quality and photoperiod, so these systems are more environmentally realistic than laboratory exposures. Artificial streams can also be more controlled and replicable than typical field studies (Dubé et al. 2002a) and can be valid alternatives in situations where the study system is too confounded or too unsafe for field surveys. Various types of artificial streams have been developed by the National Water Research Institute of Environment Canada for studying the effects of point source effluents on community-level endpoints in benthic invertebrates and periphyton (Culp et al. 2000a, Culp et al. 2003) and individual-level endpoints in fish (Dubé et al. 2002b, Dubé et al. 2004b, Dubé et al. 2004c). The benefit of many of these designs is that the systems are mobile and field based (Dubé et al. 2002a, Dubé et al. 2002b).

Much of the previous work with benthic invertebrates in artificial streams has centred on the evaluation of community-level endpoints, such as growth, changes in community composition, and total cumulative emergence (Culp et al. 2000a, Culp et al. 2000b, Culp et al. 2003). Reproductive endpoints have not been fully evaluated and their inclusion could assist with understanding multigenerational effects that a contaminant may have on a population (Allan and Daniels 1982, Postma and Davids 1995, Sibley et al. 1997), as well as improve understanding of the mechanisms of community-level

changes. In addition, most of the previous artificial stream research has modelled erosional, rather than depositional, habitats (Culp et al. 2000b, Culp et al. 2003).

Thus, there is a need to develop bioassays for benthic invertebrates that evaluate the effects of contaminants on sublethal endpoints such as reproduction under conditions more similar to those experienced in the receiving environment. There are existing bioassays that evaluate an organism's sensitivity to a toxicant throughout its life cycle. Examples of these life cycle bioassays include the fathead minnow (*Pimephales promelas*) life cycle bioassay (Borton et al. 2000, Mount and Stephan 1967) and the *Chironomus tentans* life cycle bioassay (Benoit et al. 1997). In contrast with the 10-day toxicity test using *C. tentans*, which evaluates the effects of a contaminant on survival and growth (Environment Canada 1997), the life cycle bioassay with *C. tentans* assesses the effects on survival, growth, emergence, and reproduction (ASTM 2000, USEPA 2000). In addition, the life cycle bioassay evaluates effects on all larval stages, including the most sensitive early life stages (Benoit et al. 1997, Nebeker et al. 1984). However, this bioassay uses small numbers of animals in a tightly controlled laboratory environment to assess the effects of contaminants on the organisms (Benoit et al. 1997).

This research focused on the development of a bioassay with *C. tentans* in field-based artificial streams in order to study effluent effects throughout the life cycle under more environmentally realistic conditions. This bioassay was developed by applying components of the laboratory life cycle bioassay of Benoit et al. (1997) to an artificial stream system. The objectives of this chapter are to briefly describe the development of the life cycle bioassay using *C. tentans* in artificial streams and to use the developed bioassay to test the effects of a MME *in situ*.

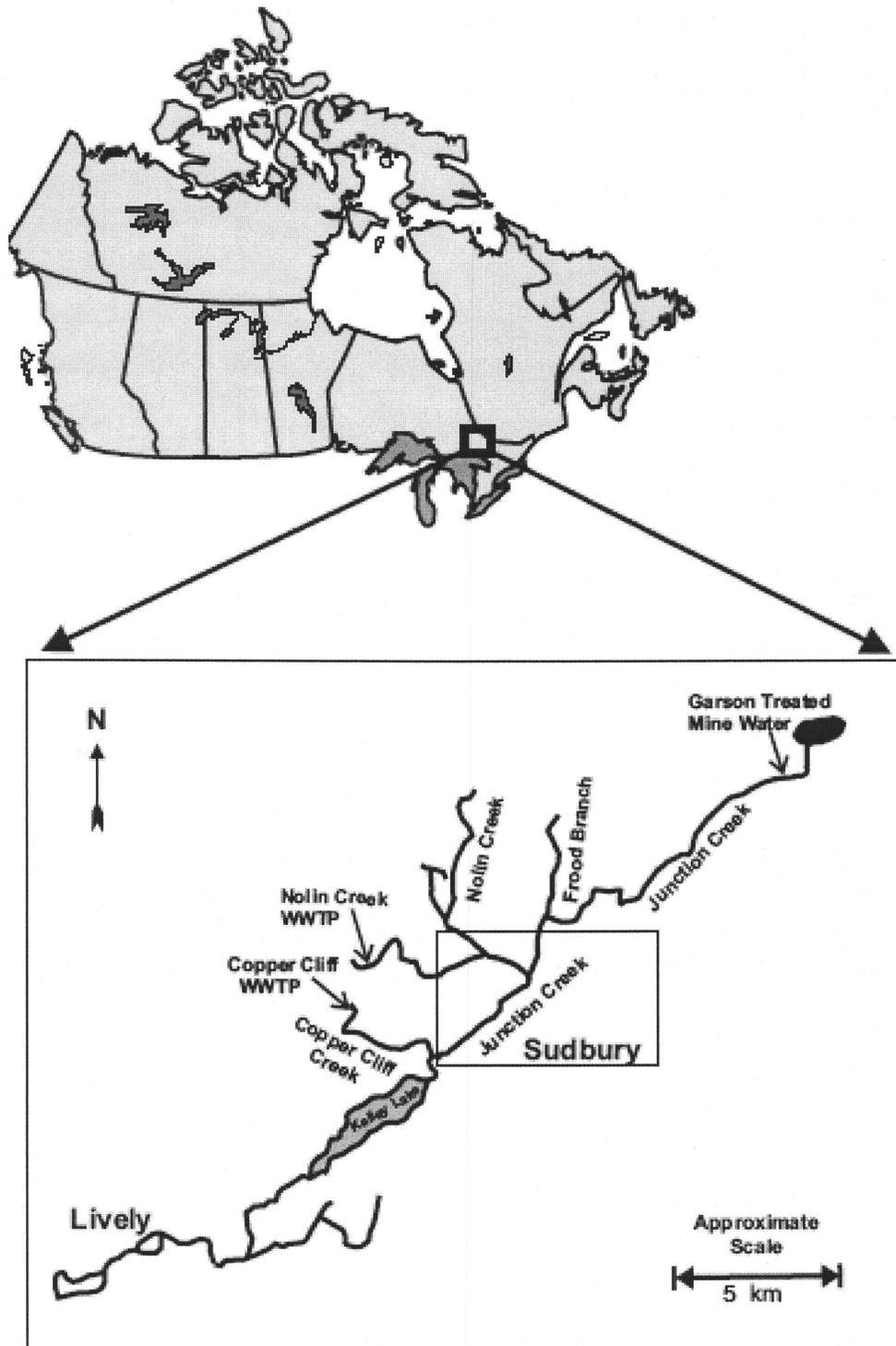
2.3 Materials and methods

2.3.1 Field site

Metal mining has taken place in the Sudbury region since 1883 (Jaagumagi and Bedard 2002) and significant effects on aquatic life have been documented (Conlon et al. 1992, Griffiths 1992, Griffiths and Keller 1992, Matuszek et al. 1992). The Copper Cliff WWTP, which is located near Sudbury, ON, Canada (Figure 2.1) and treats effluent from several metal mines in the region, discharged $8.31 \times 10^6 \text{ m}^3$ of treated effluent into Junction Creek in 2001. Effluent treatment consisting of pH adjustment and settling results in the precipitation of dissolved metals from solution prior to discharge. It has been estimated that the Copper Cliff effluent (CCE) comprises 45% [v/v] of the flow in Junction Creek at complete mix (Dubé et al. 2004b).

Junction Creek, which drains a watershed with an area of 329 km^2 , is 52 km in length and ranges from 2 to 30 m in width (Dubé et al. 2004b). The creek flows southwest through Sudbury into Kelly Lake. Further downstream, Junction Creek ends in McCharles Lake where the creek joins the Vermillion River. The Junction Creek system contains a diverse fish community including brook trout (*Salvelinus fontinalis*), fathead minnow (*Pimephales promelas*), creek chub (*Semotilus atromaculatus*), and pearl dace (*Semotilus margarita*), and a benthic invertebrate community dominated by chironomids and oligochaetes (Jaagumagi and Bedard 2002).

Junction Creek is a highly confounded system due to the number of point and non-point sources of pollution that are released into the creek. A number of mines have historically discharged effluent into the creek and several continue to operate. Atmospheric deposition from smelting activities has resulted in elevated levels of some



contaminants in surface runoff entering the creek (Jaagumagi and Bedard 2002). In addition, the Junction Creek system receives storm sewer and treated sewage discharges from the urban area. Finally, high levels of creosote (up to 4543 $\mu\text{g/g}$ in sediments) have been detected in some areas near a former wood creosoting plant (Jaagumagi and Bedard 2002). The confounded nature of Junction Creek requires the use of novel approaches such as artificial streams to elucidate the nature and magnitude of effects that individual MME might be having on benthic invertebrates

2.3.2 Artificial streams

In 2002, artificial streams were used to measure the responses of *C. tentans* to 45% CCE relative to reference water. The artificial streams were located at the Vermillion Water Treatment Plant in Lively, ON, Canada. Reference water was obtained from the Vermillion River through the raw water intake of the Vermillion Water Treatment Plant. The streams consisted of two tables (i.e., one per treatment) with six replicate 10.3 L circular high density polyethylene streams on each table (Figure 2.2). Each stream had a diameter of 0.3 m and a depth of 0.2 m. A propeller placed in the centre of each stream maintained a mean unidirectional water current of 8 cm/s. This low current velocity was selected to reduce disturbance of the sediments and, therefore, reduce stress in the animals (Lowell et al. 1995). Six replicate streams were used for each treatment and were set on a common table that drained into an 85 L dilution reservoir. The streams, table and dilution reservoir were self-contained on a shipping pallet.

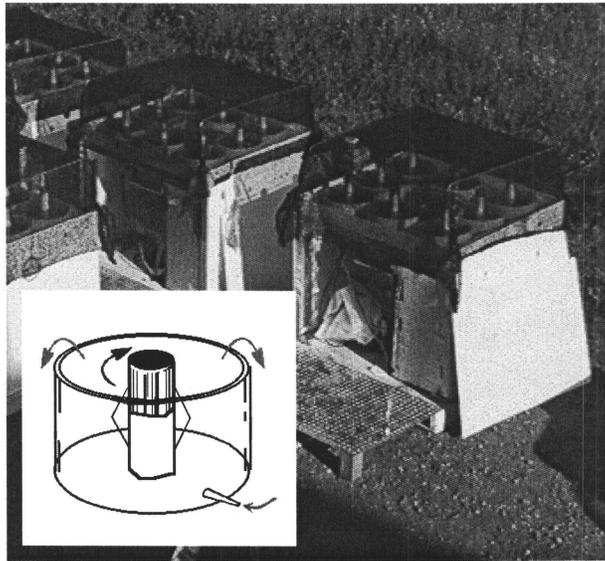


Figure 2.2: Photo of artificial stream systems. Inset is a schematic drawing of a single stream.

Treated CCE was transported to the site weekly. Effluent was stored on site in a high density polyethylene storage container. Water was added to the artificial stream reservoir for the reference water treatment at a rate of 24.5 L/hour in order to achieve a reservoir residence time of six hours. The same reservoir residence time was achieved in the 45% CCE treatment by pumping the effluent at 11.0 L/hour and the reference water at 13.5 L/hour. Chem-Feed[®] pumps (1500N, Blue-White Industries, Westminster, CA, USA) were used to deliver the treatment waters to the dilution reservoirs. The water and effluent were mixed in-line before reaching the reservoir. Using a March pump (Model LC-3CP-MD, March Manufacturing, Glenview, IL, USA), the mixture in the reservoir was pumped up to each stream on top of the table at a rate of 2 L/minute so that the residence time in each stream was five minutes. Excess water flowed over the side of the streams, onto the table, and back into the reservoir below. Excess water drained out of an overflow drain at the back of each reservoir. Shade cloth was placed over each table to reduce the amount of direct sunlight.

Each artificial stream received a 1 cm layer of washed silica sand (250-425 μm) substrate (Unimin Corporation, New Connan, CT, USA). At the beginning of the life cycle exposure, 250 μm nylon mesh screens were placed at the top edge of each stream to prevent the young larvae from being carried out of the stream with the overflowing water. Covers made of 250 μm nylon mesh were also placed over the water inflow pipes. At Day 20, the 250 μm mesh screens were replaced with emergence traps. Emergence traps were made of 500 μm nylon mesh that created a dome over each stream. A moveable plastic window allowed access to each stream.

Twelve breeding chambers (21 L covered aquariums) were set up, one for each artificial stream. Emergent adults collected from a given stream were placed into the corresponding breeding chamber. Two litres of the appropriate test solution (i.e., reference water or 45% CCE) were placed into each aquarium so eggs were laid in the treatment solution. About one litre of the solution was removed using a vacuum pump and replaced by fresh solution every two days.

An aspirator unit was used to transfer the adults from an artificial stream to its corresponding breeding chamber. The aspirator unit consisted of a 1 L Erlenmeyer flask with a two-holed rubber stopper (No. 9). Two pieces of glass tubing, each 5 cm long, were fit snugly into the two holes in the rubber stopper. One of these glass tubes was covered on the inner surface with a piece of 500 μm nylon mesh. The other end of this glass tube was attached to a piece of rubber tubing (3/8 inch i.d.) which was attached to a vacuum pump. The other glass tube was also attached to a piece of rubber tubing which was used for aspirating the adults.

Core samplers were used to collect sediment samples from the bottom of the streams during the larval stages. These core samplers were developed by modifying 60-cc plastic syringes. The core samplers (surface area = 9.62 cm^2) collected the sediments and larvae off the bottom of the artificial streams.

2.3.3 Life cycle bioassay

Twelve egg cases were obtained from Environmental Consulting and Testing (Superior, WI, USA). The egg cases were shipped overnight within 24 hours of oviposition. The number of eggs per egg case was estimated using the ring count method

(Benoit et al. 1997) so that survival could be estimated at the end of the experiment. The mean number of eggs per egg case was 927 ± 51 (mean \pm 1 SE).

Egg cases were acclimated to treatment water in individual containers by adding 25% of treatment water to each container twice a day for two days as the egg cases were starting to hatch out. The egg cases were also acclimated to ambient temperature by slowly lowering the temperature from 23°C (laboratory conditions) to 17°C (field conditions) over 36 hours. Two acclimation rates are suggested by the U.S. Environmental Protection Agency (2000). When shipping *C. tentans*, the recommended rate for adjusting water temperatures is no more than 2°C every 24 hours. When acclimating animals for testing, it is recommended that temperature be changed no faster than 1°C every one to two hours. We chose an intermediate rate of acclimation in an effort to begin exposure while the larvae were as young as possible, while attempting to prevent thermal shock.

Prior to the addition of the egg cases into the artificial streams, 5 mL of TetraMin[®] slurry was added to each stream. The TetraMin[®] slurry was prepared by blending 100 g of TetraMin[®] tropical flakes (Tetra, Blacksburgh, VA, USA) in 1 L of deionized water for 30 seconds or until the food had become a finely ground suspension (USEPA 1993). Once prepared, the food was stored frozen in 50 mL polypropylene centrifuge tubes.

One egg case was added to each stream after the initial stages of hatching were observed in all egg cases. The propellers in each stream were turned off for 24 hours following the addition of the egg cases in order to allow the larvae to finish hatching out

and become settled. Larvae were fed 10 mL of TetraMin[®] slurry per stream daily. Water inflow into the streams was turned off for one hour daily to facilitate feeding.

At Day 11, core samples were taken from the streams and examined under a dissecting microscope. It was noted that the survival of the early instar larvae was low, even in the reference streams (i.e., four larvae observed in eighteen core samples from reference streams). Therefore, 75 – 11 day old larvae were added to each stream on Day 11. These larvae originated from a culture maintained at the National Water Research Institute in Saskatoon, SK, Canada. This culture was initially started six months earlier with egg cases from Environmental Consulting and Testing (Superior, WI, USA). A sample of 75 larvae were collected from the culture at this time and preserved in 10% formalin for initial biomass measurements.

Core samples were collected from each of the streams on three occasions (Days 14, 17, and 20) after the addition of the 11 day old larvae. At least three to five individuals per stream were collected at each sampling period. Core samples were preserved in 10% formalin and taken back to the laboratory for sorting.

Emergence traps were checked daily for adults. Adults were collected with the aspirator, counted and gender determined. Females have a larger and darker abdomen than the males. Males have plumose antennae and genital claspers on the posterior end of the abdomen (USEPA 1993). Adults were then transferred to the breeding chambers. Breeding chambers were checked every morning for egg cases and dead adults. Dead adults were removed from the breeding chambers by vacuum pump. Egg cases were collected using a 60-cc syringe with a 40 cm long glass tube (2 mm i.d.) attached to the tip. The number of egg cases in each breeding chamber was recorded. Secondary egg

cases, which are smaller (up to 200 eggs) and laid by females after the primary egg case, were not counted because they are more prone to fungal infection (Benoit et al. 1997). The number of eggs per egg case was determined using the ring count method in a maximum of two egg cases per breeding chamber per day. One of these counted egg cases from each breeding chamber per day was placed into a 100- × 15-mm plastic Petri dish with 30 mL of overlying treatment solution and allowed to incubate for six days at room temperature. Hatching success was determined after six days by counting the number of eggs that had not hatched and subtracting this number from the number of eggs estimated using the ring count method (Benoit et al. 1997).

The bioassay was terminated on July 5, 2002 after 37 days of exposure. Typically, the life cycle is terminated after emergence had ceased for 7 days (ASTM 2000, USEPA 2000). This bioassay was terminated after peak emergence had already occurred in all streams. All sediments from each stream were collected and preserved in 10% formalin and taken back to the laboratory for sorting. Upon sorting the sediments, it was determined that there were very few larvae/pupae (i.e., 4.3 ± 1.1 individuals per stream) left in the streams at the end of experiment.

2.3.4 Laboratory methods

Core samples were sorted under a dissecting microscope (12×) and the number of individuals per core sample recorded and used in final survival calculations. Sorted larvae were preserved in 95% ethanol. Total body length was relatively variable due to the bending of some larvae. Therefore, head capsule length was also recorded in order to provide a less variable measure of larval body size. Length and head capsule length were

measured for each larva using a camera lucida and digitizing pad (Dubé et al. 1997). Larvae were then dried in pre-weighed aluminum weigh boats at 60°C until a constant dry weight was achieved. The larvae were then weighed with the weigh boats using a Cahn C-31 microbalance (Thermo Electron, Waltham, MA, USA). The weight of each larva was determined by subtracting the initial weight of the aluminum weigh boat from the final weight of the larva in the weigh boat. Growth curves were plotted using the weights and lengths of the larvae from the three core samples, initial biomass measurements (Day 11), and the larvae remaining in the sediments at the end of the test.

Survival was calculated by dividing the number of adults that had emerged from a given replicate by the number of adults that could have potentially emerged from that replicate. The latter was calculated by subtracting the number of individuals that were removed with either core samples or that were still in the sediments at the end of the experiment from the total number of individuals that were added to that stream.

2.3.5 Physical and chemical measurements

Temperature, dissolved oxygen, and conductivity were measured in all streams three times per week throughout the experiment. Ammonia and pH were measured three times per week in one stream per treatment. Optic stowaways[®] (Onset Computer Corporation, Bourne, MA, USA) were placed on top of both treatment tables, in both reservoirs, and in the water and the CCE head tanks to record temperature continuously throughout the experiment. Water hardness was measured once a week in one stream per treatment and in the 100% CCE head tank using a drop count titration test kit (Hach, Loveland, CO, USA).

Water and effluent samples were taken on a weekly basis from the reference water table, the 45% CCE table and the 100% CCE head tank for water chemistry analysis. Samples were collected, preserved, and analyzed following standardized methods at an accredited laboratory (Testmark Laboratories, Sudbury, ON, Canada). Total metal (i.e., aluminum, antimony, arsenic, barium, boron, beryllium, cadmium, chromium, cobalt, copper, gallium, iron, lanthanum, manganese, mercury, molybdenum, nickel, rubidium, selenium, silver, strontium, thallium, uranium, vanadium, and zinc) and general chemistry (i.e., ammonia, calcium, chloride, dissolved organic carbon, fluoride, magnesium, nitrate, nitrite, ortho-phosphorus, phosphorus, potassium, sodium, sulphate, total kjeldahl nitrogen, total organic carbon, and total phosphorus) concentrations were measured.

2.3.6 Statistics

All statistical analyses were performed with Systat[®] 9 (SPSS, Chicago, IL, USA). Linear regressions were run for each growth parameter (i.e., weight, total body length, and head capsule length) as a function of time for each treatment. Analysis of covariance was used to determine whether there were differences in slope or *y*-intercept between the two treatments. The same analysis was done on the regression of weight as a function of total body length. Repeated measures analysis of variance (ANOVA) was used to determine whether the pattern of emergence differed between the two treatments. *T*-tests were used for all other endpoints. Parametric assumptions were tested by examination of the residual patterns. If the assumptions were not met, transformations were performed on the data. All growth data was log₁₀ transformed. Percentage data

(i.e., survival and hatching success) was transformed using $\arcsin\sqrt{x}$. In order to calculate the average metal or nutrient concentrations in the water and effluent, one half of the detection limit was used any time the measurement was below the detection limits.

2.4 Results

2.4.1 Water and effluent quality

Twelve metals were measured in the 100% CCE. Eleven of these metals were measured in the 45% CCE and six were measured in the reference water (Table 2.1). All of these metals except arsenic and zinc were present at significantly higher concentrations in the 45% CCE treatment than in the reference water. Barium, cobalt, iron, and nickel were two to five times higher in the 45% effluent treatment. Boron, copper, lithium, rubidium, and strontium were about 10 to 22 times higher in the 45% CCE than in the reference water. Selenium was 64 times higher in the 45% effluent treatment compared to the reference water treatment.

Other general water quality variables were measured in the effluent (Table 2.2). Eight of these variables (i.e., ammonia, total kjeldahl nitrogen, sulphate, calcium, chloride, magnesium, potassium, and sodium) were significantly higher in the 45% CCE than in the reference water ($\alpha=0.05$).

Temperatures in the artificial streams reflected air temperatures with less extreme diel fluctuations. The mean temperature was $19.86 \pm 0.08^\circ\text{C}$ in the reference streams and $19.53 \pm 0.08^\circ\text{C}$ in the 45% CCE streams. The mean pH was 8.5 ± 0.2 and 8.2 ± 0.1 in the reference water and 45% CCE streams, respectively. The pH increased

Table 2.1: Mean metal concentrations (mean \pm SE) in reference water, 45% Copper Cliff effluent (CCE), and 100% CCE ^{a,b}

	Detection Limit ($\mu\text{g/L}$)	Reference Water ($\mu\text{g/L}$)	45% CCE ($\mu\text{g/L}$)	100% CCE ($\mu\text{g/L}$)	WQG ^c ($\mu\text{g/L}$)
Arsenic	2	1.1 \pm 0.1 A	1.6 \pm 0.3 A	3.0 \pm 0.3	5
Barium	2	12.3 \pm 0.7 A	30.9 \pm 0.6 B	52.1 \pm 0.9	
Boron	10	5.1 \pm 0.1 A	52 \pm 4 B	100 \pm 7	
Cobalt	2	1.0 \pm 0.0 A	2.3 \pm 0.4 B	4.6 \pm 0.5	
Copper	5	6 \pm 2 A	83 \pm 10 B	175 \pm 21	2-4
Iron	20	311 \pm 37 A	948 \pm 40 B	1652 \pm 79	300
Lithium	5	2.3 \pm 0.2 A	41 \pm 5 B	80 \pm 10	
Nickel	3	17 \pm 2 A	78 \pm 12 B	145 \pm 28	25-150
Rubidium	2	1.3 \pm 0.2 A	29.0 \pm 0.8 B	61.3 \pm 1.5	
Selenium	2	1 \pm 0 A	64 \pm 28 B	149 \pm 63	1.0
Strontium	2	36 \pm 2 A	576 \pm 96 B	1229 \pm 231	
Zinc	5	9 \pm 3 A	9 \pm 1 A	14 \pm 2	30

^a For concentrations below detection limits, a value of $\frac{1}{2}$ the detection limit was used to calculate the means; $n=7$

^b Values followed by the same letter are not significantly different between 45% CCE and reference water ($p < 0.05$)

^c WQG - Canadian Water Quality Guidelines for the Protection of Aquatic Life (CCME, 1999)

Table 2.2: Water quality variables (mean \pm SE) in the reference water, 45% Copper Cliff effluent (CCE), and 100% CCE ^{a,b}

	Detection Limit (mg/L)	Reference Water (mg/L)	45% CCE (mg/L)	100% CCE (mg/L)
Ammonia	0.005	0.04 \pm 0.01 A	1.39 \pm 0.07 B	2.6 \pm 0.3
Total Kjeldahl Nitrogen	0.05	0.25 \pm 0.03 A	2.11 \pm 0.05 B	4.3 \pm 0.3
Nitrate	0.1	0.3 \pm 0.1 A	0.8 \pm 0.7 A	1.6 \pm 0.7
Sulphate	0.1	14.4 \pm 0.7 A	737 \pm 22 B	1719 \pm 30
Ortho-Phosphorus	0.005	0.019 \pm 0.002 A	0.020 \pm 0.002 A	0.020 \pm 0.002
Dissolved Organic Carbon	1	4.8 \pm 0.4 A	4.2 \pm 0.1 A	3.9 \pm 0.3
Total Organic Carbon	1	5.5 \pm 0.5 A	4.7 \pm 0.5 A	5.0 \pm 0.7
Calcium	0.1	12 \pm 1 A	256 \pm 7 B	562 \pm 20
Chloride	0.1	6.6 \pm 0.3 A	75 \pm 7 B	150 \pm 15
Magnesium	0.1	2.4 \pm 0.1 A	27 \pm 3 B	55 \pm 5
Potassium	0.05	0.82 \pm 0.01 A	16.8 \pm 0.4 B	35.8 \pm 1.0
Sodium	0.1	5.0 \pm 0.2 A	58 \pm 1 B	115 \pm 3

^a For concentrations below detection limits, a value of ½ the detection limit was used to calculate the means; $n=7$

^b Values followed by the same letter are not significantly different between 45% CCE and reference water ($p \leq 0.05$)

over the course of the experiment from approximately 7.7 to 9.1. All other water quality variables remained constant throughout the experiment. Dissolved oxygen was maintained above 8 mg/L (i.e., above 90% saturation) throughout the experiment. Ammonia, water hardness, and conductivity were all higher in the 45% CCE streams than in the reference streams due to the composition of the 100% CCE. The average ammonia concentration was 0.1 ± 0 mg/L in the reference streams and 1.2 ± 0.1 mg/L in the 45% CCE streams. Water hardness was 59 ± 3 mg/L as CaCO_3 in the reference treatment and 793 ± 21 mg/L as CaCO_3 in the 45% CCE treatment. Conductivity was 91 ± 1 $\mu\text{S}/\text{cm}$ in the reference streams and 1201 ± 9 $\mu\text{S}/\text{cm}$ in the 45% CCE streams.

2.4.2 Biological endpoints

Growth of the larvae was not significantly affected by the 45% CCE treatment. Linear regressions were performed on various relationships of growth endpoints. Neither the slopes nor the y -intercepts were significantly different between the two treatments for any of the following relationships: length over time (Figure 2.3A), head capsule length over time (Figure 2.3B), weight over time (Figure 2.3C), and weight versus length (Figure 2.3D).

Mean survival in the 45% CCE treatment was reduced by 82% relative to the reference water treatment (t -test; arcsine transformed; $p=0.001$; Figure 2.4). Mean survival in the reference water treatment was low (16%) due to high mortality in the early life stages under ambient conditions. Despite the low survival rates in the reference streams, there was an average of 146 ± 7 animals that emerged from each replicate in the reference treatment.

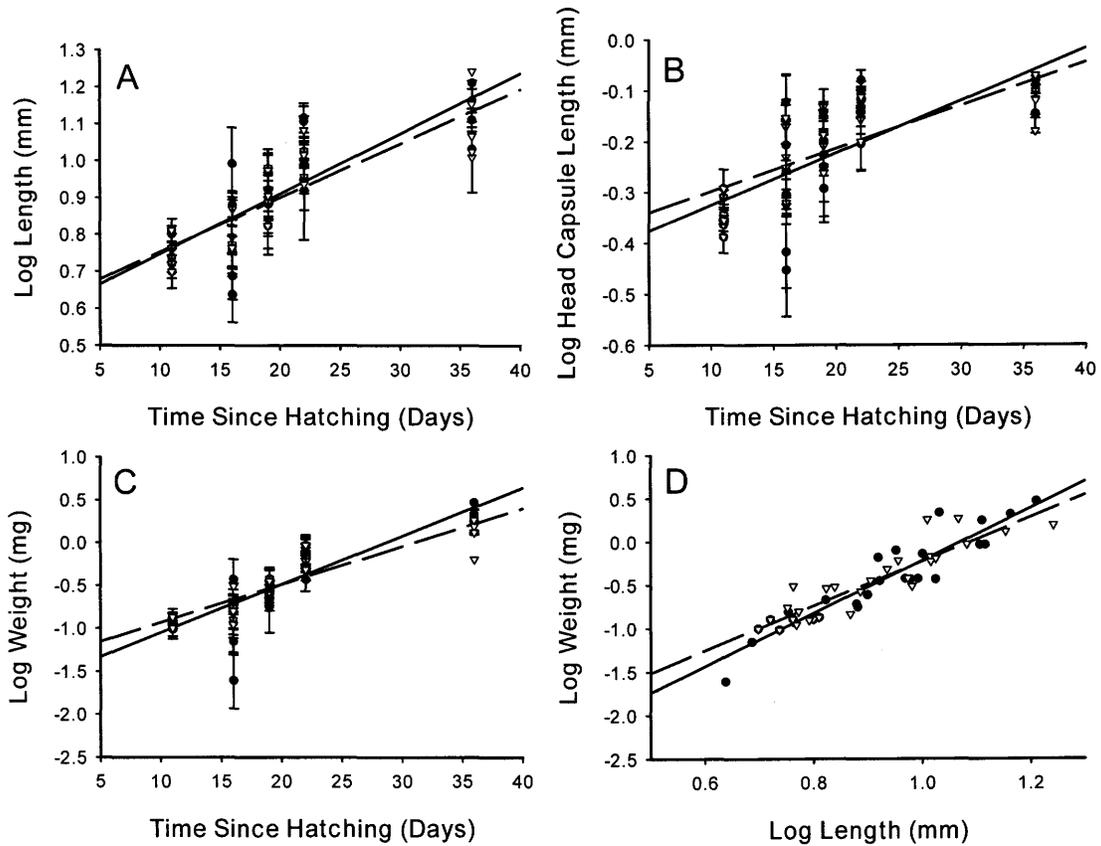


Figure 2.3: Log length (mm) over time (A), log head capsule length (mm) over time (B), log weight (mg) over time (C), and log weight as a function of log length (D) of *C. tentans* after life cycle exposure to reference water (white triangles; solid lines) or 45% Copper Cliff effluent (black circles; dashed lines) in artificial streams, Sudbury, ON, 2002. None of these four growth indices show a significant effect at $\alpha=0.05$.

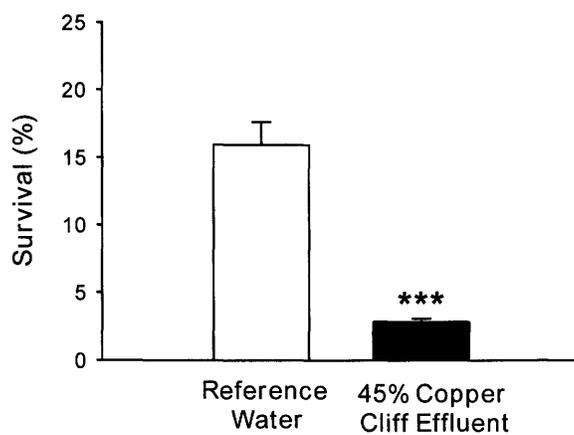


Figure 2.4: Mean ($\bar{x} \pm 1$ SE) survival (%) of *C. tentans* in reference water (white bar) and 45% Copper Cliff effluent (black bar) after life cycle exposure in artificial streams, Sudbury, ON, 2002. *** Indicates a significant difference at $p < 0.001$.

The pattern of emergence was significantly different between the two treatments (repeated measures ANOVA; $p < 0.001$) for both males (Figure 2.5A) and females (Figure 2.5B). There was a significant treatment effect, a significant time effect, and a significant interaction between these factors suggesting the effects of the CCE were time dependent.

Sex ratios (M/F) of adults were not significantly affected by the 45% CCE (data not shown). Neither the number of egg cases per female (Figure 2.6A) nor the number of eggs per egg case (Figure 2.6B) was significantly affected by the 45% CCE treatment. However, the mean number of hatched eggs was significantly reduced in the 45% CCE treatment relative to the reference water treatment (t -test; $p = 0.003$; Figure 2.6C). As a result, mean hatching success was significantly reduced from 84% in the reference treatment to 72% in the 45% CCE treatment (t -test; arcsine transformed; $p = 0.001$; Figure 2.6D).

2.5 Discussion

Additional tools are needed to measure the effects of MME on the aquatic environment. These tools should have the capacity to measure chronic, sublethal endpoints in an environmentally realistic setting. The ability to measure effects under ambient environmental conditions increases the relevance of effects assessments. There are currently a variety of methods that have been developed for testing the effects of contaminants under ambient conditions.

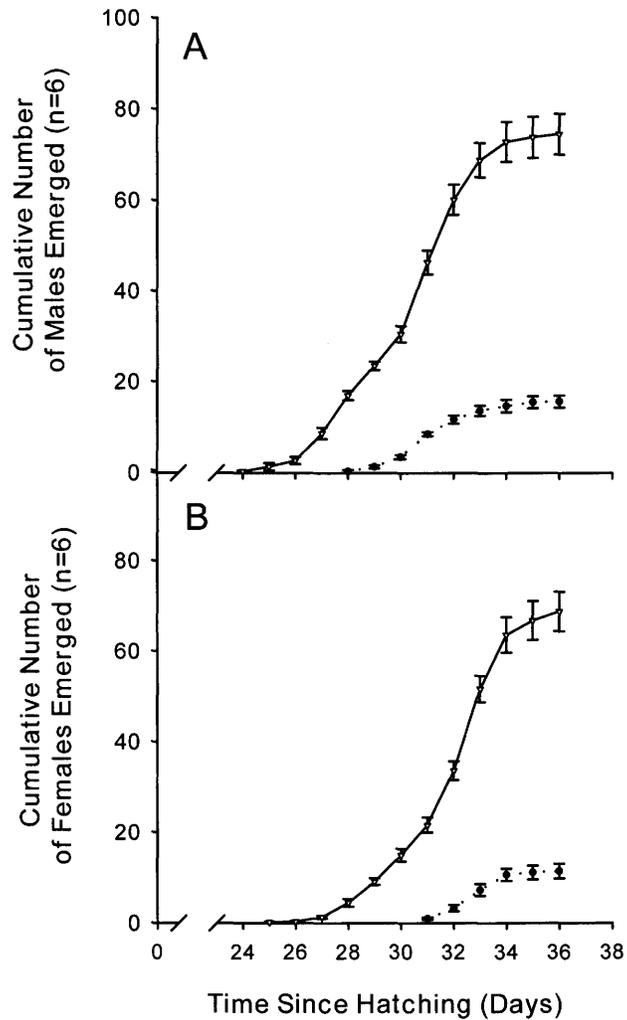


Figure 2.5: Cumulative emergence of *C. tentans* males (A) and females (B) after exposure to reference water (solid line) and 45% Copper Cliff effluent (dotted line). There was a significant treatment effect, a significant time effect, and a significant time \times treatment interaction (repeated measures ANOVA $p < 0.001$).

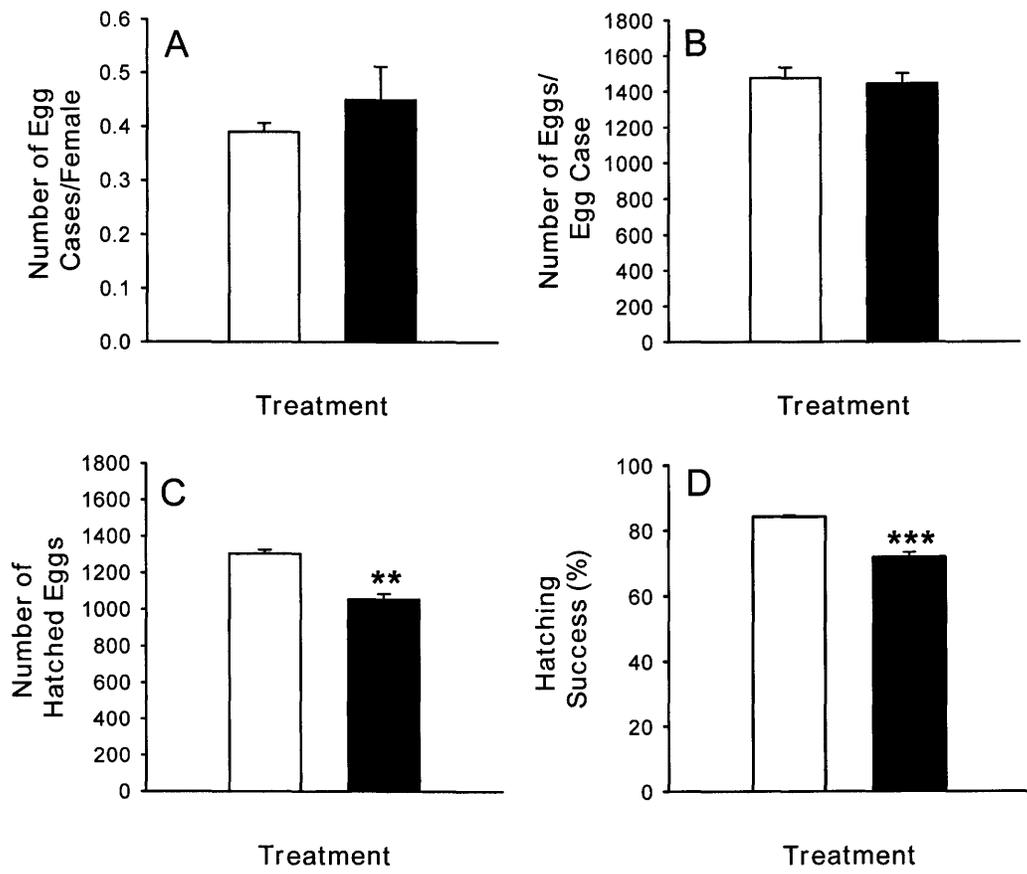


Figure 2.6: Mean number of egg cases per female (A), number of eggs per egg case (B), number of hatched eggs (C), and percent hatching success (D) in *C. tentans* after life cycle exposure to reference water (white bars) or 45% Copper Cliff effluent (black bars) in artificial streams, Sudbury, ON, 2002. ** Indicates a significant difference at $p < 0.01$; *** indicates a significant difference at $p < 0.001$.

In typical *in situ* tests, laboratory-cultured animals are placed into small, enclosed chambers which are then placed into the aquatic system at exposed and unexposed sites (Chappie and Burton 1997, Greenberg et al. 2002, Tucker and Burton 1999). Artificial streams have been used to evaluate the effects of various effluents or concentrations of effluent on invertebrate communities or fish *in situ* (Culp et al. 2000a, Culp et al. 2000b, Dubé et al. 2002a, Dubé et al. 2002b). However, invertebrate reproductive endpoints are not typically incorporated in the studies. Reproductive endpoints are often sensitive to various contaminants (Benoit et al. 1997, Pascoe et al. 1989) and changes in reproductive indicators could result in multigenerational effects on populations (Allan and Daniels 1982) as well as lead to instability in community composition.

The life cycle bioassay in artificial streams was derived from the beaker life cycle bioassay designed by Benoit et al. (1997). Both bioassays measure effects on the same types of endpoints (i.e., growth, survival, emergence, and reproduction), although the methods by which the endpoints were measured were altered in the artificial stream life cycle bioassay. First, a much larger number of individuals were used in each replicate. The beaker bioassay uses 12 animals per replicate (Benoit et al. 1997), whereas an estimated average of 1002 ± 51 animals were placed into each replicate artificial stream. This stocking density was a combined estimate based on the number of eggs in the original egg cases in addition to the 75 – 11 day old larvae. A larger number of individuals may provide greater precision in the measurement of some endpoints. In the future the two bioassays will be compared to evaluate the differences in variability and sensitivity in the endpoints (Chapter 3). Second, growth was measured over time in

the artificial stream bioassay, whereas growth is measured at only one point in the development process in the beaker bioassay (Benoit et al. 1997). This may be important if a contaminant causes the growth rate to change over time. For example, when Timmermans et al. (1992) exposed *C. riparius* to 0.010 mg/L or 0.025 mg/L of cadmium or a control treatment for part of the life cycle, they found that growth of first and second instar larvae was impaired with exposure to cadmium, but development of third and fourth instar larvae increased enough in the cadmium treatments that they emerged before the control animals.

The *C. tentans* artificial stream life cycle bioassay was successfully implemented in the field where animals were exposed to more environmentally relevant conditions. One advantage artificial streams have over most *in situ* designs is that the streams can be used to evaluate the effects of various concentrations of effluents or different effluents individually and in combination. Thus, it is possible to determine which effluents may be causing the greatest effects on the aquatic system and at what concentrations (Dubé et al. 2002a, Dubé et al. 2002b). However, unlike previous studies in artificial streams, the effects of a complex effluent were evaluated on reproductive endpoints in an invertebrate in the present study. Thus, the *C. tentans* life cycle bioassay in artificial streams provided information on effluent effects over the life cycle of the organism and under realistic environmental exposure conditions. This was also the first application using a depositional substrate in this type of artificial stream system.

2.5.1 Effluent-related effects

While previous studies have found growth to be a sensitive endpoint (Sibley et al. 1996, Sibley et al. 1997, Timmermans et al. 1992, Wentsel et al. 1977), no significant effects on *C. tentans* growth were observed in the present study. Wentsel et al. (1977) observed an inhibition of larval growth in sediments with high levels of cadmium (1030 µg/g), zinc (17 300 µg/g), and chromium (1640 µg/g). Reduced growth was also observed in *C. tentans* after life cycle exposure to zinc-spiked sediments (Sibley et al. 1996). Significant growth impairments were observed when *C. riparius* was exposed to copper concentrations as low as 0.090 mg/L (Timmermans et al. 1992). Sibley et al. (1997) have found strong correlations between growth inhibition of *C. tentans* after 10 days and other endpoints, such as emergence and reproduction. A minimum dry weight of 0.6 mg/individual must be reached for emergence to take place and reproductive output was reduced in individuals below a body mass of 1.5 mg/individual (Sibley et al. 1997).

The present study showed no significant effects on growth even though effects on both emergence and hatching success were observed. This may be because the effluent did not affect growth; a hypothesis supported by recent work that showed larvae exposed for 10 days in the beaker life cycle bioassay to 45% CCE showed no significant difference in growth relative to the larvae in the reference water treatment (Chapter 3). It is also possible that growth effects were not observed in this study because a staggered stocking of organisms in the streams at the start of the study increased the variability in growth measurements.

Exposure to 45% CCE caused a significant reduction in *C. tentans* survival relative to the reference streams. Survival is a commonly used endpoint in toxicity testing with *C. tentans* (Environment Canada 1997, Ingersoll et al. 1995, USEPA 2000). A number of studies have evaluated the lethal effects of different metals (Fargašová 2001, Nebeker et al. 1984, Williams et al. 1986) and metal contaminated sediments (Kemble et al. 1994, West et al. 1993) on aquatic invertebrates. For *C. tentans*, the LC₅₀ for copper ranges from 77.5 to 1690 µg/L, depending on the instar and length of test (Nebeker et al. 1984). Nebeker et al. (1984) found that first instars were most sensitive to copper over short term exposures with a 96 hour LC₅₀ of 298 µg/L, which is much lower than the 96 hour LC₅₀ of 1690 µg/L for the fourth instar larvae. However, after 20 days of exposure, the LC₅₀ for fourth instar larvae was 77.5 µg/L of copper (Nebeker et al. 1984). This concentration is similar to the 83 ± 10 µg/L of copper measured in the 45% effluent treatment in our study. In a *C. tentans* life cycle test, survival at 20 days was the only endpoint that showed a significant effect due to 4-nonylphenol exposure (Kahl et al. 1997). Postma and Davids (1995) also found that mortality was the most consistent endpoint when *C. riparius* was exposed to cadmium for nine successive generations.

Survival in the reference streams was lower than 65%, which is the minimum acceptable survival for *C. tentans* in the negative control in the laboratory-based life cycle bioassay (ASTM 2000, USEPA 2000). However, we do not believe the low survival affected our ability to measure effluent effects. It is not surprising that the survival was low under the variable ambient conditions encountered in the field. Newly hatching, laboratory-reared larvae were placed in the artificial streams, and there was

very high mortality in these first instar larvae. Mortality may have been caused by overcrowding. The density of animals at the start of the experiment was about 1.3 larvae/cm², which is three times higher than the density in the beaker life cycle bioassay (ASTM 2000, USEPA 2000). However, similar mortality of early instar larvae was not observed in previous trials of the artificial stream life cycle bioassay in the laboratory with similar or higher stocking densities (Hruska, unpublished data). Furthermore, natural field densities can often be several times higher than the stocking density used in this study. Coffman and Ferrington (1984) report natural field densities of up to 5 organisms/cm². Therefore, it is unlikely that mortality was caused by overcrowding.

Alternatively, high mortality may have been a result of the relatively low temperatures that were experienced at the start of the experiment (e.g., minimum water temperature of about 8°C). Egg cases were acclimated to ambient temperature and water quality conditions. However, the low night-time temperatures may have caused increased mortality. Survival of 8 to 12 day old *C. tentans* larvae was lower in 5°C and 11°C treatments compared to the 20°C treatment (Chappie and Burton 1997). This sensitivity to lower temperatures may be even greater in an earlier instar.

We are of the opinion that low survival in the reference streams did not affect our ability to measure the effluent effects because a large number of animals survived in all the reference streams (146 ± 7). In contrast, only 10 larvae are used in the standard 10-day *C. tentans* toxicity test (Environment Canada 1997) and 12 larvae are used in the *C. tentans* beaker life cycle bioassay (ASTM 2000, Benoit et al. 1997, USEPA 2000). Thus, with high levels of mortality in these beaker bioassays, it would not be possible to measure other endpoints such as growth and reproduction. The streams were restocked

on Day 11 with 11 day old larvae. The consequence of this is that some of the larvae were not exposed to the effluent during the first two instars, which may have confounded some of the effects observed in the present study. Future implementations of the artificial streams bioassay will use larvae that are about 10 days old to start the experiment.

Exposure to the 45% CCE caused both a significant decrease in the number of adults emerging and a significant increase in the time to emergence. Other studies have observed effects on emergence after exposure to various toxicants. Wentzel et al. (1978) observed a reduction in the number of adults and a delay in adult emergence from sediments with high levels of cadmium (1030 $\mu\text{g/g}$), zinc (17 300 $\mu\text{g/g}$), and chromium (1640 $\mu\text{g/g}$). Pascoe et al. (1989) saw a slight delay in emergence of *C. riparius* after chronic exposure to 0.15 mg/L of cadmium and the total numbers of adults emerging were correlated with survival during the larval stages. Ingersoll et al. (1990) observed a significant increase in the day of first emergence and emergence time for *C. riparius* exposed to >837 $\mu\text{g/L}$ of selenium, and a significant decrease in percent emergence at 6050 $\mu\text{g/L}$ of selenium.

Emergence in the reference streams was below 50%, which is the recommended level for test acceptability in the standardized laboratory procedures for the *C. tentans* life cycle bioassay (ASTM 2000, USEPA 2000). Low levels of emergence were a result of low survival of early instar larvae. The low level of emergence should not have affected the measurement of other endpoints because there were still a large number of animals that emerged from the reference streams (146 ± 7 individuals per stream). Low survival was not unexpected as the present study was carried out under variable ambient

conditions in the field, rather than under highly controlled conditions in the laboratory. Reductions in the number of emerging adults and alterations in emergence patterns observed in the present study may have important implications for a chironomid population because these factors may reduce the number of adults in a swarm, which may cause a reduction in mating success (Pascoe et al. 1989).

In the present study, the 45% CCE treatment did not have an effect on the sex ratio of emerged adults. This is consistent with other studies which have found no differential sensitivity between sexes (Kahl et al. 1997, Pascoe et al. 1989, Sibley et al. 1996). The 45% CCE treatment had no effect on the number of egg cases per female or the number of eggs per egg case. Thus, although there was a reduction in the emergence of individuals exposed to 45% CCE, the effluent did not appear to have an effect on the ability of those individuals that emerged to produce eggs. In tests using the standardized *C. tentans* life cycle bioassay in the laboratory, no significant differences in the number of eggs per egg case were observed between the treatments and the controls after exposure to concentrations of up to 91 µg/L of 4-nonylphenol (Kahl et al. 1997) or exposure to sediments contaminated with low levels of organic contaminants (Benoit et al. 1997). In contrast, Sibley et al. (1996) observed that fewer eggs were produced in zinc-spiked sediments.

The 45% CCE treatment caused a significant decrease in the hatching success of egg cases. This is consistent with Williams et al. (1987) who found decreases in the hatching success of egg cases laid directly into cadmium solutions. In contrast, other studies have found hatching success to be an insensitive endpoint (Gauss et al. 1985, Powlesland and George 1986, Sibley et al. 1996). In these latter studies, however, egg

cases were not placed into the test medium until after they were laid. Thus, the eggs may be more sensitive to contaminant exposure if they are laid directly into the test medium, as was done in the present experiment, rather than placed into the test medium after they are laid (Benoit et al. 1997).

Exposure to 45% CCE caused reduced survival, emergence, and hatching success compared to animals in the reference water. Copper Cliff effluent is a complex effluent comprised of several components that may have caused the responses observed in this study. Several metals (i.e., barium, boron, cobalt, copper, iron, lithium, nickel, rubidium, selenium, and strontium) were measured at higher concentrations in the 45% CCE treatment relative to the reference treatment (Table 2.1). In addition, other general chemistry variables (i.e., ammonia, calcium, chloride, magnesium, potassium, sodium, sulphate, and total kjeldahl nitrogen) were elevated in the effluent treatment (Table 2.2). There was also a pH increase in both treatments over the course of the study, which may have been a factor affecting metal bioavailability. Finally, chemicals, such as flocculents, added during the treatment process may be present in the effluent and may have potentially contributed to the observed effects.

Copper, selenium, and iron were present in the effluent treatment at concentrations much higher than the Canadian water quality guidelines (WQG) for the protection of aquatic life (CCME 1999), which suggests that they may be causative metals (Table 2.1). The mean copper concentration in the 45% CCE treatment (83 ± 10 $\mu\text{g/L}$) was almost 21 times higher than the WQG of 4 $\mu\text{g/L}$. In the 45% CCE treatment, the mean copper concentration is similar to the LC_{50} value for fourth instar *C. tentans* after 20 days of exposure (77.5 $\mu\text{g/L}$) (Nebeker et al. 1984). However, the water

hardness in the experiment by Nebeker et al. (1984) was much lower than it was in the 45% CCE in the present experiment, so the copper may not be taken up as readily in the CCE as it was in the Nebeker et al. study because of competition for binding sites.

The mean concentration of selenium in the 45% CCE treatment ($64 \pm 28 \mu\text{g/L}$) was 64 times higher than the WQG of $1.0 \mu\text{g/L}$. Selenium has been observed to affect emergence in *C. riparius* (Ingersoll et al. 1990); however, the concentrations causing these effects ($>837 \mu\text{g/L}$) are much higher than the concentrations measured in the 45% CCE treatment. The mean iron concentration in the 45% CCE ($948 \pm 40 \mu\text{g/L}$) was over three times higher than the WQG ($300 \mu\text{g/L}$). Survival of *C. riparius* was significantly decreased at iron concentrations of 400 mg/L (Rousch et al. 1997); however, this concentration is several orders of magnitude higher than the mean iron concentration measured in the 45% CCE treatment.

Six other metals (i.e., boron, barium, cobalt, lithium, rubidium, and strontium), which were present in the CCE, have no WQG (CCME 1999). For example, the concentration of strontium in the effluent was $576 \pm 96 \mu\text{g/L}$. However, to our knowledge there is no literature on the effects of strontium on *C. tentans*. In addition, different combinations of metals may work in additive, synergistic, or antagonistic manners, depending on the relative concentrations of the metals (Fargašová 2001). Thus, although there are several potential sources of metal toxicity in this study, it is difficult to determine the causative metal or combination of causative metals due to the complexity of the effluent.

The concentrations of several other water quality variables (i.e., ammonia, calcium, chloride, magnesium, potassium, sodium, sulphate, and total kjeldahl nitrogen)

were significantly higher in the 45% CCE treatment relative to the reference treatment. Ammonia may have contributed to the effects observed in the CCE exposed treatment. The mean ammonia concentration in the 45% CCE treatment was 1.39 ± 0.07 mg/L, which is higher than the WQG of 0.171 mg/L (pH 8.5; 20°C) (CCME 1999). However, this concentration is below the 10-day LC_{50} of 532 mg/L calculated for *C. tentans* in a water-only test (Whiteman et al. 1996). The major ions (i.e., bicarbonate, calcium, chloride, magnesium, potassium, sodium, and sulphate) in the water can be included within a measurement called total dissolved solids. Reduced dry weight and reduced survival were observed in *C. tentans* exposed to high concentrations (>1100 mg/L) of total dissolved solids (Chapman et al. 2000). The total of calcium, chloride, magnesium, potassium, sodium, and sulphate in the 45% CCE treatment was 1170 mg/L, which suggests that the total dissolved solids may have also contributed to the effects observed in this study. However, the toxicity of the total dissolved solids is not only dependent on the overall concentration, but is also dependent on the relative concentrations of the component ions (Chapman et al. 2000). Therefore, it is not possible to link the observed effects to high concentrations of any of these water quality variables.

A final factor to consider is the potential influence of the increased pH on metal bioavailability. The pH in the artificial streams increased from approximately 7.7 to 9.1 over the course of the experiment. The increase in pH was consistent across both the reference and the 45% CCE treatments. Our opinion is that the pH increase was due to the removal of carbon dioxide from the systems due to algal growth on the tables. It has been well-documented that metal bioavailability and speciation are affected at different

levels of pH (Bervoets and Blust 2000, De Schamphelaere et al. 2002). However, it is unknown how these changes in pH reflect the natural receiving water fluctuations.

The ultimate objective of this research was to develop a bioassay for assessing MME effects on a benthic invertebrate in field-based artificial streams. The bioassay was used to evaluate the effects of CCE from INCO mining operations on *C. tentans*. Review of historical data from the Sudbury area (Jaagumagi and Bedard 2002, NAR Environmental Consultants 2003) clearly illustrates that it is impossible to determine, through the use of standard field surveys, whether current MME discharges are causing impacts independent of historical effects. It is also difficult to trace effects observed in the receiving environment to the source of contamination, considering the numerous point and non-point sources of pollution that are entering Junction Creek (Jaagumagi and Bedard 2002, NAR Environmental Consultants 2003). This study provided the first controlled assessment of the effects of a single MME, independent of other pollution sources in the system, on a benthic invertebrate under more realistic exposure conditions.

2.6 Conclusions

This was a successful first-time implementation of the *C. tentans* life cycle bioassay in a field-based artificial stream system. The effects of a MME on growth, survival, emergence, and reproduction were measured under environmentally relevant conditions. Effluent-related effects include decreased survival, decreased total emergence, increased time to emergence, and decreased hatching success. The effluent

appeared to have no effect on growth, sex ratio, number of egg cases per female or number of eggs per egg case.

This experiment showed that exposure to 45% CCE has the potential to cause population-level consequences to *C. tentans*. Exposure reduced the number of animals surviving and emerging. Animals that emerged were able to reproduce. However, there was low hatching success among the offspring. These conclusions may not have been drawn using existing laboratory bioassay procedures or community-level artificial stream experiments.

In the future, studies to determine whether the *C. tentans* life cycle bioassay in artificial streams is comparable in sensitivity to the beaker life cycle bioassay will be necessary. The life cycle bioassay in artificial streams is more logistically complicated and more expensive to run, so it is not our intention to replace the existing beaker life cycle bioassay. Instead, we propose this bioassay be used in situations where it is important to develop a better understanding of effluent effects under more realistic conditions than those found in the laboratory, because an effluent may have different effects in the field than it does in the laboratory (Robinson et al. 1994). Our results show that our bioassay is sensitive enough to detect a reduction in survival, emergence, and hatching success after exposure under environmentally relevant conditions to a 45% concentration of a MME for 37 days.

2.7 Acknowledgement

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Chapter 3^a

**COMPARISON OF A PARTIAL LIFE CYCLE BIOASSAY IN ARTIFICIAL
STREAMS TO A STANDARD BEAKER BIOASSAY TO ASSESS METAL MINE
EFFLUENT EFFECTS ON THE FRESHWATER MIDGE, *CHIRONOMUS
TENTANS***

^a This chapter has been submitted to the journal of Environmental Toxicology and Chemistry under joint authorship with Monique G. Dubé (National Water Research Institute)

3.1 Abstract

A novel, partial life cycle bioassay using *Chironomus tentans* in artificial streams was developed for evaluating the effects of metal mine effluent (MME). The utility of this bioassay was compared to an existing beaker life cycle bioassay under laboratory conditions. *Chironomus tentans* larvae were exposed to 45% treated MME from Day 11 through to hatching of the next generation. Response patterns were consistent between the two bioassays for hatching success and time to emergence, but inconsistent for other endpoints. Significant effluent effects were observed for growth, survival, number of adults emerged, and number of eggs per egg case in the artificial stream bioassay, but not in the beaker bioassay. Conversely, significant effects on sex ratio and number of egg cases per female were observed in the beaker bioassay, but not in the artificial stream bioassay. These differences are believed to be a consequence of the number of organisms per replicate used in each bioassay (i.e., due to a difference in statistical power). These results provide evidence that the bioassay in artificial streams can be an effective tool for evaluating MME effects on life cycle endpoints in *C. tentans*. The results from the artificial stream bioassay were also compared with data from a field implementation of the artificial stream bioassay.

3.2 Introduction

The effects of single contaminants are often evaluated on single species under laboratory conditions where temperature, light, and water quality are tightly controlled. These methods provide rapid information on the relative toxicities of various contaminants and the relative susceptibilities of different organisms (Parrish 1995, Rand

et al. 1995). In addition, because these tests are rapid and standardized, effluent quality assessments can be performed in a relatively inexpensive manner (Clements and Kiffney 1996, USEPA 2002).

Short-term laboratory toxicity tests serve an important role for assessing contaminant toxicities. However, there are several disadvantages associated with their use. These test methods often evaluate effects on test organisms at a single life stage, which may not be the most sensitive part of the organism's life cycle. For example, 10 day old third instar *Chironomus tentans* larvae are used in the standardized 10-day toxicity tests even though first instar larvae have been shown to be the most sensitive to copper exposure (Nebeker et al. 1984). In addition, increasing control in the laboratory often is at the loss of environmental realism (Cairns 1995, Kimball and Levin 1985). Aquatic systems often receive a mixture of contaminants from multiple sources. In addition to these contaminant mixtures, aquatic organisms are challenged by interspecific and intraspecific interactions and natural fluctuations in environmental variables (e.g., diurnal temperature changes). Thus, results obtained from standardized laboratory testing often cannot predict observations made in the field (Robinson et al. 1994, Tucker and Burton 1999). Environmental realism of bioassays can be increased by evaluating the effects of whole effluents at concentrations that are likely to be measured in the receiving environment, by measuring effects under conditions similar to the receiving environment, and by measuring endpoints with greater potential for extrapolation to higher levels of biological organization.

In previous work (Chapter 2), the *C. tentans* life cycle bioassay (Benoit et al. 1997) was transferred to an artificial stream system for an *in situ* exposure to effluent

from a metal mine in Sudbury, ON, Canada. The freshwater midge, *C. tentans*, was chosen as a test species because it is commonly used for freshwater toxicity testing, is easy to handle and culture, and freshwater midges are ubiquitous in the environment and important components of freshwater food webs (ASTM 2000, Environment Canada 1997, USEPA 2000). A life cycle application was used to test effects on various endpoints, such as reproduction, throughout an organism's life. Reproductive endpoints are sensitive to toxicant exposure (Benoit et al. 1997, Langston 1990, Pascoe et al. 1989) and effects on reproduction can translate to effects on population growth rate (Allan and Daniels 1982) with potential population-level consequences. The bioassay was applied to an artificial stream system because these systems can simulate riverine conditions and effluent concentrations can be tightly controlled (Dubé and Culp 1996). Finally, a metal mine effluent (MME) was used for bioassay development because metals are persistent in the environment and are known to be toxic to invertebrates (Fargašová 2001, Nebeker et al. 1984, Timmermans et al. 1992, Williams et al. 1986). In addition, metal mines in Canada are now required to conduct environmental effects monitoring to determine the effects of the discharged effluent on fish and fish habitat in the receiving environment, including benthic invertebrate communities (Environment Canada 2002). Artificial streams are one of the alternative tools available when field studies are not possible or if the receiving system is confounded by multiple effluents (Environment Canada 2002).

The life cycle bioassay in artificial streams was used successfully in the field to measure the effects of a MME on *C. tentans* (Chapter 2). However, it is still necessary to understand how this tool compares to the standard beaker life cycle bioassay (ASTM 2000, Benoit et al. 1997, USEPA 2000). The objective of this study was to conduct a

side-by-side laboratory comparison of the artificial stream bioassay (ASB) with the standard beaker life cycle bioassay (BB) to measure the effects of MME on *C. tentans* and evaluate the strengths and weaknesses of the respective methods. The bioassays were run simultaneously using a 45% [v/v] concentration of final treated MME from the Copper Cliff WWTP in Sudbury, ON, Canada. The laboratory evaluation used effluent from the same source and at the same concentration as the field evaluation (Chapter 2), so a qualitative comparison could be made between results from the laboratory and field ASB studies.

3.3 Materials and Methods

3.3.1 Artificial stream bioassay

The artificial stream system is described in Chapter 2. For each treatment (i.e., reference and 45% Copper Cliff effluent or CCE), there were six replicate 10.3 L circular high density polyethylene streams on top of a table draining into an 85 L dilution reservoir (Figure 3.1A). Each set of six streams, table and dilution reservoir were common to a single treatment and self-contained on a shipping pallet. For the reference water treatment, water was pumped into the reservoir at a rate of 51.7 mL/min in order to achieve a 48 hour turnover rate in the reservoir. This turnover rate was used to reduce the volume of effluent shipped to the laboratory. The same turnover rate was achieved in the 45% CCE treatment by pumping the effluent at a rate of 23.3 mL/min and reference water at a rate of 28.4 mL/min into the reservoir.

From the dilution reservoir, treatment water was pumped up into streams on top of the table at a rate of 2 L/min to achieve a turnover rate of five minutes within each

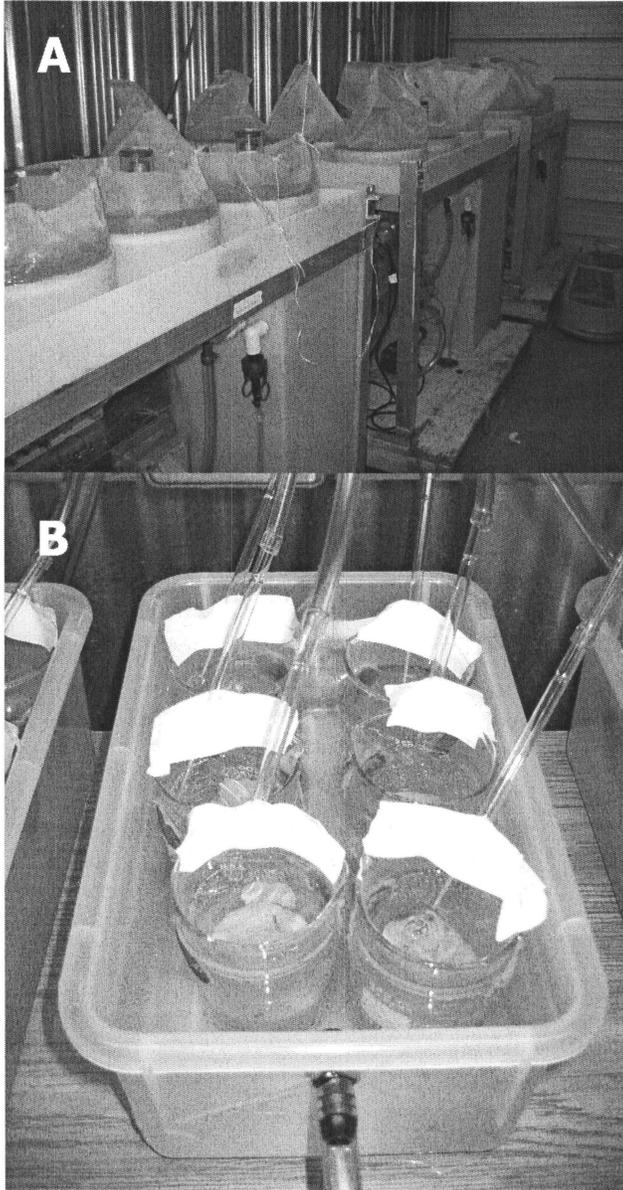


Figure 3.1: Artificial stream (A) and beaker (B) setup in laboratory exposure

stream. A propeller in the centre of each stream created a current velocity of 8 cm/s. Each artificial stream had a 1 cm layer of washed silica sand (250-425 µm) substrate (Unimin Corporation, New Connan, CT, USA). Emergence traps made of a 500 µm nylon mesh dome were fitted inside each stream. A moveable plastic window in the side of each emergence trap allowed access to the streams.

Twelve breeding chambers (21 L covered aquariums) were set up, one for each artificial stream. Emergent adults collected from a given stream were placed into the corresponding breeding chamber. Two litres of the appropriate test solution (i.e., reference water or 45% CCE) were placed in the bottom of the breeding chamber so that egg cases would be laid in the treatment solution. Every two days, one litre of the treatment solution was removed by vacuum pump and replaced by fresh solution (i.e., 50% water change).

The experimental design for the ASB is displayed in Table 3.1. The ASB was originally designed as a full life cycle bioassay. However, in the first implementation of the bioassay there was high mortality of laboratory-reared first instar larvae in the field (Chapter 2). As a result, the protocol was modified to use older animals (11 days old) to increase survivorship in the reference treatment. To start the exposure, 250 – 11 day old *C. tentans* larvae were placed into each stream. The density of larvae (~0.36 individuals/cm²) used in the ASB was similar to that used in the BB (~0.37 individuals/cm²) (ASTM 2000, Benoit et al. 1997, USEPA 2000). Larvae were obtained from cultures maintained at the National Water Research Institute in Saskatoon, SK, Canada, following procedures of the U.S. Environmental Protection Agency (1993). At the start of the exposure, 36 larvae (~15% of total initial colonization) were also

Table 3.1: Experimental setup for the beaker bioassay and the artificial stream bioassay in the laboratory and in the field

	Beaker Bioassay	Artificial Stream Bioassay Laboratory	Artificial Stream Bioassay Field ^a
Treatments	Reference water; 45% CCE	Reference water; 45% CCE	Reference water; 45% CCE
Design	Static renewal; 12 hour turnover (2 hours @ 2×/day)	Partial flow through; 48 hour turnover	Continuous flow through; 6 hour turnover
# Animals	12 – 11 day old larvae	250 – 11 day old larvae	1002 ± 51 larvae
Duration	32 days	32 days	37 days
Endpoints	Growth at day 10 (<i>n</i> =4) Survival (<i>n</i> =8) Emergence (<i>n</i> =8) Reproduction (<i>n</i> =8): Sex Ratios Number of egg cases/female Number of eggs/egg case Hatching success	Growth rates – days 5, 9, and 13 (<i>n</i> =6) Survival (<i>n</i> =6) Emergence (<i>n</i> =6) Reproduction (<i>n</i> =6): Sex Ratios Number of egg cases/female Number of eggs/egg case Hatching success	Growth rates – days 14, 17, and 20 (<i>n</i> =6) Survival (<i>n</i> =6) Emergence (<i>n</i> =6) Reproduction (<i>n</i> =6): Sex Ratios Number of egg cases/female Number of eggs/egg case Hatching success

^a From Chapter 2

collected and preserved in 10% formalin for initial length and weight measurements. Upon addition of the larvae, and daily throughout the exposure period, 2 mL of TetraMin[®] (Tetra, Blacksburgh, VA, USA) slurry was added to each stream as a food source. Water flow into the streams was shut off for one hour after each feeding.

Core samples of stream sediments were taken on Days 5, 9, and 13 of the exposure period and preserved in 10% formalin. Core samples were sorted under a dissecting microscope (12×) and larvae preserved in 95% ethanol. The number of individuals taken from each stream was recorded and used in survival calculations. Total body length and head capsule length were measured using a camera lucida and digitizing pad (Dubé et al. 1997). Larvae were then placed in pre-weighed aluminum weigh boats and dried at 60°C until they reached a constant dry weight. The weigh boats and larvae were then weighed using a Cahn C-31 microbalance (Thermo Electron, Waltham, MA, USA). Larval weight was determined by subtracting the weight of the weigh boat from the final weight of the larva in the weigh boat.

Streams were checked daily for adults. Adults were collected, counted (by gender), and transferred to the corresponding breeding chamber. Breeding chambers were checked every morning for egg cases and dead adults. Dead adults were removed and discarded. The number of egg cases, excluding secondary egg cases, in each breeding chamber was recorded daily. Egg cases were removed from the breeding chambers using a 60-cc syringe with a glass tube (40 cm long; 2 mm i.d.) affixed to the tip. The number of eggs per egg case was determined using the ring count method (Benoit et al. 1997) for a maximum of two egg cases per replicate daily. One of these egg cases was placed in a 100- × 15-mm plastic Petri dish with 30 mL of overlying

treatment solution for six days, after which time the number of unhatched eggs was counted. The number of unhatched eggs was subtracted from the estimated number of eggs to calculate the number of hatched eggs. Hatching success was calculated by dividing the number of hatched eggs by the estimated number of eggs.

3.3.2 *Beaker bioassay*

The flow-through system used for the beaker bioassay was based on that described in Benoit et al. (1993). Four polypropylene trays (30.5 cm × 22.9 cm × 12.7 cm) were placed on a table; two trays were designated for each treatment (Figure 3.1B). Each tray could hold up to 8 – 300 ml tall-form glass beakers. Each beaker had two holes (1.6 cm diameter) drilled in opposite sides at a height of 7 cm from the inside beaker bottom. The holes were covered with mesh (3.35 mm) attached with aquarium-safe silicone glue. Water renewal took place for two hours twice daily, so water in the beakers was renewed every 12 hours. This renewal rate was different from that in the ASB, where water in the dilution reservoirs was turned over every 48 hours. Reference water or 45% CCE was pumped into one end of each tray using a peristaltic pump at a rate of 32 mL/minute. Excess water drained out of the trays through a drain hole placed 6.4 cm from the bottom at the opposite end of the tray.

The beaker life cycle bioassay was modified from Benoit et al. (1997), with respect to the age of the larvae used at the start of the exposure period. In the life cycle bioassay of Benoit et al. (1997) the larvae were less than 24 hours old at the start of exposure. In the present study, 11 day old larvae were used to maintain a consistent

exposure duration between the ASB and the BB so a better comparison between the bioassays could be made.

The experimental design for the BB is described in Table 3.1. Twelve – 11 day old larvae were placed into each of the 24 beakers (i.e., 12 beakers per treatment; 6 beakers per tray) (Benoit et al. 1997). Each beaker was prepared with 100 mL of washed silica sand (250 to 425 μm) substrate (Unimin Corporation, New Connan, CT, USA). Eight more beakers (i.e., 4 beakers per treatment; 2 beakers per tray) were prepared in the same manner ten days after the start of exposure to provide auxiliary males for both treatments (Benoit et al. 1997). On Day 10, four of the original beakers from each treatment were removed and the contents were preserved in 10% formalin. Sediments were sorted and larvae were measured for growth as in the ASB.

The remaining beakers were covered with emergence traps. The emergence traps were made of inverted plastic drink cups, which had the bottoms removed and covered with nylon mesh (Benoit et al. 1997). The emergence traps were checked each morning for adults. Adults were counted (by gender), collected and transferred to a corresponding reproduction/oviposition chamber. The reproduction/oviposition chambers were of the same design as the emergence traps except they were inverted into the bottom of a 100 \times 20-mm plastic Petri dish containing 50 mL of overlying treatment solution (Benoit et al. 1997). The number of egg cases in each reproduction/oviposition chamber was recorded and egg cases were removed every morning. The number of eggs per egg case and hatching success were determined for each egg case following the methods described for the ASB.

3.3.3 *Physical and chemical measurements*

Effluent used in the exposure was obtained from the Copper Cliff WWTP in Sudbury, ON, Canada, which treats tailings from several metal mines in the region prior to discharge to Junction Creek, ON, Canada. The effluent contains high concentrations of various metals, including copper (mean concentration of 104 µg/L in 2002) and nickel (mean concentration of 182 µg/L in 2002) (NAR Environmental Consultants 2003). In the treatment process, dissolved metals are precipitated from solution by pH adjustment with lime and settling. Suspended solids are removed by the addition of a flocculent. After treatment, sulphuric acid is added to the effluent to achieve neutral pH prior to discharge (G. Watson, INCO Limited, Sudbury, ON, Canada, personal communication). The Copper Cliff WWTP discharged 8.31×10^6 m³ of treated effluent to Junction Creek in 2001 (Dubé et al. 2004b).

Treated effluent was shipped weekly in lined, food-grade high-density polyethylene plastic pails from Sudbury, ON, Canada to Saskatoon, SK, Canada by refrigerated truck. The effluent was stored in the laboratory in a 380 L high-density polyethylene container. Dechlorinated tap water from the Saskatoon municipal water supply, which was used as the reference water, was stored in a 1200 L high-density polyethylene container for 24 hours to acclimate to room temperature (23°C). The water was then transferred to a 380 L high-density polyethylene storage container from which the water was pumped to the bioassays.

The photoperiod was set to 16:8 hours (light:dark). Temperature, dissolved oxygen, conductivity, pH, and ammonia were measured three times per week in one stream per treatment and one beaker per treatment. Optic stowaways[®] (Onset Computer

Corporation, Bourne, MA, USA) recorded temperatures throughout the experiment. Water hardness and total chlorine were measured weekly in one stream per treatment and in one beaker per treatment using drop count titration test kits (Hach, Loveland, CO, USA).

At the start, middle, and end of the exposure period, water and effluent samples were taken from the reference ASB, the 45% CCE ASB, one of the reference beaker trays, one of the 45% CCE beaker trays, and the 100% CCE head tank. Samples were sent to the National Laboratory for Environmental Testing in Burlington, ON, Canada, where total metal concentrations (i.e., aluminum, antimony, arsenic, barium, boron, cadmium, chromium, cobalt, copper, gallium, iron, lanthanum, lead, lithium, manganese, molybdenum, nickel, rubidium, selenium, thallium, uranium, vanadium, and zinc) were measured by inductively coupled plasma - mass spectrometry. Other variables (i.e., ammonia, calcium, chloride, dissolved organic carbon, magnesium, nitrate, nitrite, ortho-phosphorus, particulate organic carbon, particulate organic nitrogen, potassium, sodium, sulfate, total kjeldahl nitrogen, and total phosphorus) were also measured.

3.3.4 Statistical methods

All statistical analyses were performed using Systat[®]9 (SPSS, Chicago, IL, USA). Prior to analysis, assumptions of normality and homogeneity of variance were tested by examination of residual patterns and Levene's test, respectively. If these assumptions were not met, transformations were performed on the data. Percentage data (i.e., survival and hatching success) were arcsin \sqrt{x} transformed. All other data were log₁₀ transformed if necessary.

Growth was the only endpoint that was measured differently in the two bioassays. For the analysis of growth data in the ASB, linear regressions were performed for weight, total body length, and head capsule length as a function of time for each treatment. Analysis of covariance was used to determine whether there were differences in slope or y -intercept between the two treatments in the ASB. The same analysis was performed on weight as a function of total body length (i.e., condition factor). In the BB, mean growth was analysed using a t -test to detect differences between the two treatments for length, weight, and head capsule length. For both the ASB and the BB, repeated measures ANOVAs were used to determine whether the pattern of emergence differed between the two treatments. T -tests were used for all other endpoints (i.e., survival, sex ratios, number of egg cases per female, number of eggs per egg case, hatching success, and metal/nutrient concentration in treatment solution). In the calculation of mean metal or nutrient concentrations, one half the detection limit was used if concentrations were below the detection limit.

Coefficients of variation (CV) were calculated for each endpoint in the two treatments to measure the relative amount of variability in each of the bioassays. A Z -test was then used to determine whether the CVs for each endpoint were significantly different between the reference and 45% CCE treatments (Zar 1999). For all endpoints except hatching success there were no significant differences between treatments within each bioassay. Therefore, the CVs were pooled across treatments within each bioassay (Zar 1999) to facilitate variability comparisons between the ASB and the BB. For hatching success, the CVs could not be pooled because of a significant difference

between the two treatments. Thus, the CVs were compared separately for each treatment across the two bioassays for hatching success.

Effect sizes (ES) were used to determine the minimum difference that could be detected between two treatments based on the amount of variability and number of replicates in the two bioassays. Effect sizes were calculated using the following equation:

$$ES = SD/\sqrt{(n/(2 \times (t_{\alpha} + t_{\beta})^2))} \quad (3.1)$$

(Environment Canada 2002), where SD = standard deviation; n = the number of replicates; t_{α} = critical t value at α level of significance; t_{β} = critical t value at β level of significance. Effect sizes were calculated using $\alpha(2) = 0.10$ and $\beta = 0.10$ because these are error levels commonly used in environmental effects monitoring (Environment Canada 2002). For the number emerged (total, females, and males), SD was converted to a percentage of the total adults expected to emerge (i.e., total numbers in the ASB = SD/250; total numbers in the BB = SD/12). This conversion was done so that a direct comparison could be made between the ESs in the two bioassays.

Magnitude of effect is a measure of the amount of difference in an endpoint between an exposed and a reference treatment. Magnitudes of effect were calculated using the following equation:

$$(\text{Exposed} - \text{Reference})/\text{Reference} \times 100\% \quad (3.2)$$

for various endpoints to facilitate comparison between the ASB in the laboratory and the ASB in the field.

3.4 Results

3.4.1 Water and effluent quality

Twenty-four metals were present in the reference water, 45% CCE, and/or 100% CCE in the artificial streams and/or beakers (Table 3.2). Thirteen of these metals (i.e., arsenic, boron, cadmium, cobalt, copper, iron, lithium, manganese, nickel, rubidium, selenium, strontium, and thallium) show a consistent trend in which the concentrations in the 45% CCE were significantly higher than in the reference water for both the ASB and the BB.

Barium, chromium, lanthanum, lead, and molybdenum showed inconsistent patterns between the two bioassays. For example, barium was measured at higher concentrations in the 45% CCE than in the reference water in both bioassays; however, treatment differences were only statistically significant ($p=0.039$) in the ASB (BB: $p=0.084$). Molybdenum showed a similar pattern with significantly higher concentrations in the 45% CCE treatment of the ASB ($p=0.029$), but non-significant treatment differences measured in the BB ($p=0.162$). Lead showed a reverse trend with significant treatment differences measured in the BB ($p=0.014$), but not the ASB ($p=0.849$).

Calcium, chloride, conductivity, magnesium, nitrate, potassium, sodium, sulphate, total kjeldahl nitrogen, and water hardness were significantly higher in the 45% CCE treatment than in the reference water treatment in both bioassays (Table 3.3). One of the variables (i.e., pH) was significantly lower in the 45% CCE than in the reference water in both bioassays. There were no significant differences between treatments in both bioassays for dissolved organic carbon, dissolved oxygen, nitrite,

Table 3.2: Mean metal concentrations (mean \pm SE^a; $\mu\text{g/L}$) in reference water (Ref Water), 45% Copper Cliff effluent (CCE), and 100% CCE during the *C. tentans* bioassays in artificial streams and beakers^{b,c}

^a SE = standard error of the mean

^b For concentrations below detection limits, a value of 1/2 the detection limit was used to calculate the means; $n=3$ for all metals except As and Se where $n=2$

^c Significant difference between reference water and 45% CCE within the bioassay: * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$

^d Metal concentrations in 100% CCE during field implementation of *C. tentans* life cycle bioassay in artificial streams (Chapter 2)

^e WQG - Canadian Water Quality Guidelines for the Protection of Aquatic Life (CCME, 1999)

^f ND = concentration below detection limit

^g $10^{(0.86(\log[\text{hardness}]) - 3.2)}$

	Detection Limit	Artificial Streams		Beakers		Lab	Field	WQG ^e
		Ref Water	45% CCE	Ref Water	45% CCE	100% CCE	100% CCE ^d	
Aluminum	0.1	53 ± 7	37 ± 8	59 ± 6	52 ± 20	78 ± 66	34 ± 9	100
Antimony	0.01	0.16 ± 0.01	0.18 ± 0.02	0.18 ± 0.03	0.16 ± 0.01	0.12 ± 0.00	ND ^f	
Arsenic	0.0001	0.25 ± 0.03	2.17 ± 0.51	0.35 ± 0.04	2.31 ± 0.49	4.96 ± 0.77	3.00 ± 0.31	5
Barium	0.05	36 ± 1	62 ± 7*	41 ± 6	58 ± 5	76 ± 3	52 ± 1	
Boron	0.1	24 ± 3	94 ± 1***	28 ± 2	100 ± 2***	175 ± 9	100 ± 7	
Cadmium	0.005	0.02 ± 0.01	0.99 ± 0.23*	0.09 ± 0.00	1.28 ± 0.13***	2.26 ± 0.38	0.86 ± 0.36	g
Chromium	0.02	0.36 ± 0.03	0.28 ± 0.02	0.425 ± 0.003	0.30 ± 0.02**	0.12 ± 0.01	ND	1.0-8.9
Cobalt	0.002	0.04 ± 0.00	15.03 ± 0.47***	0.08 ± 0.01	16.83 ± 0.29***	37.17 ± 2.11	4.57 ± 0.53	
Copper	0.02	13.4 ± 1.5	63.7 ± 8.6**	2.8 ± 0.4	47.9 ± 10.4***	98.3 ± 24.3	175.4 ± 21.3	2-4
Gallium	0.002	0.155 ± 0.010	0.092 ± 0.002**	0.174 ± 0.014	0.093 ± 0.002**	0.009 ± 0.001	ND	
Iron	1	1.1 ± 0.5	23.7 ± 3.3**	2.8 ± 0.9	54.4 ± 11.1*	160.4 ± 57.8	1652.1 ± 79.3	300
Lanthanum	0.001	0.005 ± 0.001	0.022 ± 0.006	0.003 ± 0.002	0.058 ± 0.031*	0.334 ± 0.229	ND	
Lead	0.005	0.06 ± 0.02	0.06 ± 0.02	0.50 ± 0.09	2.38 ± 0.79*	0.18 ± 0.03	ND	1-7
Lithium	0.02	11.4 ± 0.2	59.5 ± 9.4**	13.2 ± 2.0	57.9 ± 5.5**	110.3 ± 4.9	80.0 ± 10.2	
Manganese	0.005	0.3 ± 0.0	13.0 ± 3.9***	1.7 ± 0.7	28.4 ± 11.4**	48.8 ± 5.2	21.3 ± 4.2	
Molybdenum	0.01	2.1 ± 0.1	3.3 ± 0.5*	2.6 ± 0.3	4.4 ± 1.0	3.5 ± 0.6	2.0 ± 0.5	
Nickel	0.05	7 ± 4	199 ± 25**	2 ± 1	198 ± 27**	445 ± 55	145 ± 28	25-150
Rubidium	0.002	1.3 ± 0.1	47.7 ± 5.6***	1.5 ± 0.1	47.0 ± 2.8***	101.5 ± 3.1	61.3 ± 1.5	
Selenium	0.0001	0.40 ± 0.02	6.84 ± 0.36	0.44 ± 0.03	7.55 ± 0.12	16.60 ± 2.37	148.71 ± 63.11	1.0
Strontium	0.05	209 ± 7	722 ± 82***	234 ± 31	697 ± 40**	1227 ± 15	1229 ± 231	
Thallium	0.001	0.002 ± 0.001	0.664 ± 0.052***	0.004 ± 0.001	0.672 ± 0.076***	1.3 ± 0.2	1.86 ± 0.26	0.8
Uranium	0.001	1.16 ± 0.04	0.66 ± 0.08**	1.30 ± 0.16	0.66 ± 0.03*	0.06 ± 0.01	ND	
Vanadium	0.01	0.24 ± 0.04	0.17 ± 0.02	0.27 ± 0.05	0.21 ± 0.01	0.11 ± 0.04	1.00 ± 0.27	
Zinc	0.05	2.1 ± 0.9	4.5 ± 0.7	2.0 ± 0.3	4.9 ± 1.5	6.5 ± 1.9	13.9 ± 1.9	30

Table 3.3: Mean concentrations (mean \pm 1 SE ^a) of water quality parameters in reference water, 45% Copper Cliff effluent (CCE), and 100% CCE during the *C. tentans* bioassays in artificial streams and beakers ^{b,c}

^a SE = standard error of the mean

^b For concentrations below detection limits, a value of 1/2 the detection limit was used to calculate the means; $n=3$ for all parameters

^c Significant difference between reference water and 45% CCE within the bioassay: * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$

^d DOC = dissolved organic carbon; POC = particulate organic carbon; TP = total phosphorus; OP = ortho-phosphorus; TKN = total kjeldahl nitrogen; PON = particulate organic carbon; DO = dissolved oxygen

^e all parameters are measured in mg/L except ortho-phosphorus ($\mu\text{g/L}$), temperature ($^{\circ}\text{C}$), DO (%), pH, conductivity ($\mu\text{S/cm}$), and hardness (mg/L as CaCO_3)

^f Concentrations in 100% CCE during field implementation of *C. tentans* life cycle bioassay in artificial streams (Chapter 2)

^g nm = not measured

^h ND = not detected

Parameter ^{d,e}	Detection	Artificial Streams		Beakers		Lab	Field
	Limit	Reference Water	45% CCE	Reference Water	45% CCE	100% CCE	100% CCE ^f
DOC	0.1	3.5 ± 0.7	5.6 ± 1.7	3.5 ± 0.4	3.9 ± 0.7	5.3 ± 2.6	3.9 ± 0.3
POC	0.005	0.22 ± 0.03	0.43 ± 0.14	0.21 ± 0.03	0.39 ± 0.08	0.29 ± 0.03	nm ^g
TP	0.0002	0.022 ± 0.003	0.027 ± 0.005	0.023 ± 0.002	0.028 ± 0.002	0.016 ± 0.001	0.016 ± 0.004
OP	0.2	5.6 ± 3.9	4.1 ± 2.1	2.2 ± 0.2	2.5 ± 1.5	4.1 ± 0.6	19.7 ± 2.4
TKN	0.014	0.5 ± 0.1	2.7 ± 1.0*	0.8 ± 0.3	4.5 ± 0.8*	7.5 ± 0.1	4.3 ± 0.3
Ammonia	0.001	0.2 ± 0.1	2.3 ± 0.9	0.4 ± 0.2	3.2 ± 0.3**	6.5 ± 0.1	2.6 ± 0.3
Nitrite	0.001	0.07 ± 0.04	1.09 ± 0.84	0.09 ± 0.04	0.22 ± 0.09	0.29 ± 0.06	ND ^h
Nitrate	0.005	0.4 ± 0.1	2.1 ± 0.9*	0.3 ± 0.1	1.1 ± 0.1**	1.9 ± 0.1	1.6 ± 0.7
PON	0.002	0.036 ± 0.006	0.088 ± 0.036	0.035 ± 0.007	0.068 ± 0.013	0.055 ± 0.007	nm
Sulphate	0.2	89 ± 2	1123 ± 43***	87 ± 3	1150 ± 21***	2407 ± 39	1719 ± 30
Calcium	0.05	34.5 ± 0.9	284.0 ± 13.9***	33.9 ± 1.2	296.3 ± 5.2***	590.0 ± 0.6	562.2 ± 20.3
Chloride	0.05	10.67 ± 0.03	59.07 ± 5.92**	10.33 ± 0.03	60.40 ± 5.06**	114.67 ± 6.69	150.1 ± 15.2
Magnesium	0.01	17.2 ± 0.5	71.2 ± 1.1***	16.8 ± 0.5	71.9 ± 1.8***	134.7 ± 1.2	54.7 ± 5.2
Potassium	0.01	3.10 ± 0.10	26.77 ± 1.04***	3.08 ± 0.04	27.83 ± 0.32***	56.80 ± 0.91	35.79 ± 0.97
Sodium	0.01	23 ± 1	91 ± 4***	22 ± 1	95 ± 4***	173 ± 11	115 ± 3
Temperature	–	23.1 ± 0.3	22.9 ± 0.3	21.9 ± 0.4	21.4 ± 0.3	nm	nm
DO	–	90 ± 1	87 ± 1	80 ± 4	73 ± 6	nm	nm
pH	–	8.2 ± 0.0	7.8 ± 0.1**	8.0 ± 0.1	7.5 ± 0.1**	6.8 ± 0.2	nm
Conductivity	–	377 ± 4	1864 ± 48***	334 ± 17	1624 ± 124***	nm	nm
Hardness	–	170 ± 10	1035 ± 116***	175 ± 5	1030 ± 171***	2175 ± 180	nm

ortho-phosphorus, particulate organic nitrogen, temperature, total organic carbon, and total phosphorus. There was an inconsistency between the two bioassays for ammonia which was significantly higher in the 45% CCE treatment than in the reference water treatment in the BB ($p=0.002$) but non-significant ($p=0.051$) in the ASB.

Effluent quality was not identical in the laboratory and field exposures (Tables 3.2 and 3.3). Barium, boron, lithium, and rubidium were one to two times higher in the laboratory exposures compared to concentrations measured during the 2002 field study (Chapter 2). Concentrations of cobalt and nickel were eight and three times higher during laboratory exposures, respectively. Copper, iron, selenium, and zinc had lower concentrations in the laboratory exposure. Copper and zinc were about two times lower in the laboratory than in the field exposure. Concentrations of iron and selenium were ten and nine times lower during the laboratory exposure, respectively (Chapter 2).

3.4.2 Biological endpoints

In the ASB, the slopes of the regressions for log length over time (Figure 3.2A), log head capsule length over time (Figure 3.2B), log weight over time (Figure 3.2C), and log weight as a function of log length (Figure 3.2D) were all significantly different from zero for both the 45% CCE treatment and the reference water treatment ($\alpha=0.05$) illustrating growth of *C. tentans* over the experiment in both treatments. Effluent exposure did not affect growth relationships as there were no significant differences in slope between the two treatments for any of the comparisons. There were significant differences between treatment y -intercepts for log length over time (repeated measures ANOVA; $p=0.007$; Figure 3.2A) and log weight as a function of log length (repeated

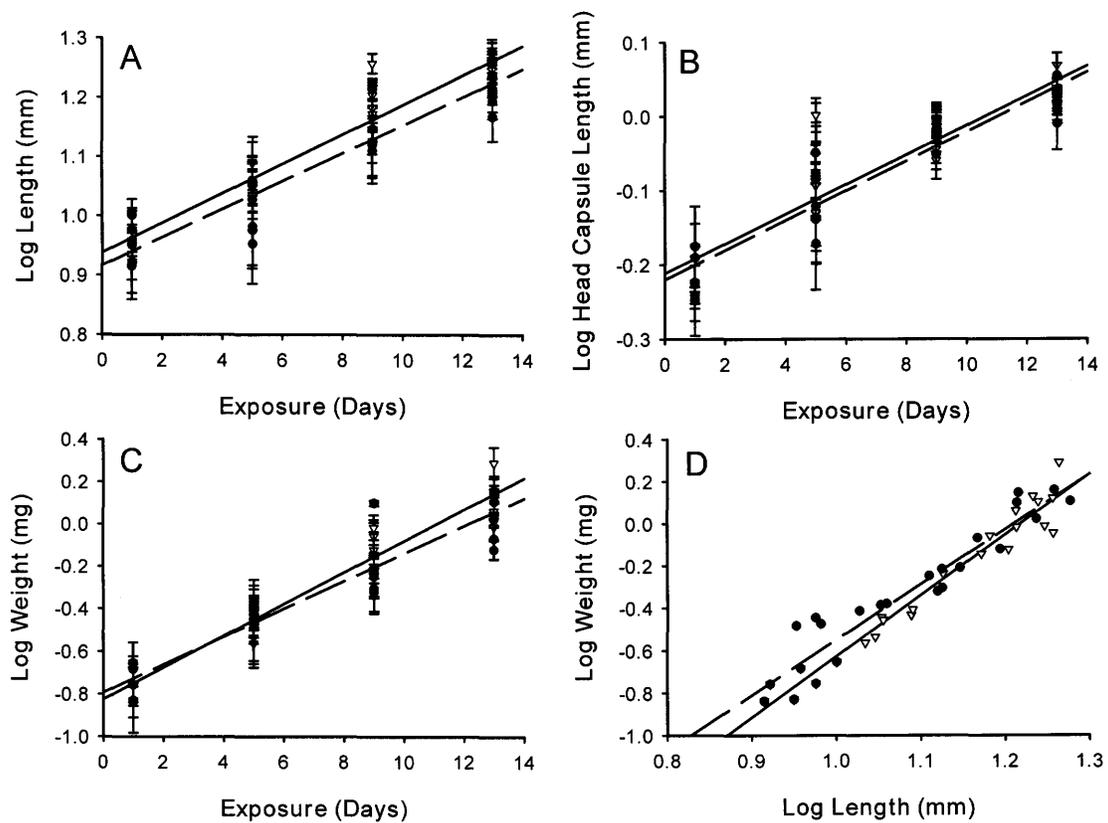


Figure 3.2: Log length (mm) over time (A), log head capsule length (mm) over time (B), log weight (mg) over time (C), and log weight as a function of log length (D) of *C. tentans* during partial life cycle exposure to reference water (white triangles; solid lines) or 45% Copper Cliff effluent (black circles; dashed lines) in artificial streams. There were no significant differences in the slopes of these relationships at $\alpha=0.05$; however, there were significant differences in the y-intercepts for log length over time and log weight as a function of log length ($p<0.05$). Each point represents the mean \pm 1 SE for one replicate on one sampling occasion.

measures ANOVA; $p=0.042$; Figure 3.2D) suggesting larvae in the 45% CCE treatment were shorter and of higher condition than the larvae from the reference water treatment. In order to examine these effects on larval growth more closely, treatment effects were evaluated at each time period. Larvae from the 45% CCE treatment were significantly shorter than the larvae from the reference water treatment at Day 5 (t -test on log length; $p=0.032$), but there was no significant difference between treatments at Day 9 (t -test; $p=0.052$) or Day 13 (t -test; $p=0.371$). There were also no significant differences between treatments for log weight or log head capsule length at any time period.

In the BB, there was no significant difference between the CCE treatment and the reference water treatment for larval length, weight, or head capsule length after 10 days of exposure (t -test; Figure 3.3).

Mean percent survival was significantly lower in the CCE treatment (61 ± 3) relative to the reference water treatment (73 ± 3) in the ASB (t -test; arcsine transformed; $p=0.017$; Figure 3.4A). There was no significant difference in mean percent survival between the treatments in the BB. In the BB, the mean percent survival was 75 ± 4 in the reference water treatment and 71 ± 6 in the CCE treatment (Figure 3.4B).

Patterns of emergence were significantly different between the two treatments for both the ASB and the BB (repeated measures ANOVA; $p<0.001$; Figure 3.5). There was a significant delay in emergence (repeated measures ANOVA; $p<0.001$) for females, males, and the total number of adults emerging in both effluent treatments of the two bioassays. There were significantly reduced numbers of males (repeated measures ANOVA; $p=0.012$) and total adults (repeated measures ANOVA; $p=0.034$) from the 45% CCE treatment relative to the reference water treatment in the ASB.

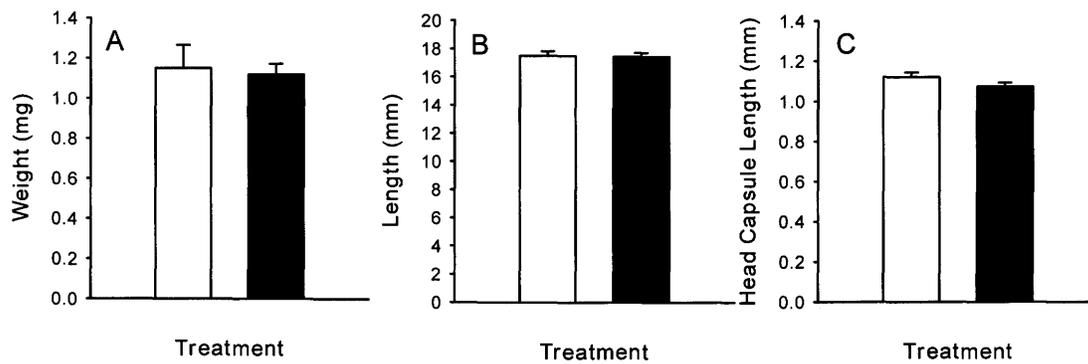


Figure 3.3: Weight (mg) (A), length (mm) (B), and head capsule length (mm) (C) of *C. tentans* after 10 days of exposure to reference water (white bars) or 45% Copper Cliff effluent (black bars) in beaker bioassay. There were no significant differences between the two treatments ($\alpha=0.05$). Each bar represents the mean ± 1 SE.

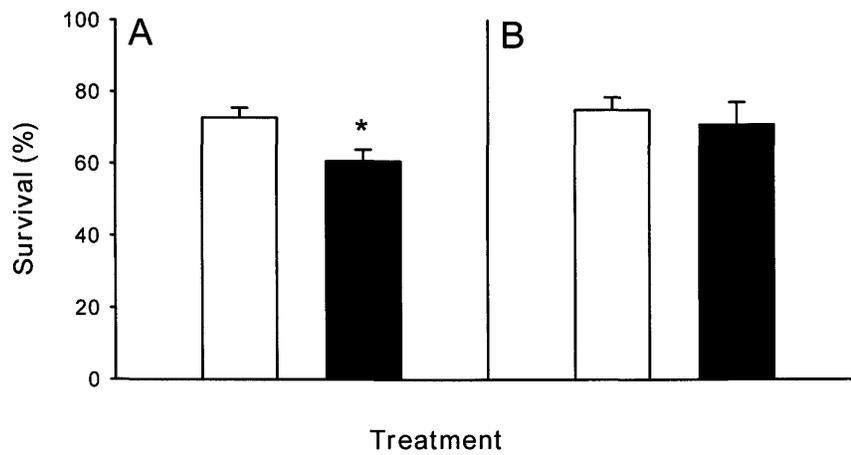


Figure 3.4: Percent survival of *C. tentans* in reference water (white bars) and 45% Copper Cliff effluent (black bars) after partial life cycle exposure in artificial streams (A) or beakers (B). * Indicates a significant difference at $p < 0.05$. Each bar represents the mean \pm 1 SE.

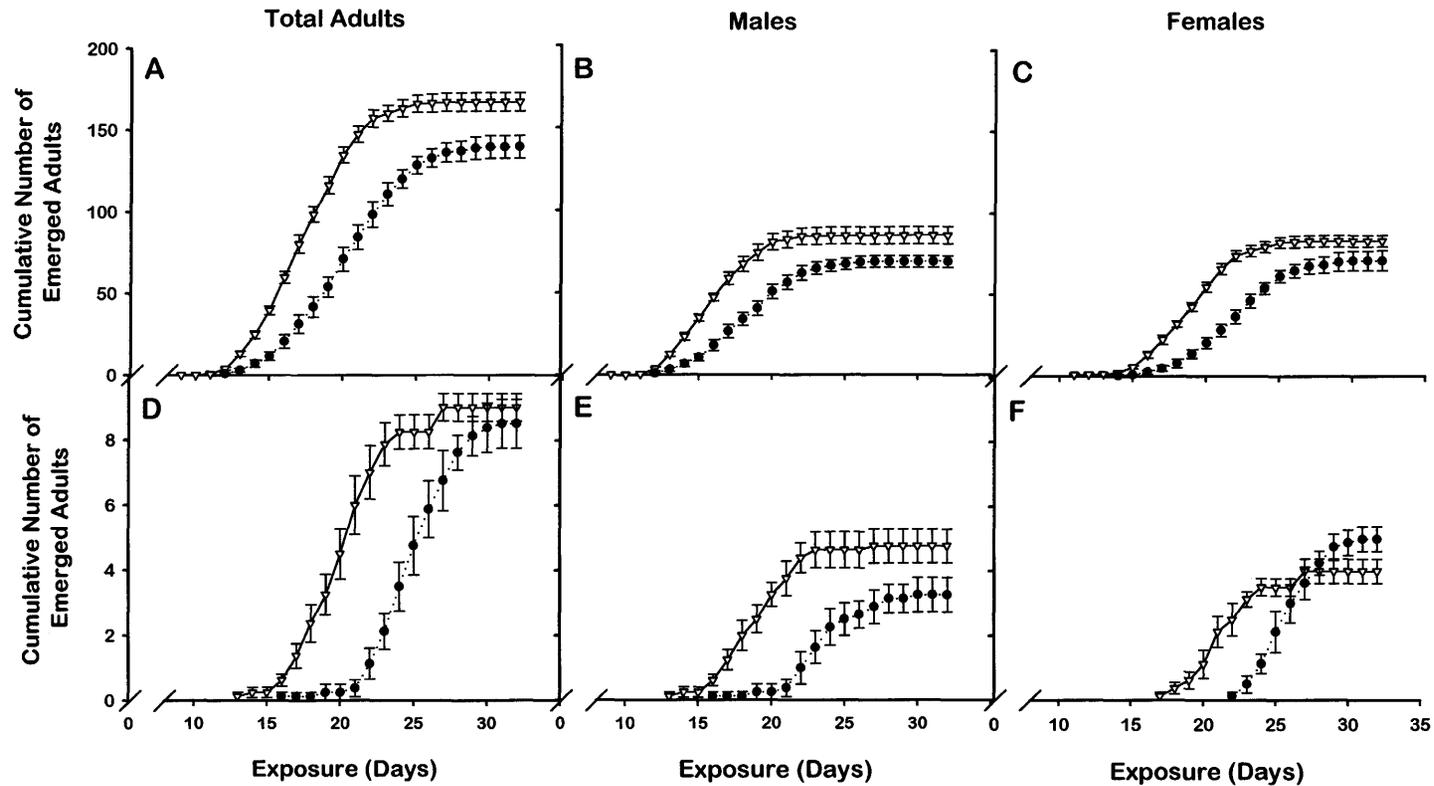


Figure 3.5: Cumulative emergence of *C. tentans* adults after exposure to reference water (white triangles; solid line) or 45% Copper Cliff effluent (black circles; dotted line) in artificial streams (A-C) and beakers (D-F). There was a significant treatment effect, a significant time effect, and a significant time \times treatment interaction for both total adults (A) and males (B) in artificial streams. For females in artificial streams (C) and for total adults (D), males (E) and females (F) in beakers, there was a significant time effect and a significant time \times treatment interaction but no significant treatment effect (repeated measures ANOVA; $p=0.05$). Each point represents the mean ± 1 SE.

However, there was no significant difference in the number of emergent females in the ASB or the number of females, males, or total adults in the BB.

There was no significant difference between treatments for sex ratios in the ASB (Figure 3.6A). In the BB, sex ratios were significantly different between the two treatments (*t*-test; $p=0.018$; Figure 3.6B) with a greater proportion of females emerging from the 45% CCE treatment than from the reference water treatment.

Egg production in females was measured in both bioassays as the number of egg cases per female and the number of eggs per egg case. In the ASB, there was no significant difference between treatments for the number of egg cases per female (Figure 3.7A). However, the number of eggs per egg case was significantly lower in the CCE treatment relative to the reference water treatment (*t*-test; $p=0.044$; Figure 3.8A). Conversely, in the BB, there were significantly fewer egg cases per female in the 45% CCE treatment than in the reference water treatment (*t*-test; $p=0.027$; Figure 3.7B), but there was no significant difference between the two treatments for the number of eggs per egg case (Figure 3.8B).

Hatching success was significantly lower in the 45% CCE treatment than in the reference water treatment in both the ASB (*t*-test; $p=0.000$; Figure 3.9A) and in the BB (*t*-test; $p=0.002$; Figure 3.9B). The hatching success in the ASB was $96.4\% \pm 0.2\%$ in the reference water treatment and $79.8\% \pm 0.7\%$ in the 45% CCE treatment. In the BB, hatching success was $96.4\% \pm 2.1\%$ and $75.4\% \pm 6.3\%$ in the reference water and 45% effluent treatments, respectively.

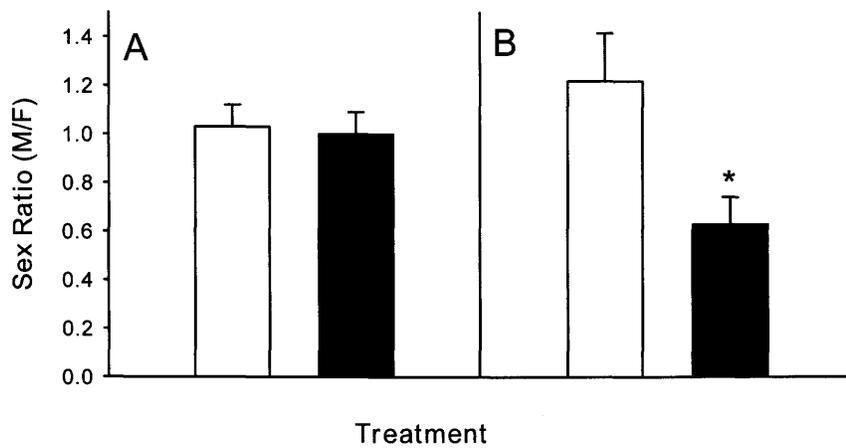


Figure 3.6: Sex ratios of emergent adults after partial life cycle exposure to reference water (white bars) or 45% Copper Cliff effluent (black bars) in artificial streams (A) or beakers (B). * Indicates a significant difference at $p < 0.05$. Each bar represents the mean \pm 1 SE.

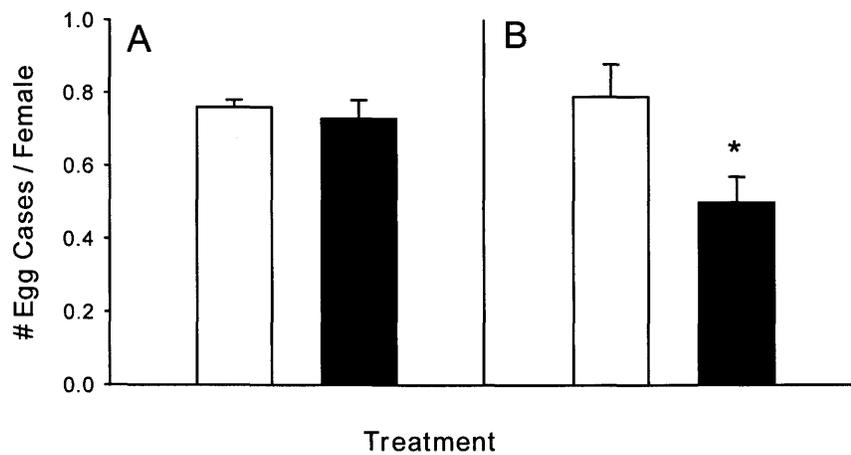


Figure 3.7: Number of egg cases per female after partial life cycle exposure to reference water (white bars) or 45% Copper Cliff effluent (black bars) in artificial streams (A) or beakers (B). * Indicates a significant difference at $p < 0.05$. Each bar represents the mean \pm 1 SE.

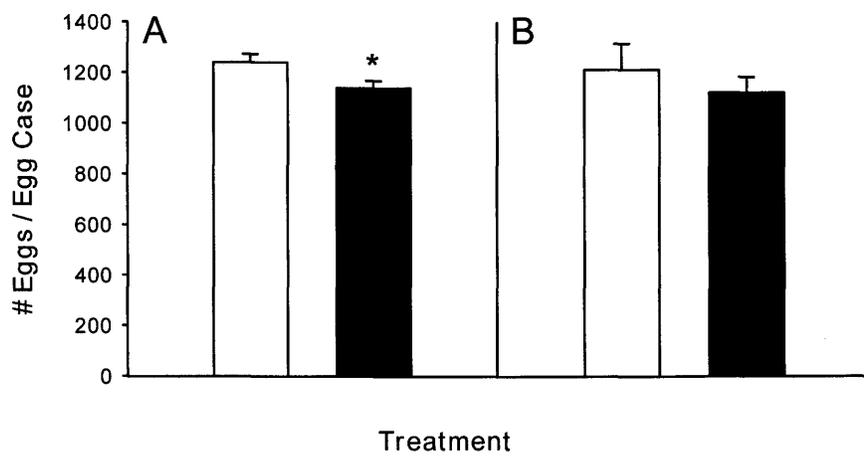


Figure 3.8: Number of eggs per egg case after partial life cycle exposure to reference water (white bars) or 45% Copper Cliff effluent (black bars) in artificial streams (A) or beakers (B). * Indicates a significant difference at $p < 0.05$. Each bar represents the mean ± 1 SE.

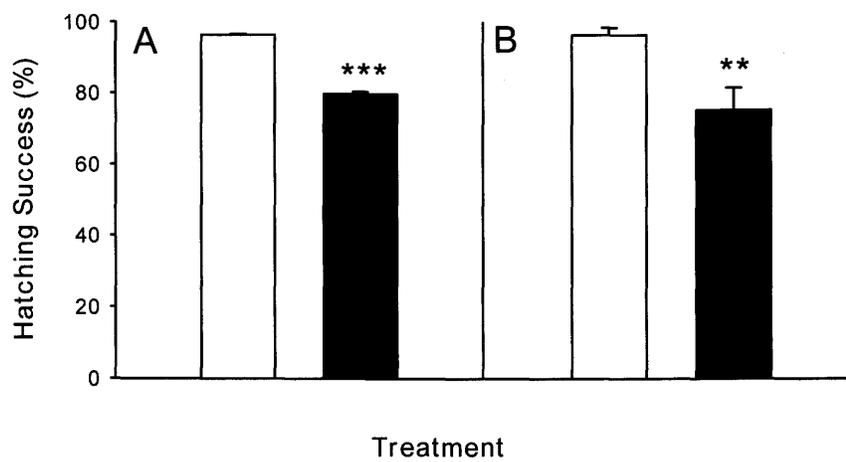


Figure 3.9: Hatching success of *C. tentans* egg cases after partial life cycle exposure to reference water (white bars) or 45% Copper Cliff effluent (black bars) in artificial streams (A) or beakers (B). ** Indicates a significant difference at $p < 0.01$; *** Indicates a significant difference at $p < 0.001$. Each bar represents the mean \pm 1 SE.

3.4.3 Evaluation of endpoint variability between bioassays

Coefficients of variation were used to compare the amount of variability in the two bioassays (i.e., ASB vs. BB; Table 3.4). The CVs were significantly lower in the ASB than in the BB for number of adults emerged, number of males emerged, sex ratios, number of egg cases per female, and number of eggs per egg case (Z-test; Table 3.4), which indicates that there was lower variability in the ASB than in the BB for these endpoints. For the number of emerged females and survival, the CVs were lower in the ASB than in the BB although these differences were not significant. For the three growth endpoints (i.e., weight, length, and head capsule length), the CVs were non-significantly higher in the ASB than in the BB. For hatching success, the CVs could not be pooled across treatments within each bioassay. Nonetheless, CVs were significantly lower in the ASB versus the BB when each treatment was compared separately across bioassays (i.e., reference ASB vs. reference BB and 45% CCE ASB vs. 45% CCE BB; Z-test; Table 3.4).

Effect sizes were lower in the ASB than in the BB for weight, survival, number emerged (total, males, and females), sex ratios, number of egg cases per female, number of eggs per egg case, and hatching success (Table 3.5), which indicates that the ASB would be able to detect smaller differences between treatments than the BB for these endpoints. Effect sizes were the same for head capsule length and higher in the ASB than in the BB for larval length.

Table 3.4: Coefficients of variation (CV) for each endpoint in the artificial stream bioassay (ASB) and the beaker bioassay (BB) ^a

Endpoint	CV ^b		Z-value ^c
	ASB	BB	
Weight	0.301	0.147	1.76
Length	0.074	0.035	1.96
Head Capsule Length	0.067	0.039	1.44
Survival	0.111	0.191	1.77
Number Emerged - Total	0.104	0.193	1.99*
Number Emerged - Female	0.161	0.241	1.32
Number Emerged - Male	0.145	0.386	2.81**
Sex ratios	0.225	0.469	2.03*
Number of Egg Cases per Female	0.118	0.375	3.21**
Number of Eggs per Egg Case	0.064	0.271	3.88***
Hatching Success - Reference	0.015	0.062	2.65**
Hatching Success - 45% CCE	0.050	0.237	2.77**

^a In ASB $n=6$ for all endpoints; in BB $n=4$ for weight, length, and head capsule length, $n=8$ for all other endpoints

^b Coefficients of variation were calculated using the following formula: $CV = SD/\text{mean}$, where SD=standard deviation

^c Significant difference in CV between ASB and BB: * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$

Table 3.5: Effect sizes for endpoints in the artificial stream bioassay and the beaker bioassay

Endpoint	Artificial Stream Bioassay	Beaker Bioassay
Larval Weight	0.26	0.30
Larval Length	1.26	1.03
Larval Head Capsule Length	0.08	0.08
Survival	9.5	15.8
Number Emerged - Total ^b	0.08	0.16
Number Emerged - Female ^b	0.13	0.20
Number Emerged - Male ^b	0.12	0.27
Sex ratios	0.307	0.518
Number of Egg Cases per Female	0.120	0.262
Number of Eggs per Egg Case	99	357
Hatching Success	3.91	14.62

^a In ASB $n=6$ for all endpoints; in BB $n=4$ for weight, length, and head capsule length, $n=8$ for all other endpoints

^b Effect sizes for numbers emerged were calculated as a percentage of the expected emergence

3.4.4 Laboratory and field artificial stream bioassay applications

Effects on the biological endpoints measured in the two bioassays are summarized in Table 3.6 along with the effects observed in the field application of the ASB in 2002 (Chapter 2). Effects observed in larvae exposed to 45% CCE in the field application of the ASB included decreased survival, decreased emergence, increased time to emergence, and decreased hatching success relative to the reference water treatment (Table 3.6). All of the effects observed in the field were also observed in the laboratory ASB. However, the magnitude of the observed effect was much higher in the field than in the laboratory for three of the four endpoints (i.e., survival, time to emergence, and numbers emerged). For survival, the magnitude of effect of effluent exposure was almost five times higher in the field (-82.4%) than in the laboratory (-16.6%). There was a similar difference between the field and the laboratory in magnitude of effect for numbers emerged (-80.8% and -16.7%, respectively). The magnitude of effect for time to emergence was over two times higher in the field (14.6%) than in the laboratory (6.8%). In contrast, the magnitude of effect for hatching success was slightly larger in the laboratory (-17.2%) than in the field (-14.8%). However, hatching success was lower in both treatments in the field than in the laboratory. Hatching success in the laboratory was $96.4 \pm 0.2\%$ in the reference water treatment and $79.8 \pm 0.7\%$ in the 45% CCE treatment. Hatching success in the field was $84.3 \pm 0.6\%$ and $71.8 \pm 1.5\%$ in the reference water and 45% CCE treatments, respectively (Chapter 2).

There were significant effects on growth (i.e., decreased length and increased condition factor) and a significant reduction in the number of eggs per egg case in the

Table 3.6: Summary of effects ^a measured in the beaker bioassay, the artificial stream bioassay in the laboratory, and the artificial stream bioassay in the field after exposure to 45% Copper Cliff effluent

Endpoint	Beaker Bioassay	Artificial Stream Bioassay Laboratory	Artificial Stream Bioassay Field ^b
Larval Weight	N.S. ^c (↓; <i>p</i> =0.90)	N.S. (nt; <i>p</i> =0.54; <i>p</i> =0.25) ^d	N.S. (nt; <i>p</i> =0.28; <i>p</i> =0.20)
Larval Length	N.S. (nt; <i>p</i> =0.89)	<i>y</i> -intercept ↓; slope N.S. (nt; <i>p</i> =0.29)	N.S. (nt; <i>p</i> =0.88; <i>p</i> =0.39)
Larval Head Capsule Length	N.S. (↓; <i>p</i> =0.17)	N.S. (nt; <i>p</i> =0.67; <i>p</i> =0.43)	N.S. (nt; <i>p</i> =0.43; <i>p</i> =0.75)
Larval Condition (weight vs. length)	nm ^e	<i>y</i> -intercept ↓; slope N.S. (nt; <i>p</i> =0.16)	N.S. (nt; <i>p</i> =0.35; <i>p</i> =0.82)
Survival	N.S. (↓; <i>p</i> =0.57)	↓	↓
Emergence - time	↑	↑	↑
- number	N.S. (↓; <i>p</i> =0.57)	↓ ^f	↓
Sex Ratios	females > males	N.S. (nt; <i>p</i> =0.82)	N.S. (males>females; <i>p</i> =0.16)
Number of Egg Cases per Female	↓	N.S. (↓; <i>p</i> =0.64)	N.S. (↑; <i>p</i> =0.30)
Number of Eggs per Egg Case	N.S. (↓; <i>p</i> =0.40)	↓	N.S. (↓; <i>p</i> =0.71)
Hatching Success	↓	↓	↓

^a Differences between treatments are indicated in terms of 45% CCE relative to reference water treatment (i.e. ↑ = endpoint is significantly higher in 45% CCE than in reference water treatment, ↓ = endpoint is significantly lower in 45% CCE than in reference water treatment)

^b From Chapter 2

^c N.S. = no significant difference between reference water and 45% CCE treatments; non-significant trends (nt = no trend) and *p*-values are given in parentheses

^d For growth endpoints in artificial stream bioassay, the first *p*-value is for slope and the second is for *y*-intercept, unless otherwise stated

^e nm = not measured

^f Significant decrease in number of males and total number; no significant difference in number of females

effluent exposed treatment detected in the laboratory ASB study but not in the field study. The CV for number of eggs per egg case in the field was 0.099 (Chapter 2), which was non-significantly higher than the CV for the same endpoint in the laboratory ASB (0.064). The CVs for length and condition were 0.188 and 0.475, respectively, in the field ASB, which were higher than the CVs measured for the same endpoints in the laboratory ASB (0.074 and 0.271, respectively). These differences in CV between the field and the laboratory were significant for length (Z -test; $p < 0.01$), but not significant for condition factor.

3.5 Discussion

3.5.1 Comparison between artificial stream and beaker bioassays

Two endpoints showed consistent responses to 45% CCE exposure across the two laboratory bioassays. Time to emergence (total, males, and females) increased and hatching success decreased in the 45% CCE treatment relative to the reference water treatment in both bioassays. However, significant decreases in survival, number emerged (total and males), and number of eggs per egg case and significant changes in length and condition factor were observed in the ASB but not in the BB. In contrast, there were significant changes in sex ratios and a significant decrease in the number of egg cases per female in the BB that were not observed in the ASB. Differences in bioassay response to effluent can be largely explained by two factors. First, there were many more individuals used per replicate in the ASB (i.e., 250 larvae) than in the BB (i.e., 12 larvae). Second, growth was measured over several time periods in the ASB, but only once in the BB.

Differences in the number of larvae per replicate affected the sensitivity of the endpoints in the two bioassays. Fewer individuals used in the BB may have resulted in “significant effects” for endpoints that were calculated as a ratio of two measurements (e.g., sex ratio). It is assumed at the start of each bioassay that larvae are added to each replicate in a 1:1 (male:female) ratio. However, this assumption may not be accurate in situations where only a small number of individuals are used per replicate. Increased number of animals per replicate increases the probability that the assumption of a 1:1 ratio holds. In the ASB, where there were 250 larvae placed into each replicate, the sex ratio was very close to 1:1 in both the reference and the 45% CCE treatments. In contrast, the sex ratio in the reference treatment in the BB was 1.3:1. This value was similar to the sex ratio of 1.27:1 observed by Benoit et al. (1997) in the control treatment of a life cycle beaker bioassay with *C. tentans*. Increased number of animals per replicate in the ASB likely reduced the probability of a skewed sex ratio at the start of the experiment, thus allowing for an unconfounded evaluation of effluent effects on sex ratio.

Differences in the number of individuals per replicate also appeared to affect endpoint variability between the bioassays. Coefficients of variation measure the amount of variability in a sample relative to the mean of the sample. Thus, CVs can be used to compare the amount of variability in two populations with different means (Zar 1999). The CVs in the BB were significantly higher than the CVs in the ASB for six of the eleven endpoints measured, which indicates higher endpoint variability measured in the BB compared to the ASB. Increased variability could result in a reduced probability of detecting changes in a particular endpoint (Zar 1999). This result is illustrated in the

present study in the number of eggs per egg case and hatching success. For the former endpoint, the magnitude by which the number of eggs per egg case was reduced in the effluent-exposed replicates was similar in both the ASB and the BB (Figure 3.8). However, due to the smaller variability in the ASB the decrease was found to be significant ($p=0.044$), whereas no significant effect was detected in the BB ($p=0.398$). The magnitude of decrease in hatching success in the 45% CCE treatments relative to the reference water treatments was similar between the two bioassays (Figure 3.9), but the effect seen in the ASB was at a higher level of significance ($p<0.001$) than the effect seen in the BB ($p=0.002$). The higher level of significance in the ASB is not related to the level of replication. In fact, the level of replication was higher in the BB ($n=8$) than in the ASB ($n=6$) for all endpoints except growth. Instead, the larger number of animals per replicate in the ASB reduced the variability because there were a greater number of subsamples used to estimate the mean for each replicate, which increased the precision of the estimates.

As a result of the reduced variability in the ASB, one would expect to detect smaller changes in an endpoint. Effect sizes were calculated to determine the minimum difference between two treatments that could be detected with a given amount of variability, number of replicates, α and β (Environment Canada 2002). Effect sizes were smaller in the ASB than in the BB for all endpoints except length and head capsule length. Thus, the ASB was more sensitive at detecting smaller significant changes than the BB for all but two endpoints.

There were no effects on growth observed in the BB where growth was measured after 10 days of exposure. However, in the ASB, where growth was measured multiple

times during larval exposure, significant effects on growth were observed. Although the relationship of increasing length over time (i.e., slope) did not change with exposure, the overall length (i.e., y -intercept) was less in the 45% CCE treatment than in the reference water treatment. Therefore, effluent exposure caused a reduction in length. Condition factor was also higher in the effluent treatment (i.e., significantly higher y -intercept) suggesting effluent-exposed larvae were heavier at a given body length relative to the reference water larvae. This significant effect may be driven by the effects on length in the younger larvae. Effluent-exposed larvae at Day 5 of exposure were significantly shorter than the larvae in the reference water. At later sampling periods however (Days 9 and 13) this significant effect was not observed. This result is consistent with Timmermans et al. (1992) who found that cadmium had an effect on growth and survival of early instar larvae. However, the growth rate of the remaining cadmium-exposed larvae caught up and exceeded the growth rate of the controls so adults emerged from cadmium treatments sooner than control animals. When growth is measured at only one point in time, as it is in the BB, these changes in growth rate over time were not detected.

3.5.2 Effluent-related effects

In the ASB, the 45% CCE-exposed larvae showed a significant decrease in length and an increase in condition (i.e., log weight vs. log length) relative to the reference larvae. These changes were not observed in the BB. Previous studies have shown chironomid growth is inhibited by exposure to various metals, including cadmium, chromium (Wentzel et al. 1977), zinc (Sibley et al. 1996, Wentzel et al. 1977),

and copper (Timmermans et al. 1992). The growth of larval *C. riparius* was impaired at copper concentrations of 90 µg/L, which is slightly higher than the mean concentration of 64 ± 9 µg/L detected in the 45% CCE treatment in the ASB.

A significant decrease in survival was observed in the 45% CCE treatment in the ASB, but not in the BB. Metals (Fargašová 2001, Nebeker et al. 1984, Williams et al. 1986) and metal-contaminated sediments (West et al. 1993) have been shown to be lethal to aquatic invertebrates. After a 20-day exposure, the copper LC₅₀ for fourth instar *C. tentans* larvae was 77.5 µg/L (Nebeker et al. 1984), which is slightly higher than the mean copper concentration in the effluent treatment in the ASB (64 ± 9 µg/L).

Emergence has been shown to be affected by exposure to several metals (Ingersoll et al. 1990, Pascoe et al. 1989, Wentsel et al. 1978). In the present study, time to emergence was increased in both bioassays for males, females, and total adults, and there was a significant reduction in the number of adults emerged (total and males) in the ASB. Delays in emergence have been observed in *C. riparius* after exposure to 0.15 mg/L of cadmium (Pascoe et al. 1989) and > 837 µg/L of selenium, and significant decreases in percent emergence were observed in larvae exposed to 6050 µg/L of selenium (Ingersoll et al. 1990). The concentrations of cadmium and selenium used in these studies are much higher than the values measured in the 45% CCE treatments in the present study. In the ASB, the concentrations of these metals were 1.0 ± 0.2 µg/L of cadmium and 6.8 ± 0.4 µg/L of selenium. In the BB, these values were 1.3 ± 0.1 µg/L of cadmium and 7.6 ± 0.1 µg/L of selenium.

Sex ratios of chironomids have not been found to be sensitive to toxicant exposure in previous studies (Kahl et al. 1997, Pascoe et al. 1989, Sibley et al. 1996).

The results of the ASB showed no significant difference in sex ratio between the two treatments, which is consistent with the above studies. However, there was a significant difference between treatments in the BB, which may have been an artefact of the number of animals in each replicate.

Exposure to 45% CCE caused a significant decrease in the number of egg cases per female in the BB. In the ASB, effluent exposure had no significant effect on the number of egg cases per female. There was a trend towards fewer numbers of eggs per egg case from the 45% CCE treatment, but this trend was not significant in the BB ($p=0.398$) and was only slightly significant in the ASB ($p=0.044$). Sibley et al. (1996) observed that fewer eggs were produced after exposure to zinc-spiked sediments. However, there was no difference in the number of eggs per egg case after life cycle exposure to sediments with low levels of contaminants (Benoit et al. 1997) or up to 91 $\mu\text{g/L}$ of 4-nonylphenol (Kahl et al. 1997).

Hatching success was lower in the 45% CCE treatments relative to the reference water treatments in both the ASB and the BB. Decreased hatching success was observed in egg cases laid directly into cadmium solutions (Williams et al. 1987). Other studies have shown hatching success to be insensitive to zinc (Sibley et al. 1996), copper (Gauss et al. 1985), and nickel (Powlesland and George 1986) when egg cases were not laid directly in the treatment solution.

Two endpoints (i.e., emergence and hatching success) showed significant effects in both the ASB and the BB. Effects on either of these endpoints could translate into population-level effects. Reductions in the number of adults emerging would have direct effects on population levels and number of reproducing adults. In addition, if the number

of adults emerging became low enough the adults would not be able to form successful swarms for reproduction (Pascoe et al. 1989). Decreased hatching success could reduce recruitment in the next generation, which may also negatively affect population levels.

In both bioassays, there were several metals that were higher in the 45% CCE treatment than in the reference water treatment. However, only four metals (i.e., cadmium, copper, nickel, and selenium) were detectable in the effluent treatment at concentrations above the Canadian water quality guidelines (WQG) for the protection of aquatic life (CCME 1999). Of these metals, the copper concentration in the effluent was most similar to concentrations in the literature where effects on survival have been observed (Nebeker et al. 1984). However, the water hardness in the present study was much higher than in the Nebeker et al. (1984) study. It is not possible to definitively determine what component of the effluent may be causing the toxicity based on the results of this study because of the complex nature of the effluent. Several metals may act by the same mechanism so effects would be observed at concentrations lower than that of a single metal in isolation. Some metals also have been shown to work synergistically or antagonistically (Fargašová 2001), which makes it difficult to determine the causative metal(s) in a complex effluent such as this one. There were other parameters (e.g., ammonia, calcium, chloride, conductivity, magnesium, nitrate, pH, potassium, sodium, sulphate, total kjeldahl nitrogen, and water hardness) that were measured at higher concentrations in the effluent and may have contributed to the observed toxicity. In addition, chemicals, such as flocculents, that are added during the effluent treatment process may have been present and may have also contributed to the observed effects.

3.5.3 Laboratory and field artificial stream bioassay comparison

There were some similarities in the responses to 45% CCE exposure in the laboratory and field applications of the ASB. All four endpoints (i.e., survival, emergence, time to emergence, and hatching success) that showed significant differences in the field also responded significantly in the laboratory. However, the magnitude of effect of the responses was not always consistent in the two applications of the ASB. These results are consistent with Sibley et al. (1999) who observed similar response patterns in both laboratory sediment toxicity tests and *in situ* tests using *C. tentans*, but different magnitudes of response were observed in the laboratory and field. In the ASB, there were much larger magnitudes of effect for survival, time to emergence, and numbers emerged in the field than in the laboratory. One reason for the larger response in the field may be that the animals are more susceptible to effects from the effluent when tested under ambient conditions. Or, the toxicity of the effluent may be reduced during the time taken to ship the effluent from the field to the laboratory. In contrast, the magnitude of effect for hatching success was slightly greater in the laboratory than in the field. However, hatching success was, on average, lower in the field than in the laboratory. The lower hatching success in the field may be due to the more variable and less than optimal conditions in the field exposure. Although there are no previous studies that have evaluated hatching success of laboratory-reared *C. tentans* larvae in the field, some studies have shown that survival of older larvae is reduced under field conditions (Tucker and Burton 1999).

There were two endpoints (i.e., growth and number of eggs per egg case) for which significant differences were detected in the laboratory ASB application but not in

the field study. First, there were significant effects on log length over time and condition in the laboratory, but neither of these trends was evident in the field. There was significantly more variability in the length data from the field exposure than in the laboratory exposure, which is consistent with other studies that have found greater variability in field data (Sibley et al. 1999). This increased variability may have made it possible to detect significant effects in the laboratory but not in the field. Second, there was a significant decrease in the number of eggs per egg case in the laboratory ($p=0.044$), but there was no significant effect in the field ($p=0.710$). The lack of a significant trend in the field may be due to higher variability in the field. Although, there was no significant difference in CV between the laboratory and the field for this endpoint, there was a trend towards decreased variability in the laboratory. Alternatively, these significant effects seen in the laboratory but not in the field may be a result of differences in exposure (e.g., effluent quality, water quality, or ambient conditions) between the two experiments.

There were differences in effluent quality between the field and laboratory exposures, which may have contributed to the different results between the two ASB studies. The concentrations of some metals were as much as ten times higher in the 100% CCE from one exposure to the other. However, these variations in effluent composition were consistent with the typical range of variability reported for the CCE (NAR Environmental Consultants 2003). Metal mine effluent is a complex mixture that is continually changing due to several factors (e.g., composition of the ore). Despite the variability in the effluent composition, there was a high degree of consistency in the nature of responses observed in the laboratory and field ASB studies. In order for a more

direct comparison to be made between the laboratory and the field, the two experiments would have had to be run simultaneously so the effluent would be more similar in the two exposures.

There were also differences in the quality of reference water used in the two exposures. For example, water hardness in the reference water was almost three times higher in the laboratory (170 ± 10 mg/L) than in the field exposure (59 ± 3 mg/L). Water quality differences in the two experiments may have influenced the ability of the test animals to take up contaminants from the treatment solutions.

One other factor to consider in evaluating results for the bioassay studies was the difference in the turnover rates. The laboratory and field implementations of the ASB had turnover rates of 48 and 6 hours, respectively. This longer turnover rate in the laboratory was necessary to reduce the volume of effluent that was shipped to the laboratory. In long-term exposure systems, the treatment solution must be renewed regularly for several reasons, including the maintenance of dissolved oxygen levels and the prevention of waste product build-up. Dissolved oxygen levels were maintained at high levels in the artificial streams due to the five minute treatment solution turnover rate within the streams. There was also no evidence of a build-up of nitrogenous wastes in the laboratory ASB as a result of the longer turnover rate in the system. However, even if there were increases in waste products, these increases would have been similar across both treatments.

Turnover rates may also have contributed to differences in exposure between the BB and the ASB in the laboratory. The BB used a static renewal system, whereas the treatment solution was turned over more continuously in the artificial stream systems.

Ammonia was significantly higher in the 45% CCE treatment than in the reference water treatment in the BB ($p=0.002$), but there was no significant difference between treatments in the ASB ($p=0.051$). Higher ammonia concentrations in the 45% CCE treatments can be attributed mostly to the composition of the 100% CCE. However, there may have also been a build up of waste products in the beakers due to the intermittent exchange of treatment solution.

3.5.4 Beaker bioassay performance evaluation

In order to provide an appropriate comparison for the ASB, it was necessary to evaluate the BB using performance criteria for the standardized protocol. The USEPA (2000) provides criteria for evaluating *C. tentans* life cycle bioassay acceptability, including criteria for age of initial larvae, mean dry weight, survival, emergence, mean number of eggs per egg case, and percent hatching success. These performance criteria, along with brief explanations of whether these criteria were met in the BB, are listed in Table 3.7. The BB did not meet the performance criteria for the initial age of the animals due to an alteration in the protocol that was made in the present experiment (see section 3.3.2). Although survival was not directly measured at Day 20 and at the end of the test, it can be inferred that both of these criteria were achieved based on the high percent emergence. All other performance criteria were met in the BB.

Table 3.7: Comparison of the beaker bioassay with test acceptability criteria for the standardized *Chironomus tentans* life cycle bioassay protocol ^a

Test acceptability criteria	Acceptable?	Comments ^b
Start with < 24 h old larvae	No	Protocol altered for this study (see section 3.3.2)
Mean dry weight at 20 d \geq 0.6 mg/surviving individual	Yes	1.15 \pm 0.12 at Day 10 of test (21 d old)
Percent survival at Day 20 \geq 70%	Yes	Not measured
Percent survival at end \geq 65%	Yes	Not measured
Emergence \geq 50%	Yes	75 \pm 4%
Mean # eggs per egg case \geq 800	Yes	1213 \pm 103
Percent hatch \geq 80%	Yes	96.41 \pm 2.10%

^a Test acceptability from USEPA 2000

^b Values listed were measured in the reference water treatment of the beaker bioassay

3.6 Conclusions

This study compared the results of two laboratory bioassays used to measure the effects of an environmentally relevant concentration of a MME (45%) on the life cycle of *C. tentans*. The ASB showed increased sensitivity (i.e., reduced endpoint variability and smaller effect sizes) in the detection of effects for most of the endpoints measured compared to the BB. Increased sensitivity in the ASB was due to greater numbers of animals used in this protocol as well as improved methodology to measure endpoints such as growth over time. However, the ASB is more logistically complicated and more expensive to run than the BB. Therefore, it was not our intention to replace the laboratory-based BB. Instead, our aim was to provide a new tool for assessing the effects of a MME on a benthic invertebrate in both laboratory and field settings. The results of this study suggest the ASB is quite sensitive in the measurement of endpoints with population-level consequences.

In the laboratory, the ASB showed significant responses for all of the endpoints in which a significant response was observed in the field. However, there were differences in magnitude of effect between the field and the laboratory. Other endpoints showed significant effects in the laboratory but not in the field. Although there was agreement in the responses observed in the laboratory and the field, the inconsistencies encountered in these two exposure environments could complicate the prediction of effects in the field based on data collected in the laboratory.

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Chapter 4

GENERAL DISCUSSION AND CONCLUSIONS

4.1 Synthesis of results

The overall objective of this research was to develop a more environmentally relevant toxicity bioassay for evaluating metal mine effluent (MME) effects in the laboratory or *in situ*. The end product of this research was a partial life cycle bioassay with *Chironomus tentans* in artificial streams. This research was undertaken in three phases: (1) development of the artificial stream bioassay (ASB); (2) evaluation of the ASB in the field; and (3) evaluation of the ASB in the laboratory with a comparison to the standardized beaker life cycle bioassay (ASTM 2000, Benoit et al. 1997, USEPA 2000) and a qualitative comparison of the ASB in the laboratory and the field. In the first phase, methodology was developed for the new *C. tentans* life cycle bioassay in artificial streams by transferring the standardized *C. tentans* life cycle bioassay (ASTM 2000, Benoit et al. 1997, USEPA 2000) to artificial streams under controlled conditions in the laboratory.

In the second phase, the ASB was evaluated in the field using an environmentally relevant concentration of an effluent from a metal mine in Sudbury, ON, Canada. The field study conducted in 2002 was the first implementation of the life cycle bioassay with *C. tentans* in artificial streams under ambient environmental conditions (Chapter 2). Exposure of *C. tentans* larvae to the effluent resulted in decreased survival, decreased emergence, increased time to emergence, and decreased hatching success relative to a reference water treatment. The effluent did not appear to have an effect on growth, sex ratios, number of egg cases per female, or number of eggs per egg case. The observed effluent-related effects could have population-level consequences because of effects on the number of reproducing adults and recruitment in

the next generation. These results provided evidence that the *C. tentans* life cycle bioassay in artificial streams could be used to measure effects on growth, survival, emergence, and reproduction under ambient field conditions.

In phase three, the *C. tentans* life cycle bioassay in artificial streams (ASB) was compared to an existing beaker life cycle bioassay (BB) in the laboratory (Chapter 3). This evaluation used the same concentration of MME from the same source as the field implementation so a qualitative comparison could be made between the field and laboratory ASB exposures. In the laboratory, the response patterns were consistent between the ASB and the BB for hatching success and time to emergence, but there were inconsistencies between the two bioassays for other endpoints. There was increased sensitivity in the detection of effects (i.e., reduced variability and smaller effect sizes) for most of the endpoints in the ASB compared to the BB likely because of the greater number of animals per replicate in the ASB. These results are evidence that the *C. tentans* life cycle bioassay in artificial streams may have a greater ability to measure the effects of a MME than typical laboratory exposures.

There were significant responses in the laboratory ASB for all endpoints that showed significant effects in the field. However, there were differences in magnitude of effect between the laboratory and the field for some endpoints. Much greater magnitudes of effect were seen in the field for survival, numbers emerged, and time to emergence, which supports the hypothesis that effects on some endpoints are worse in the field because the animals are challenged by stressors in the natural receiving environment. This may suggest the prediction of field effects based on laboratory data may underestimate some *C. tentans* responses to MME.

4.2 Significance of research

This research was significant because a novel, environmentally-relevant bioassay was developed that could be used to evaluate the effects of MME in the laboratory and *in situ*. This bioassay uses artificial streams, which means that effects can be measured under environmentally relevant physicochemical conditions in the field using systems that are replicable and in which conditions, like effluent concentration, can be tightly controlled. However, unlike previous artificial stream research, the ASB evaluates reproductive effects of a MME on a benthic invertebrate *in situ*, which has not previously been done. Reproductive processes can be more sensitive to some contaminants than other endpoints. For example, pulp mills have been shown to affect reproduction in wild fish, while the same effluent does not affect other physiological endpoints measured in the laboratory (Robinson et al. 1994). In addition, if the only individual-level endpoints measured in benthic invertebrates are growth and survival, then effects cannot be fully extrapolated to the population-level. The ASB is also the first mobile, modular artificial stream study to model effects in depositional habitats, rather than in the riffle habitats that have previously been used (Culp et al. 2003).

The ASB was shown to be sensitive to MME exposure both in the field and in the laboratory. The field experiment showed effluent-related effects on endpoints that could result in population-level effects. The laboratory experiment illustrated that the ASB could be more sensitive in the detection of effects than a standardized *C. tentans* beaker life cycle bioassay. For example, there was significantly less variability in the ASB than in the BB for six of eleven endpoints and there were smaller effect sizes in the ASB for all but two endpoints. Therefore, even though the ASB is more logistically

complicated and more expensive to run, it was, at least in this case, also more sensitive than the BB in the detection of effects.

In addition, the types of *C. tentans* responses showed a high degree of similarity between the laboratory and field experiments. However, the variable ambient conditions had two important consequences for the detection of effects. First, the magnitudes of effect were greater in the field than in the laboratory for three of the four endpoints that showed significant effects in both experiments. Second, there were two endpoints for which significant results were observed in the laboratory but not in the field, which may have been due to the increased variability in the field. These results indicate that the bioassay in the laboratory may be more sensitive in the detection of effects than the bioassay in the field. However, the laboratory bioassay appears to have underestimated the effects observed in the field.

This project was also significant because the effects of a MME were measured on life cycle endpoints in a benthic invertebrate. Most previous work has focused on the evaluation of MME effects on community-level endpoints in artificial streams (Perrin et al. 1992) and in the natural receiving environment (Clements et al. 1992, Farag et al. 1998). Research on individual-level endpoints has typically measured the effects of one or two metals in isolation (Ingersoll et al. 1990, Nebeker et al. 1984, Pascoe et al. 1989, Powlesland and George 1986) or the effects of contaminated sediments (Sibley et al. 1996, Wentsel et al. 1977). Thus, this project is one of the first to study MME effects in a bioassay that evaluates effects on growth, survival, emergence, and reproduction. The results from these studies illustrated MME-related effects on survival, emergence, and hatching success, which may result in population-level consequences.

Finally, the ASB can be transferred for application to the Canadian environmental effects monitoring (EEM) program, which is now required for all metal mines and pulp mills in Canada (*Pulp and Paper Effluent Regulations* 1992, *Metal Mining Effluent Regulations* 2002). Endpoints required for EEM include population-level endpoints in fish and community-level endpoints in benthic invertebrates (Environment Canada 2002). The endpoints measured in the ASB are individual-level endpoints in a benthic invertebrate with potential for extrapolation to the population-level. Thus, the ASB endpoints are not specifically part of the monitoring protocol as they do not measure community-level effects. However, this bioassay could be useful in EEM in several situations, including the following: (1) when the receiving environment has benthic communities dominated by one or two taxonomic groups; (2) to explore the mechanisms behind community-level changes; and (3) to help identify the individual component(s) in the effluent responsible for the observed effects.

First, some benthic invertebrate communities are dominated by one or two taxonomic groups, which, in some receiving environments, may be due to historical impacts by anthropogenic processes, such as the release of metal mining or other industrial effluents. For example, Junction Creek, ON, Canada is dominated by chironomids and oligochaetes as a result of over 100 years of mining in the area (Jaagumagi and Bedard 2002). In these communities, any impact on the population of a dominant species could directly affect the density of organisms, which may result in a reduction in the food availability to fish in that receiving environment.

Second, if effects are observed in benthic invertebrates at the community-level, then a potential next step could be to determine the mechanisms underlying these

effects. Community-level changes are typically the result of changes in the populations of component species. Therefore, even though community-level endpoints are more environmentally relevant than endpoints at lower levels of biological organization, they are often less sensitive than population-level endpoints because they are a composite of the responses of the component species in the community. For example, Versteeg et al. (1999) illustrated that the chronic toxicity data from the most sensitive laboratory species tested was often more sensitive than data collected in multi-species mesocosm toxicity tests. Studying effects at the population-level may allow researchers to develop a better understanding of effects observed at the community-level.

Finally, if the effluent is found to have an effect, then the next step in the EEM program is investigation of cause (Environment Canada 2002). In this phase, different components of the final effluent are evaluated individually and together in order to determine which component may be responsible for the observed effects. Once the causative component has been identified, it is possible to make more educated management decisions about reducing the impact of the effluent on the environment (Dubé and MacLatchy 2000, Environment Canada 2002). This phase of the EEM program could be conducted using the *C. tentans* artificial stream life cycle bioassay.

4.3 Difficulties encountered

The major difficulty encountered in the field implementation of the life cycle bioassay in artificial streams was low survival of laboratory-reared, early instar larvae. The observed low survival may have been the result of overcrowding within the streams. The density of animals placed into the streams at the start of the experiment was about

three times higher than the typical density in the standardized beaker life cycle bioassay (Benoit et al. 1997, USEPA 2000). However, comparable levels of mortality were not observed in earlier laboratory trials with similar or greater stocking densities (Hruska, unpublished data). In addition, natural densities of chironomids in the field can often be several times higher than the stocking density used in the artificial streams in the field (Coffman and Ferrington 1984). Therefore, the high mortality was unlikely caused by overcrowding.

The stress of the variable ambient conditions on the young, laboratory-reared larvae was a more likely cause of the high mortality. The larvae were acclimated to ambient field temperatures at a slower acclimation rate than is recommended by the USEPA (2000), which should have helped to prevent thermal shock. Older (8 to 12 days old) *C. tentans* larvae have been shown to be sensitive (i.e., lower survival) to temperatures of 5°C and 11°C (Chappie and Burton 1997). However, first instar larvae may be even more sensitive to the transition from laboratory to field conditions than older larvae. There is no literature available on exposing laboratory-reared, first instar larvae *in situ*. Future work will be necessary to develop protocols for acclimating first instar larvae to ambient conditions.

The purpose of the ASB was to determine whether the effluent caused effects under the variable conditions in the field. The animals in the field implementation of the ASB were stressed by factors in the environment (e.g., temperature and water quality) that are held at optimal levels in the laboratory. Thus, it may not always be possible to maintain high survival in the reference treatment in field exposures. Despite the low survival of larvae in the field, there were still a large number of animals (146 ± 7) that

emerged from the reference streams, which enabled the measurement of effects. Thus, it may not be appropriate to use survival criteria for laboratory-based bioassays to evaluate *in situ* experiments. Effects in the ASB were always determined by comparing results in the exposed treatment to the reference treatment run under similar exposure conditions.

As a result of the low survival in the field implementation, the artificial stream bioassay was shortened to a partial life cycle bioassay. The protocol was changed so that third instar larvae (about 10 days old), rather than newly hatched larvae (less than 24 hours old), would be used to start the exposure. The measurement of all other endpoints remained the same. By starting the exposure with older larvae, there was no measurement of effects during the first two instars, during which time contaminant exposure would primarily affect growth and survival. However, in the second generation, hatching success of the first instar larvae was measured, which includes a measure of effects on embryo survival.

4.4 Future directions

4.4.1 Chironomus tentans life cycle bioassay

As the *C. tentans* bioassay in artificial streams is to be used primarily for testing the effects of complex effluents on sublethal endpoints *in situ*, it would be useful to understand the types of responses expected from different effluent types. Other effluents, such as pulp mill effluents, should be evaluated to determine endpoint responses to various complex effluents, so that it may be possible to develop a more complete understanding of the responses expected from exposure to the various complex effluents.

The animals in the ASB are exposed through water only. Other routes of exposure could be incorporated into future implementations of the bioassay. First, contaminated sediments, rather than silica sand, could be used in the streams. Second, exposure through the food source, in addition to exposure through the water, could be evaluated. Organisms in the environment are exposed to toxicants through their food source, as well as through bioconcentration (Munger and Hare 1997, Roy and Hare 1999). In the ASB, the TetraMin[®] food could be mixed using the appropriate concentration of effluent for the effluent treatments to test exposure through food.

There should also be continued attempts to transfer laboratory-reared first instar larvae to the field for exposure. The current ASB protocol starts exposure with larvae that are about 10 days old, which means that effects on the younger animals (i.e., first and second instar larvae) are not evaluated directly. If appropriate acclimation procedures could be developed so that survival of young larvae in the reference water treatments in the field could be maintained at higher levels, then it may be possible to re-incorporate younger larvae into the bioassay.

4.4.2 Multiple species bioassay

The ecological implications of complex effluents could also be tested by combining the *C. tentans* artificial stream bioassay with a bioassay that evaluates effects on similar endpoints in a predator species. The fathead minnow (*Pimephales promelas*) partial life cycle bioassay has been used to assess the effects of contaminants on survival, physiological endpoints (e.g., liversomatic index, condition factor, gonadosomatic index), and reproduction in adults, and hatching success and deformities

in eggs (Ankley et al. 2001). Preliminary work has been done to transfer the fathead minnow bioassay to artificial streams so that effects can be evaluated *in situ*. The next stage of the research will involve combining the fathead minnow bioassay with the *C. tentans* artificial stream bioassay in order to determine the effects of effluents on multiple trophic levels in the same bioassay (Rickwood and Dubé, National Water Research Institute, Saskatoon, SK, Canada, unpublished). Effects can be evaluated on *C. tentans* and fathead minnows, both separately and together, to determine whether the presence of another species influences responses to the effluent.

4.5 Conclusions

A new method for measuring the effects of metal mine effluents (MME) discharged to aquatic receiving environments was developed. This life cycle bioassay in artificial streams using *Chironomus tentans* was developed and tested both *in situ* and in the laboratory using treated MME. This bioassay can be used to test the effects of complex effluents, such as MME and pulp mill effluent, on endpoints with population-level consequences under ambient environmental conditions. The results obtained from this bioassay can be used to better understand effluent-related effects so that environmental impacts can be reduced.

Chapter 5

REFERENCES

- Allan JD, Daniels RE. 1982. Life table evaluation of chronic exposure of *Eurytemora affinis* (Copepoda) to kepone. *Mar Biol* 66:179-184.
- ASTM. 2000. Standard test methods for measuring the toxicity of sediment-associated contaminants with freshwater invertebrates. E 1706-00b. American Society for Testing and Materials, Philadelphia, PA, USA.
- Ankley GT, Jensen KM, Kahl MD, Korte JJ, Makynen EA. 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 20:1276-1290.
- Armitage P, Cranston PS, Pinder LCV. 1995. *The Chironomidae: the biology and ecology of non-biting midges*. Chapman and Hall, London, UK.
- Barnthouse LW. 1993. Population-level effects. In Suter GW Jr, ed, *Ecological Risk Assessment*. Lewis Publishers, Boca Raton, FL, USA, pp 247-274.
- Benoit DA, Phipps GL, Ankley GT. 1993. A sediment testing intermittent renewal system for the automated renewal of overlying water in toxicity tests with contaminated sediments. *Water Res* 27:1403-1412.
- Benoit DA, Sibley PK, Juenemann JL, Ankley GT. 1997. *Chironomus tentans* life-cycle test: design and evaluation for use in assessing toxicity of contaminated sediments. *Environ Toxicol Chem* 16:1165-1176.
- Bervoets L, Blust R, Verheyen R. 1996. Uptake of zinc by the midge larvae *Chironomus riparius* at different salinities: role of speciation, acclimation, and calcium. *Environ Toxicol Chem* 15:1423-1428.
- Bervoets L, Blust R. 2000. Effects of pH on cadmium and zinc uptake by the midge larvae *Chironomus riparius*. *Aquat Toxicol* 49: 145-157.
- Besser JM, Kubitz JA, Ingersoll CG, Braselton WE, Giesey JP. 1995. Influences on copper bioaccumulation, growth, and survival of the midge, *Chironomus tentans*, in metal-contaminated sediments. *J Aquat Ecosyst Health* 4:157-168.
- Boone MD, Bridges CM. 1999. The effect of temperature on the potency of carbaryl for survival of tadpoles of the green frog (*Rana clamitans*). *Environ Toxicol Chem* 18:1482-1484.
- Borton D, Streblow W, Bousquet T, Cook D. 2000. Fathead minnow (*Pimephales promelas*) reproduction during multi-generation life-cycle tests with kraft mill effluents. *Proceedings*, 4th International Conference on Environmental Impacts of the Pulp and Paper Industry Helsinki, Finland, June 12-15, 2000, pp 152-157.

- Brazner JC, Heinis LJ, Jensen DA. 1989. A littoral enclosure for replicated field experiments. *Environ Toxicol Chem* 8:1209-1216.
- Burton GA Jr, Norberg-King TJ, Ingersoll CG, Benoit DA, Ankley GT, Winger PV, Kubitz J, Lazorchak JM, Smith ME, Greer E, Dwyer FJ, Call DJ, Day KE, Kennedy P, Stinson M. 1996. Interlaboratory study of precision: *Hyaella azteca* and *Chironomus tentans* freshwater sediment toxicity assays. *Environ Toxicol Chem* 15:1335-1343.
- Cairns J Jr. 1995. The genesis of ecotoxicology. In Cairns J Jr, Niederlehner, eds, *Ecological Toxicity Testing*. Lewis Publishers, Boca Raton, FL, USA, pp 1-10.
- Cash KJ, Culp JM, Dubé MG, Lowell RB, Glozier NE, Brua RB. 2003. Integrating mesocosm experiments with field and laboratory studies to generate weight-of-evidence risk assessments for ecosystem health. *Aquat Ecosyst Health Manage* 6:177-183.
- CCME. 1999. Canadian environmental quality guidelines. Canadian Council of Ministers of the Environment, Winnipeg, MB, Canada.
- Chapman PM, Farrell MA, Brinkhurst RO. 1982. Relative tolerances of selected aquatic oligochaetes to combinations of pollutants and environmental factors. *Aquat Toxicol* 2:69-78.
- Chapman PM, Bailey H, Canaria E. 2000. Toxicity of total dissolved solids associated with two mine effluents to chironomid larvae and early life stages of rainbow trout. *Environ Toxicol Chem* 19:210-214.
- Chappie DJ, Burton GA Jr. 1997. Optimization of in situ bioassays with *Hyaella azteca* and *Chironomus tentans*. *Environ Toxicol Chem* 16:559-564.
- Clements WH, Cherry DS, Cairns J Jr. 1988. Structural alterations in aquatic insect communities exposed to copper in laboratory streams. *Environ Toxicol Chem* 7:715-722.
- Clements WH, Cherry DS, Van Hassel JH. 1992. Assessment of the impact of heavy metals on benthic communities at the Clinch River (Virginia): evaluation of an index of community sensitivity. *Can J Fish Aquat Sci* 49:1686-1694.
- Clements WH, Kiffney PM. 1996. Validation of whole effluent toxicity tests: integrated studies using field assessments, microcosms, and mesocosms. In Grothe DR, Dickson KL, Reed-Judkins DK, eds, *Whole Effluent Toxicity Testing: An Evaluation of Methods and Prediction of Receiving System Impacts*. SETAC Pellston Workshop on Whole Effluent Toxicity, September 16-25, 1995, Pellston, MI, USA, SETAC Press, Pensacola, FL, USA, pp 229-244.

- Coffman WP, Ferrington LC Jr. 1984. Chironomidae. In Merritt RW, Cummins KW, eds, *An Introduction to the Aquatic Insects of North America*, 2nd ed. Kendall/Hunt Publishing Company, Dubuque, IA, USA, pp 551-652.
- Conlon M, Gunn JM, Morris JR. 1992. Prediction of lake trout (*Salvelinus namaycush*) presence in low-alkalinity lakes near Sudbury, Ontario. *Can J Fish Aquat Sci* 49(Suppl 1):95:101.
- Cotter AJR, Phillips DHJ, Ahsanullah M. 1982. The significance of temperature, salinity, and zinc as lethal factors for the mussel *Mytilus edulis* in a polluted estuary. *Mar Biol* 68:135-144.
- Culp JM, Podemski CL. 1996. Design and application of a novel stream microcosm system for assessing effluent impacts to large rivers. In Servos M, Munkittrick KR, Carey JH, van der Kraak G, eds, *Environmental Fate and Effects of Pulp and Paper Mill Effluents*. St. Lucie Press, Delray Beach, FL, USA, pp 549-555.
- Culp JM, Podemski CL, Cash KJ, Lowell RB. 1996. Utility of field-based artificial streams for assessing effluent effects on riverine ecosystems. *J Aquat Ecosyst Health* 5:117-124.
- Culp JM, Lowell RB, Cash, KJ. 2000a. Integrating mesocosm experiments with field and laboratory studies to generate weight-of-evidence risk assessments for large rivers. *Environ Toxicol Chem* 19:1167-1173.
- Culp JM, Podemski CL, Cash KJ. 2000b. Interactive effects of nutrients and contaminants from pulp mill effluents on riverine benthos. *J Aquat Ecosyst Stress Recovery* 8:67-75.
- Culp JM, Cash KJ, Glozier NE, Brua, RB. 2003. Effects of pulp mill effluent on benthic assemblages in mesocosms along the Saint John River, Canada. *Environ Toxicol Chem* 22:2916-2925.
- Culp JM, Glozier NE, Cash KJ, Dubé MG, MacLatchy DL, Brua B, Firth BK, Wilson GM. 2004. Cumulative effects investigation of pulp mill and sewage effluent impacts on benthic food webs: a mesocosm example. In Borton DL, Hall TJ, Fisher RP, Thomas JF, eds, *Pulp & Paper Mill Effluent Environmental Fate & Effects*. DesTech Publications, Lancaster, PA, USA, pp 464-472.
- De Schamphelaere KAC, Heijerick DG, Janssen CR. 2002. Refinement and field validation of a biotic ligand model predicting acute copper toxicity to *Daphnia magna*. *Comp Biochem Physiol* 133C:243-258.
- Dubé MG, Culp JM. 1996. Growth responses of periphyton and chironomids exposed to biologically treated bleached-kraft pulp mill effluent. *Environ Toxicol Chem* 15:2019-2027.

- Dubé MG, Culp JM, Scrimgeour GJ. 1997. Nutrient limitation and herbivory: processes influenced by bleached kraft pulp mill effluent. *Can J Fish Aquat Sci* 54: 2584-2595.
- Dubé MG, MacLatchy DL. 2000. Endocrine responses of *Fundulus heteroclitus* to effluent from a bleached-kraft pulp mill before and after installation of reverse osmosis treatment of a waste stream. *Environ Toxicol Chem* 19:2788-2796.
- Dubé MG, Culp JM, Cash KJ, Glozier NE, MacLatchy DL, Podemski CL, Lowell RB. 2002a. Artificial streams for environmental effects monitoring (EEM): development and application in Canada over the past decade. *Water Qual Res J Can* 37:155-180.
- Dubé MG, MacLatchy DL, Culp JM, Gillis G, Parker R, Courtenay S, Gilman C. 2002b. Utility of mobile, field-based artificial streams for assessing effects of pulp mill effluents on fish in the Canadian environmental effects monitoring (EEM) program. *J Aquat Ecosyst Stress Recovery* 9:85-102.
- Dubé MG, MacLatchy DL, Firth BK, Culp JM, Glozier NE, Cash KL. 2004a. Using mesocosms to explore confounding factors influencing longnose dace (*Rhinichthys cataractae*) responses to kraft mill effluent on the Wapiti River, AB, Canada. In Borton DL, Hall TJ, Fisher RP, Thomas JF, eds, *Pulp & Paper Mill Effluent Environmental Fate & Effects*. DesTech Publications, Lancaster, PA, USA, pp 481-490.
- Dubé MG, MacLatchy DL, Hruska KA, Glozier NE. 2004b. Assessing the response of creek chub (*Semotilus atromaculatus*) and pearl dace (*Semotilus margarita*) to metal mine effluents using in situ artificial streams in Sudbury, Ontario, Canada. *Environ Toxicol Chem* In review.
- Dubé MG, MacLatchy DL, Kieffer JD, Glozier NE, Culp JM, Cash KJ. 2004c. Effects of metal mining effluent on Atlantic salmon (*Salmo salar*) and slimy sculpin (*Cottus cognatus*): using mesocosms to assess existing effects and predict future consequences. *Sci Total Environ* In review.
- Environment Canada. 1992a. Biological test method: fertilization assay with echinoids (sea urchins and sand dollars). EPS 1/RM/25. Ottawa, ON, Canada.
- Environment Canada. 1992b. Biological test method: test of larval growth and survival using fathead minnows. EPS 1/RM/22. Ottawa, ON, Canada.
- Environment Canada. 1992c. Biological test method: test of reproduction and survival using the cladoceran *Ceriodaphnia dubia*. EPS 1/RM/21. Ottawa, ON, Canada.
- Environment Canada. 1997. Biological test method: test for survival and growth in sediment using the larvae of freshwater midges (*Chironomus tentans* or *Chironomus riparius*). EPS 1/RM/32. Ottawa, ON, Canada.

- Environment Canada. 1998. Pulp and paper technical guidance for aquatic environmental effects monitoring. EEM/1998/1. National EEM Office, Science Policy and Environmental Quality Branch, Burlington, ON, Canada.
- Environment Canada. 2000a. Biological test method: reference method for determining acute lethality of effluents to *Daphnia magna*. EPS 1/RM/14. Ottawa, ON, Canada.
- Environment Canada. 2000b. Biological test method: reference method for determining acute lethality of effluents to rainbow trout. EPS 1/RM/13. Ottawa, ON, Canada.
- Environment Canada. 2002. Metal mining guidance document for aquatic environmental effects monitoring. EEM/June 2002. National EEM Office, Science Policy and Environmental Quality Branch, Burlington, ON, Canada.
- Farag AM, Woodward DF, Goldstein JN, Brumbaugh W, Meyers JS. 1998. Concentrations of metals associated with mining waste in sediments, biofilm, benthic macroinvertebrates, and fish from the Coeur d'Alene River Basin, Idaho. *Arch Environ Contam Toxicol* 34:119-127.
- Fargašová A. 2001. Winter third- to fourth-instar larvae of *Chironomus plumosus* as bioassay tools for assessment of acute toxicity of metals and their binary combinations. *Ecotoxicol Environ Saf* 48:1-5.
- Forrow DM, Maltby L. 2000. Toward a mechanistic understanding of contaminant-induced changes in detritus processing in streams: direct and indirect effects on detritivore feeding. *Environ Toxicol Chem* 19:2100-2106.
- Gauss JD, Woods PE, Winner RW, Skillings JH. 1985. Acute toxicity of copper to three life stages of *Chironomus tentans* as affected by water hardness-alkalinity. *Environ Pollut* 37:149-157.
- Gerhardt A, Janssens de Bisthoven L. 1995. Behavioural, developmental and morphological responses of *Chironomus* gr. *thummi* larvae (Diptera, Nematocera) to aquatic pollution. *J Aquat Ecosyst Health* 4:205-214.
- Giesy JP, Graney RL, Newsted JL, Rosiu CJ, Benda A, Kreis RG Jr, Horvath FJ. 1988. Comparison of three sediment bioassay methods using Detroit River sediments. *Environ Toxicol Chem* 7:483-498.
- Greenberg MS, Burton GA Jr, Rowland CD. 2002. Optimizing interpretation of in situ effects of riverine pollutants: impacts of upwelling and downwelling. *Environ Toxicol Chem* 21:289-297.
- Griffiths RW. 1992. Effects of pH on community dynamics of Chironomidae in a large river near Sudbury, Ontario. *Can J Fish Aquat Sci* 49(Suppl 1):76-86.

- Griffiths RW, Keller W. 1992. Benthic macroinvertebrate changes in lakes near Sudbury, Ontario, following a reduction in acid emissions. *Can J Fish Aquat Sci* 49(Suppl 1):63-75.
- Guckert JB. 1993. Artificial streams in ecotoxicology. pp 350-356. In Lamberti GA, Steinman AD, eds, Research in artificial streams: applications, uses, and abuses. *J North Am Benthol Soc* 12:313-384.
- Gunn J, Negusanti J, Potvin R, Beckett P, Winterhalder K. 1995. Ecosystem recovery after emission reductions: Sudbury, Canada. *Water Air Soil Pollut* 85:1783-1788.
- Harrahy EA, Clements WH. 1997. Toxicity and bioaccumulation of a mixture of heavy metals in *Chironomus tentans* (Diptera: Chironomidae) in synthetic sediment. *Environ Toxicol Chem* 16:317-327.
- Havas M, Woodfine DG, Lutz P, Yung K, MacIsaac HJ, Hutchinson TC. 1995. Biological recovery of two previously acidified, metal-contaminated lakes near Sudbury, Ontario, Canada. *Water Air Soil Pollut* 85:791-796.
- Ingersoll CG, Dwyer FJ, May TW. 1990. Toxicity of inorganic and organic selenium to *Daphnia magna* (Cladocera) and *Chironomus riparius* (Diptera). *Environ Toxicol Chem* 9:1171-1181.
- Ingersoll CG, Ankley GT, Benoit DA, Brunson EL, Burton GA Jr, Dwyer FJ, Hoke RA, Landrum PF, Norberg-King TJ, Winger PV. 1995. Toxicity and bioaccumulation of sediment-associated contaminants using freshwater invertebrates: a review of methods and applications. *Environ Toxicol Chem* 14:1885-1894.
- Jaggumagi R, Bedard D. 2002. Junction Creek System (Sudbury) Environmental Monitoring Study September 1999. Ontario Ministry of the Environment, Northern Region, Sudbury District Office, Sudbury, ON, Canada.
- Kahl MD, Makynen EA, Kosian PA, Ankley GT. 1997. Toxicity of 4-nonylphenol in a life-cycle test with the midge *Chironomus tentans* *Ecotoxicol Environ Saf* 38:155-160.
- Keller W. 1992. Introduction and overview to aquatic acidification studies in the Sudbury, Ontario, Canada, area. *Can J Fish Aquat Sci* 49(Suppl 1):3-7.
- Kemble NE, Brumbaugh WG, Brunson EL, Dwyer FJ, Ingersoll CG, Monda DP, Woodward DF. 1994. Toxicity of metal-contaminated sediment from the upper Clark Fork River, Montana, to aquatic invertebrates and fish in laboratory exposures. *Environ Toxicol Chem* 13:1985-1997.

- Kimball KD, Levin SA. 1985. Limitations of laboratory bioassays: the need for ecosystem-level testing. *BioScience* 35:165-171.
- Kovacs TG, Megraw SR. 1996. Laboratory responses of whole organisms exposed to pulp and paper mill effluents:1991-1994. In Servos M, Munkittrick KR, Carey JH, van der Kraak GJ, eds, *Environmental Fate and Effects of Pulp and Paper Mill Effluents*. St. Lucie Press, Delray Beach, FL, USA, pp 459-472.
- Langston WJ. 1990. Toxic effects of metals and the incidence of metal pollution in marine ecosystems. In Furness RW, Rainbow PS, eds, *Heavy Metals in the Marine Environment*. CRC Press, Boca Raton, FL, USA, pp 101-122.
- Liber K, Call DJ, Dawson TD, Whiteman FW, Dillon TM. 1996. Effects of *Chironomus tentans* larval growth retardation on adult emergence and ovipositing success: implications for interpreting freshwater sediment bioassays. *Hydrobiol* 323:155-167.
- Liber K, Solomon KR, Carey JH. 1997. Persistence and fate of 2,3,4,6-tetrachlorophenol and pentachlorophenol in limnocorrals. *Environ Toxicol Chem* 16:293-305.
- Liber K, Gangl JA, Corry TD, Heinis LJ, Stay FS. 1999. Lethality and bioaccumulation of 4-nonylphenol in bluegill sunfish in littoral enclosures. *Environ Toxicol Chem* 18:394-400.
- Lohner TW, Fisher SW. 1990. Effects of pH and temperature on the acute toxicity and uptake of carbaryl in the midge, *Chironomus riparius*. *Aquat Toxicol* 16: 335-354.
- Lowell RB, Culp JM, Wrona FJ. 1995. Toxicity testing with artificial streams: effects of differences in current velocity. *Environ Toxicol Chem* 14:1209-1217.
- Luoma SN, Carter JL. 1991. Effects of trace metals on aquatic benthos. In Newman MC, McIntosh AW, eds, *Metal Ecotoxicology: Concepts and Applications*. Lewis Publishers, Chelsea, MI, USA, pp 261-300.
- Matuszek JE, Wales DL, Gunn JM. 1992. Estimated impacts of SO₂ emissions from Sudbury smelters on Ontario's sportfish populations. *Can J Fish Aquat Sci* 49(Suppl 1):87-94.
- Metal Mining Effluent Regulations. 2002. *Fisheries Act*. 6 June, 2002. SOR/2002-222.
- Moriarty F. 1999. *Ecotoxicology: The Study of Pollutants in Ecosystems*. Academic Press, San Diego, CA, USA.
- Mount DI, Stephan CE. 1967. A method for establishing acceptable toxicant limits for fish - malathion and the butoxyethanol ester of 2,4-D. *Trans Am Fish Soc* 96:185-193.

- Munger C, Hare, L. 1997. Relative importance of water and food as cadmium sources to an aquatic insect (*Chaoborus punctipennis*): implications for predicting Cd bioaccumulation in nature. *Environ Sci Technol* 31:891-895.
- NAR Environmental Consultants. 2003. Review of historical information INCO Limited - Sudbury operations: Junction Creek and Monk Lake watersheds Sudbury, Ontario. Technical Report. Sudbury, ON, Canada.
- Nebeker AV, Cairns MA, Wise CM. 1984. Relative sensitivity of *Chironomus tentans* life stages to copper. *Environ Toxicol Chem* 3:151-158.
- Nebeker AV, Onjukka ST, Cairns MA. 1988. Chronic effects of contaminated sediments on *Daphnia magna* and *Chironomus tentans*. *Bull Environ Contam Toxicol* 41:574-581.
- Nriagu JO, Wong HKT, Lawson G, Daniel P. 1998. Saturation of ecosystems with toxic metals in the Sudbury basin, Ontario, Canada. *Sci Total Environ* 223:99-117.
- Parrish PR. 1995. Acute toxicity tests. In Rand GM, ed, *Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment*, 2nd ed. Taylor and Francis, Philadelphia, PA, USA, pp 947-973.
- Pascoe D, Williams KA, Green DWJ. 1989. Chronic toxicity of cadmium to *Chironomus riparius* Meigen - effects upon larval development and adult emergence. *Hydrobiol* 175:109-115.
- Perrin CJ, Wilkes B, Richardson JS. 1992. Stream periphyton and benthic insect responses to additions of treated acid mine drainage in a continuous-flow on-site mesocosm. *Environ Toxicol Chem* 11:1513-1525.
- Postma JF, Davids C. 1995. Tolerance induction and life cycle changes in cadmium-exposed *Chironomus riparius* (Diptera) during consecutive generations. *Ecotoxicol Environ Saf* 30:195-202.
- Powlesland C, George J. 1986. Acute and chronic toxicity of nickel to larvae of *Chironomus riparius* (Meigen). *Environ Pollut* 42:47-64.
- Pulp and Paper Effluent Regulations. 1992. *Fisheries Act*. 7 May, 1992. SOR/92-269.
- Rand GM, Wells PG, McCarty LS. 1995. Introduction to aquatic toxicology. In Rand GM, ed, *Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment*, 2nd ed. Taylor and Francis, Philadelphia, PA, USA, pp 3-67.
- Robinson RD, Carey JH, Solomon KR, Smith IR, Servos M, Munkittrick KR. 1994. Survey of receiving-water environmental impacts associated with discharges from

- pulp mills. 1. Mill characteristics, receiving-water chemical properties and lab toxicity tests. *Environ Toxicol Chem* 13:1075-1088.
- Roline RA. 1988. The effects of heavy metals pollution of the upper Arkansas River on the distribution of aquatic macroinvertebrates. *Hydrobiol* 160:3-8.
- Rouch JM, Simmons TW, Kerans BL, Smith BP. 1997. Relative acute effects of low pH and high iron on the hatching and survival of the water mite (*Arrenurus manubriator*) and the aquatic insect (*Chironomus riparius*). *Environ Toxicol Chem* 16:2144-2150.
- Roy I, Hare L. 1999. Relative importance of water and food as cadmium sources to the predatory insect *Sialis velata* (Megaloptera). *Can J Fish Aquat Sci* 56: 1143-1149.
- Sasson-Brickson G, Burton GA Jr. 1991. In situ and laboratory sediment toxicity testing with *Ceriodaphnia dubia*. *Environ Toxicol Chem* 10:201-207.
- Sibley PK, Ankley GT, Cotter AM, Leonard EN. 1996. Predicting chronic toxicity of sediments spiked with zinc: an evaluation of the acid-volatile sulfides model using a life-cycle test with the midge *Chironomus tentans*. *Environ Toxicol Chem* 15:2102-2112.
- Sibley PK, Benoit DA, Ankley GT. 1997. The significance of growth in *Chironomus tentans* sediment toxicity tests: the relationship to reproduction and demographic endpoints. *Environ Toxicol Chem* 16:336-345.
- Sibley PK, Benoit DA, Balcer MD, Phipps GL, West CW, Hoke RA, Ankley GT. 1999. In situ bioassay chamber for assessment of sediment toxicity and bioaccumulation using benthic invertebrates. *Environ Toxicol Chem* 18:2325-2336.
- Sibley PK, Ankley GT, Benoit DA. 2001 Factors affecting reproduction and the importance of adult size on reproductive output in the midge *Chironomus tentans*. *Environ Toxicol Chem* 20:1296-1303.
- Timmermans KR, Peeters W, Tonkes M. 1992. Cadmium, zinc, lead and copper in *Chironomus riparius* (Meigen) larvae (Diptera: Chironomidae): uptake and effects. *Hydrobiol* 241:119-134.
- Tucker KA, Burton GA Jr. 1999. Assessment of nonpoint-source runoff in a stream using *in situ* and laboratory approaches. *Environ Toxicol Chem* 18:2797-2803.
- USEPA. 1993. Standard operating procedures for laboratory cultures of *Chironomus tentans*. ERL-D-SOP-CTI-015. U.S. Environmental Protection Agency, Environmental Research Laboratory, Duluth, MN, USA.

- USEPA. 1994. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms. 3rd ed. EPA 600/4-91/003. U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Cincinnati, OH, USA.
- USEPA. 2000. Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. EPA 600/R-99/064. U.S. Environmental Protection Agency, Environmental Research Laboratory, Duluth, MN, USA.
- USEPA. 2002. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. 5th ed. EPA 821/R-02/012. U.S. Environmental Protection Agency, Washington DC, USA.
- Versteeg DJ, Belanger SE, Carr GJ. 1999. Understanding single-species and model ecosystem sensitivity: data-based comparison. *Environ Toxicol Chem* 18:1329-1346.
- Watts MM, Pascoe D. 2000. A comparative study of *Chironomus riparius* Meigen and *Chironomus tentans* Fabricus (Diptera: Chironomidae) in aquatic toxicity tests. *Arch Environ Contam Toxicol* 39:299-306.
- Wentzel R, McIntosh A, Atchison G. 1977. Sublethal effects of heavy metal contaminated sediment on midge larvae (*Chironomus tentans*). *Hydrobiol* 56:153-156.
- Wentzel R, McIntosh A, McCafferty W. 1978. Emergence of the midge *Chironomus tentans* when exposed to heavy metal contaminated sediment. *Hydrobiol* 57:195-196.
- West CW, Mattson VR, Leonard EN, Phipps GL, Ankley GT. 1993. Comparison of the relative sensitivity of three benthic invertebrates to copper-contaminated sediments from the Keweenaw Waterway. *Hydrobiol* 262:57-63.
- Whiteman FW, Ankley GT, Kahl MD, Rau DM, Balcer MD. 1996. Evaluation of interstitial water as a route of exposure for ammonia in sediment tests with benthic macroinvertebrates. *Environ Toxicol Chem* 15: 794-801.
- Williams KA, Green DWJ, Pascoe D, Gower DE. 1986. The acute toxicity of cadmium to different larval stages of *Chironomus riparius* (Diptera: Chironomidae) and its ecological significance for pollution regulation. *Oecologia* 70:362-366.
- Williams KA, Green DWJ, Pascoe D, Gower E. 1987. Effect of cadmium on oviposition and egg viability in *Chironomus riparius* (Diptera: Chironomidae). *Bull Environ Contam Toxicol* 38:86-90.
- Zar JH. 1999. *Biostatistical Analysis*. Prentice Hall, Upper Saddle River, NJ, USA.