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Toxicity Assessment Of The Antimicrobial Triclocarban Using Sub-Lethal Behaviour And Reproduction Endpoints

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TOXICITY ASSESSMENT OF THE ANTIMICROBIAL TRICLOCARBAN USING SUB-LETHAL BEHAVIOUR AND REPRODUCTION ENDPOINTS

by

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Abstract

Toxicity assessment of the antimicrobial triclocarban using sub-lethal behaviour and reproduction endpoints

Master of Applied Science, 2013

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Environmental Applied Science and Management

Ryerson University

Aquatic environments have long been used as disposal sites for domestic and industrial wastes, resulting in increasing chemical contamination, decreased water quality, and concern for ecosystem health and drinking water sources. This study utilized bioassays, the "golden standard" method to measure biological impact, to assess the toxicity of the widely found surface water contaminant, the antimicrobial triclocarban. Culturing protocols were implemented to provide healthy, age-synchronized organisms for bioassays. Behaviour and reproduction were demonstrated as useful endpoints while refining these methods using 4-chlorophenol and were successfully implemented in the toxicity assessment of triclocarban. While no sub-lethal behavioural impact was seen, 10.0 ppb triclocarban was found to delay reproduction in *Daphnia magna*. This delay could result in population, community, and ecosystem-level responses.

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Table of Contents

List of Tables

List of Figures

List of Appendices

1.0 INTRODUCTION

1.1 Overview

Aquatic environments have long been used as disposal sites for domestic and industrial wastes, resulting in increasing chemical contamination, decreased water quality, and concern for ecosystem health and drinking water sources (Streeter, 1931; Hubbs, 1933; Wright and Tidd, 1933; Middleton and Rosen, 1956; Beeton, 1965; Tsai, 1970; Esvelt *et al*., 1973; Anderson and D'Apollonia, 1978; Chambers *et al*., 1997; Servos *et al*., 2001). When chemical contaminant levels are in excess of the ecosystem's attenuation capacity, serious implications for endemic biota may occur (Anderson and D'Apollonia, 1978; Cooney, 1995). Adverse effects may directly cause death, or be sub-lethal and affect the organism's ability to be mobile, grow, develop and reproduce (Cooney, 1995). Biological assays, or bioassays, are necessary for assessing these adverse effects and the impact of contaminants on our environment.

Analytical methods have greatly decreased limits of detection; however, external standards for every possible contaminant and its daughter metabolites do not currently exist. Chemical analysis is therefore only a partial analysis; our current technologies do not allow us to detect and measure every contaminant. Even if full characterization of environmental samples were possible, chemical analysis can only suggest a toxic *potential* and "is insufficient to provide a realistic appraisal of *actual* toxicity" (Samoiloff, 1989). Chemical analysis does not consider the bioavailability, biological activity nor the complex interactions between chemicals as well as between chemicals and the environment (Wang *et al*., 2003). Cairns and Mount (1990) state "that no instrument has yet been devised that will measure toxicity and while chemical concentrations can be measured with an instrument, only living material can be used to measure toxicity." It is critical to employ bioassays to evaluate the toxicity of contaminants because it is the only *true* way to measure environmental impact.

A number of biological endpoints have been used to assess toxicity in biota, with the most popular being the easiest to observe: lethality and immobilization. However, sub-lethal endpoints such as animal behaviour (Warner *et al*., 1966; Hellou *et al*., 2008; Robinson, 2009; Hellou, 2011) and reproductive impairment (Beisinger and Christensen, 1972; Cooper, 1995) are far more sensitive endpoints. Behaviour, or the animal's overt activity, is a cumulative response

that integrates the environmental conditions with the organism's biochemical and physiological processes (Warner *et al*., 1966). Reproductive endpoints measure the effect of toxicants on the reproductive cycle. Since many contaminants in our natural systems are found in sub-lethal levels, assessing endpoints such as behaviour and reproduction gives a more accurate representation of the potential hazard of these contaminants to our ecosystem. Presented in this research are methods for employing behaviour and reproduction as endpoints of aquatic toxicity. Building on existing knowledge, these protocols are refined with the use of a positive toxicant, and are demonstrated in assessing the toxicity of the antimicrobial triclocarban (TCC).

1.2 Objectives

The overall objective was to further the use of bioassays to assess the impact of the antimicrobial triclocarban, rather than depending on chemical analysis.

Comprising this overall objective are several sub-objectives:

- To further refine behaviour toxicity protocols based on existing knowledge;
- To use reproductive endpoints in conjunction with behavioural endpoints;
- To implement culturing procedures that produce healthy, age-synchronized organisms for toxicity testing.

This project will contribute to the existing body of knowledge regarding triclocarban toxicity as well as help to determine if the chosen organisms and related responses are suitable for toxicity testing.

1.3 Importance of Bioassays

Bioassays are routinely used to assess the toxicity of environmental contaminants. In addition to being much more cost-effective than comprehensive chemical analysis, bioassays "provide a direct functional response that relates to the overall toxic properties of the mixture of compounds present in a sample" (Baun and Nyholm, 1996). Chemical analysis can, with many limitations, provide us with a chemical characterization of environmental samples; however, it cannot predict the ecological effect of contaminants in those samples to the environment (Wang *et al*., 2003). While bioassays and chemical analysis can work hand-in-hand, bioassays should guide the chemical analysis and not vice-versa. Only living material, such as through the use of bioassays, can be used to measure toxicity and therefore attempt to understand the effect of a

pollutant such as triclocarban on the environment (Cairns and Mount, 1990). It is important to use bioassays to evaluate the toxicity of contaminants such as triclocarban because it is the only *true* way to measure environmental impact.

1.3.1 Review of Bioassay Organisms in WWTP Effluent Toxicity Research

The antimicrobial triclocarban (see section 1.7), a domestic pollutant found in personal care products, enters the aquatic environment mostly through the discharge of wastewater treatment plant (WWTP) effluent. Toxicity of WWTP effluent has been assessed using various aquatic organisms, including the green algae *Pseudokirchneriella subcapitata*, formerly known as *Selenastrum capricornutum* (Bailey *et al*., 2000; Manusadzianas *et al*., 2003; Hernando *et al*., 2005; Ra *et al*., 2007; Pignata *et al*., 2012) and *Scenedesmus quadricauda* (Di Marzio *et al*., 2005), the pondweed algae *Nitellopsis obtusa* (Manusadzianas *et al*., 2003), the fairy shrimp *Thamnocephalus platyurus* (Manusadzianas *et al*., 2003), the protozoate *Tetrahymena thermophila* (Manusadzianas *et al*., 2003), and the bacteria *Vibrio fischeri* (Manusadzianas *et al*., 2003; Wang *et al*., 2003; Pignata *et al*., 2012; Hernando *et al*., 2005), and *Photobacterium phosphoreum* (Logue *et al*., 1989; Rutherford *et al*., 1994). Invertebrates include the water fleas *Daphnia magna* (Hernando *et al*., 2005; Manusadzianas *et al*., 2003; Pignata *et al*., 2012; Schroder *et al*., 1991; Rutherford *et al*., 1994; Ra *et al*., 2007; Maltby *et al*., 2000; Pessala *et al*., 2004), *Daphnia pulex* (Logue *et al*., 1989), *Daphnia spinulata* (Di Marzio *et al*., 2005) and *Ceriodaphnia dubia* (Bailey *et al*., 2000; Neiheisel *et al*., 1988; Schroder *et al*., 1991), and the amphipods *Gammarus pulex* (Maltby *et al*., 2000) and *Hyalella curvispina* (Di Marzio *et al*., 2005). Vertebrates were fish including the golden shiner *Notemigonous chrysoleucas* (Esvelt *et al*., 1973), the stickleback *Gasterosteus aculeatus* (Esvelt *et al*., 1973; Rutherford *et al*., 1994), the rainbow trout *Oncorhynchus mykiss* (Rutherford *et al*., 1994) and the fathead minnow *Pimephales promelas* (Neiheisel *et al*., 1988; Birge *et al*., 1989). Overall, the most popular test organisms for assessing the toxicity of WWTP effluent were *Pseudokirchneriella subcapitata* and *Daphnia* sp.

1.3.2 Single-Species Bioassays

This study employs multiple single-species toxicity bioassays to assess the toxicity of triclocarban potentially found in WWTP effluent. Single-species bioassays are relatively easy to perform in a laboratory and can provide a reliable way of estimating toxicity; however, it is difficult to predict responses at higher levels of organization from single species bioassays alone (Cairns, 1984). Single-species bioassays often use one of the most sensitive species in the ecosystem and therefore may be overprotective and overestimate toxicity (Cairns, 1984). The sensitivity of species to different toxicants has been well demonstrated. For example, Phipps *et al*. (1995) examined the relative sensitivity of three benthic macroinvertebrates to ten contaminants and found differing sensitivities among the species to different contaminants. Phipps *et al*. (1995) stresses the importance of testing with multiple species, especially when unknown contaminants are present. Due to this inherent difference in species' sensitivities, and to attempt to minimize over- or under-estimation of toxicity, this study utilizes four species, each from different taxa, to further refine behaviour toxicity protocols and estimate toxicity of TCC. They include the zooplankton *Daphnia magna*, the amphipod *Hyalella azteca*, the aquatic worm *Lumbriculus variegatus* and the duckweed *Lemna minor* (see sections 1.6.2 – 1.6.5).

1.4 Protocol Development

Standardized toxicity test protocols, such as those published by Environment Canada, United States Environmental Protection Agency (EPA), American Association for Public Health (APHA), American Society for Testing and Materials (ASTM) and Organization for Economic Co-operation and Development (OECD), provide very specific factors and detailed methods for toxicity test design. This high level of specificity reduces the chance that extraneous test factors, such as water chemistry, substrate or organism age or developmental stage may have an effect on organism response (Buikema Jr., 1982). While standardized protocols theoretically increase data repeatability, accuracy, comparability and efficiency (Davis, 1977; Buikema Jr., 1982; Chapman, 1995), in practice interlaboratory variation can be high (Buikema Jr., 1982). Not all laboratories have the equipment and fiscal capability to undertake rigorous standardized methods. In addition, over-standardization of methods may stifle "innovative and creative" work (Davis, 1977). The purpose of the toxicity test must inform the test design (Buikema Jr., 1982). If the purpose is comparison of toxicity data between toxicants, laboratories or personnel, then standardization is important and should be high to minimize variation. Comparatively, if the purpose is to describe

or predict the behaviour of a toxicant in a particular system, over-standardization of protocols could hinder rather than aid (Buikema Jr., 1982).

An objective of this study is to design usable behaviour bioassay protocols that can be tweaked, within reason, to fit the confines and capabilities of the laboratory. Because the nature of the proposed behaviour bioassay is inherently quantitative relative to the control or reference water, the capacity for inter-laboratory comparison of results is low. With the use of positive controls or reference toxicants, results could be normalized to the reference toxicant to aid in comparison between laboratories.

1.4.1 Reference Toxicants

Reference or positive toxicants are materials used in toxicity tests to estimate the condition or sensitivity of a group of test organisms (Lee, 1980). Factors such as water quality, genetic history of test organisms and technician training and experience can differ between laboratories as well as within one laboratory over time (Environment Canada, 1990). These factors may lead to differences in organism sensitivity and thus doubts as to whether the results are in fact due to the toxicant of study or the condition of the test organism (Lee, 1980). Reference toxicants are important because they ultimately provide experimental control (Lee, 1980). They can be used in multiple ways depending on the objective of control. The most common objective is to determine the condition or sensitivity of a group of test organisms, whether for comparison between laboratories or within a single laboratory over time (Environment Canada, 1990). Bioassays are conducted using the reference toxicant to generate a series of endpoints including the lethal concentration for 50% of the organisms (LC_{50}), the effective concentration at which 50% of the organisms display a particular endpoint (EC_{50}), the no observed effect concentration (NOEC) or lowest observed effect concentration (LOEC) (Environment Canada, 1990). A mean chart, also termed a "warning chart," is prepared by plotting the results of successive bioassays with different groups of organisms over time against the endpoint concentrations (Environment Canada, 1990). Figure 1 presents an example of a warning chart. "Acceptable" variability is defined as results within the 95% confidence interval, or within two times the standard deviation above and below the mean for a sufficiently large sample size of >15 successive bioassays (Environment Canada, 1990). If the result of a reference toxicant bioassay falls outside of the accepted limits this indicates variability, e.g., differences in

test organism health, genetic tolerance to toxicants or water quality (Environment Canada, 1990). Warning charts can also be used to assess training or re-training of personnel (Environment Canada, 1990).

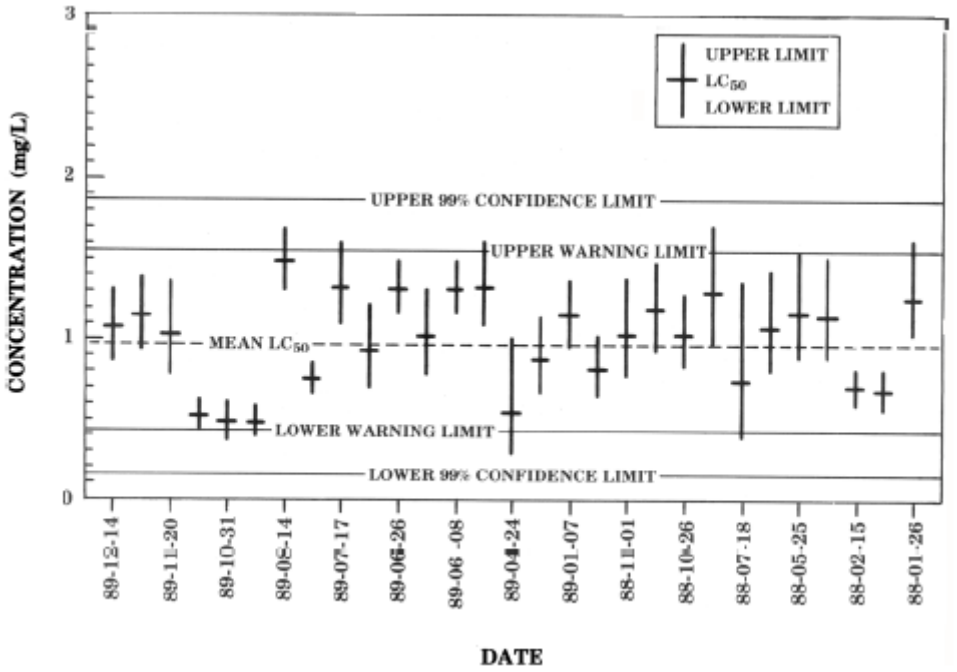

Figure 1 Example of a mean chart or warning chart (Environment Canada, 1990)

A second approach to using reference toxicants is as an experimental control within the design of the bioassay (Lee, 1980). The reference toxicant is tested along with the toxicant in question, with the objective of confirming the response induced by the reference toxicant with historical data. Any deviation from what is considered "normal" behaviour, as established through repeated behaviour bioassays with the reference toxicants over time, is then investigated for discrepancies with the test organism, solution preparation or environmental factors.

While there is no one standardized reference toxicant for toxicity testing bioassays, Environment Canada (1990) has evaluated four organic and seven inorganic chemicals and recommended suitable reference toxicants for each of their published test protocols. Reference toxicants were evaluated on the basis of the following criteria: (1) the existence of an established toxicity database and previous studies detecting abnormal organisms, (2) whether the chemical was readily available in pure form, (3) soluble in reference water, (4) stable in solution, e.g. in

reference water, and (5) stable on the shelf as well as easily analyzed and with limited interlaboratory water quality effects. This study will utilize 4-chlorophenol as a reference toxicant; zinc is also discussed as an example of an inorganic positive toxicant.

4-Chlorophenol

4-chlorophenol, also called parachlorophenol, is a synthetic organic compound commonly used as an antiseptic, fungicide and general disinfectant (Czaplicka, 2004). Monochlorophenols can also be produced as byproducts of paper production, coking, wood distillation or drinking water disinfection by chlorination (Czaplicka, 2004). They can also be released along the biodegradation pathway of many pesticides (Czaplicka, 2004). The mechanism of toxicity to aquatic organisms is mainly by non-specific polar narcosis, but can also be through uncoupling of the oxidative phosphorylation pathway (Penttinen, 1995).

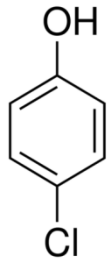

Figure 2 Structure of 4-chlorophenol (Sigma-Aldrich, 2013)

In the scientific literature 4-chlorophenol has been used as a reference toxicant in both freshwater and marine toxicology studies. Its use in freshwater studies has so far been restricted to use with the invertebrate *Hydra* sp. (Pollino and Holdway, 1999; Mitchell and Holdway, 2000; Ganeshakumar, 2009; Ginou, 2010). In marine studies, 4-chlorophenol has been used as a reference toxicant for the brown algae *Hormosira banksii* (Gunthorpe *et al*., 1995), the scallop *Chlamys (Mimachlamys) asperrima* L. (Krassoi *et al*., 1997), the octopus *Octopus pallidus* (Long and Holdway, 2002), and the brine shrimp *Artemia salina* (Svensson *et al*., 2005). Alongside zinc sulphate, da Cruz *et al*. (2007) determined 4-chlorophenol to be one of the best reference toxicants for the marine oyster *Crassostrea rhizophorae* bioassay, again due to the high reproducibility and low variability.

4-chlorophenol passed all Environment Canada criteria for a reference toxicant with the exception of: (1) the existence of an established toxicity database, and (2) the existence of studies detecting abnormal organisms (Environment Canada, 1990). While relatively little has been published regarding the effect of 4-chlorophenol on freshwater biota, it is otherwise an acceptable reference toxicant and this study hopes to add to the toxicological database. 4-chlorophenol is readily available in pure form, is soluble and stable in solution, has a stable shelf

life, exhibits limited interlaboratory water quality effects and is easily analyzed (Environment Canada, 1990). It was subsequently recommended for use alongside lethality tests, including the 96-hour rainbow trout lethality, 48-hour *Daphnia* spp. lethality and 96-hour threespine stickleback lethality tests. At the time, its use for chronic and sublethal tests had not been investigated, and thus its use in these tests was not recommended.

The use of 4-chlorophenol as a reference toxicant in this study is supported by its evaluation and acceptance by Environment Canada (1990).

Zinc

Although zinc is not used in this study as a reference toxicant, it is described here as an example of a suitable inorganic positive toxicant.

Zinc is an essential metal for all living organisms as it is required in many necessary enzymes; however, it is toxic at elevated concentrations (Eisler, 1993). The primary mechanism of toxicity in aquatic organisms is a disturbed calcium balance, caused by competition between zinc and calcium, both divalent cations, for the same ion channels (Santore *et al*., 2002). Muyssen and colleagues (2006) hypothesized that zinc toxicity in the zooplankton *D. magna* is caused by a disturbed calcium balance, much as in fish. In freshwater fish, elevated zinc concentrations impair the branchial calcium influx, leading to decreased calcium levels, or hypocalcaemia (Spry and Wood, 1985). Muyssen *et al*. (2006) also observed reduced movement and filtration rate leading to reduced growth and reproduction in *D. magna* with increasing chronic zinc exposures, with the exception of very low (80 μg/L) concentrations.

In the scientific literature, zinc, as a sulfate, sulfate heptahydrate or chloride salt, has been used as a reference toxicant for both freshwater and marine toxicology studies. In marine studies zinc has been used as a reference toxicant for various sea urchin species (Phillips *et al*., 1998; Cesar *et al*., 2004) and other marine invertebrate studies (Hunt and Anderson, 1989; Martin *et al*., 1989; Gulec *et al*., 1997; Nipper *et al*., 1997). Zinc has been used as a reference toxicant for freshwater *D. magna* (Johnson and Delaney, 1998; Johnson *et al*., 2006; Lazorchak *et al*., 2009) as well as freshwater fish (Dawson *et al*., 1988; Lazorchak and Smith, 2007) and frog (Dawson *et al*., 1988; Bantle *et al*., 1989) studies. In 2007, Da Cruz and colleagues determined zinc

sulphate to be one of the best reference toxicants for the marine oyster *Crassostrea rhizophorae* bioassay due to the high reproducibility and low variability achieved through repeated testing.

Zinc sulphate passed all Environment Canada criteria for a reference toxicant and scored the highest of all the reference toxicants evaluated. It was subsequently recommended for use alongside most of Environment Canada's toxicity tests, including the 96-hour rainbow trout lethality, 7-day fathead minnow larval survival and growth, 48-hour *Daphnia* sp. lethality, 96-hour *Selenastrum capricornutum* growth and inhibition and 96-hour threespine stickleback lethality tests (Environment Canada, 1990). It was not recommended for use in the Microtox™ standardized test, which utilizes the marine bacterium *Vibrio fischeri*, due to low reproducibility (Thomas *et al*., 1986; Environment Canada, 1990). At the time, its use for the 3-brood *Ceriodaphnia dubia* survival and reproduction test had not been investigated, and thus, zinc sulphate was not recommended for this test.

1.5 Bioassay Endpoints

1.5.1 Rationale for Selection of Test Endpoints

Bioassays are designed to "provide a quantitative measure of an adverse effect on some biological endpoint" (Samoiloff, 1989). This study used two endpoints, behaviour and reproduction, to assess the toxicity of triclocarban. As discussed in section 1.5.2, behaviour is a sensitive, ecologically-relevant endpoint that reflects the cumulative stress on the whole organism. Toxic conditions can also cause reduced or delayed reproduction and consequently affect population growth rates such as a change in clutch size or sex ratio (Baird *et al*., 1990; Dodson and Hanazato, 1995; Schmidt *et al*., 2005). Many chemical contaminants, including triclocarban, are found widespread in the environment at low levels, and their exposure time to biota is long. Behaviour and reproduction are therefore more useful endpoints than lethality because they are more sensitive and provide a more accurate estimation of toxicity at environmentally-relevant concentrations (Blaxter and Hallers-Tjabbes, 1992; Gerhardt, 1996; McWilliam and Baird, 2002; Green *et al*., 2003).

1.5.2 Behaviour

What is Behaviour?

Behaviour is "a series of overt, whole body observable activities which operate through the nervous system and assist animals to survive, grow and reproduce" (Beitinger, 1990). An animal's behaviour is governed by both its internal processes and the external effects or ecological consequences of its behaviour (Figure 3) (Real, 1994; Grue *et al*., 2002). The internal processes, such as the animal's hormonal processes, neurobiology and development and decision processes in turn result in external or ecological effects in the environment (Grue *et al*., 2002). These effects are acted upon by natural selection, which in turn shapes the animal's internal processes and ultimately its behaviour (Grue *et al*., 2002).

Behaviour is not a random process and has been selected through evolutionary processes to be a highly structured and predictable series of activities with the goal of ensuring maximum fitness and survival of the species (Kane *et al*., 2005). Each species has typical behaviour patterns that are a result of adaptations to the environment and that are favourable to that species' survival (Little, 2002). These behaviour patterns can change in response to a contaminant or other stressful condition (Little, 2002).

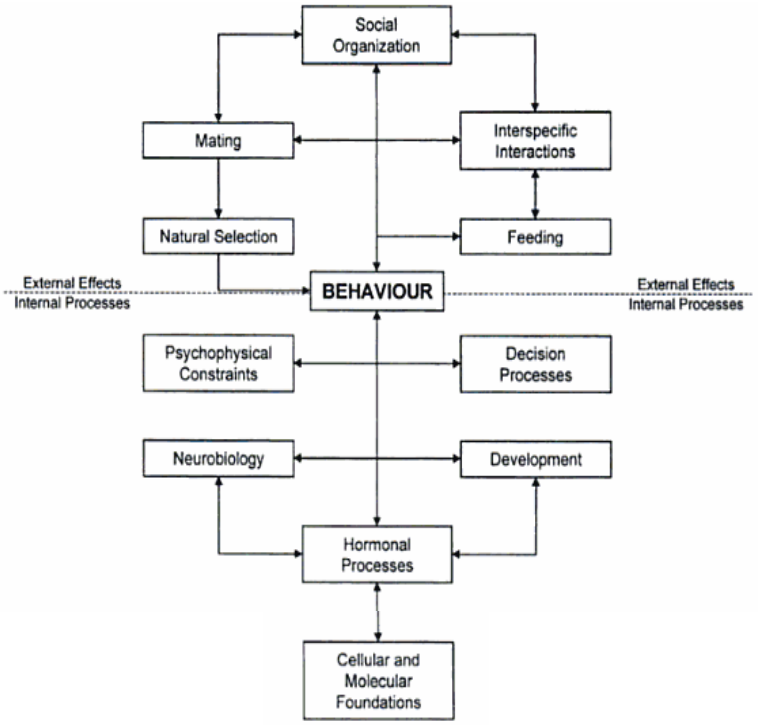

Figure 3 Interrelationships governing behaviour (Grue *et al*., 2002 after Real, 1994)

Behavioural stress response patterns can vary in complexity, from simple reflexes such as phototaxis or foraging behaviour to complex social interactions such as territoriality, courtship and mating (Little, 2002; Gerhardt, 2007).

Why Study Behaviour?

Behaviour is a valuable response for toxicity bioassays because it is an integration of an organism's molecular, physiological, nervous, sensorial and muscular system changes in response to a change in environment (Little, 1990; Scherer, 1992; Gerhardt, 1996; Grue *et al*., 2002; Untersteiner *et al*., 2003; Kane *et al*., 2005; Martin and Bateson, 2007). In short, behaviour is an integrated, whole-organism response (Kane *et al*., 2005). It can be observed as a single endpoint that represents the cumulative environmental stress on an organism. A change in behaviour can have organism-level biological effects such as decreased survival or reproduction, as well as ecological effects such as changes to populations, community structure, and whole ecosystems function (Grue *et al*., 2002). Behavioural responses "rest on biochemical processes but also reflect the fitness of the individual organism as well as potential effects on the population level, such as altered abundance of the species in the ecosystem" (Gerhardt *et al*., 1994). Behavioural observations are therefore ecologically-relevant, as they link together and can help predict higher level organization responses (Doving, 1991; Bunn, 1995; Duquesne and Kuster, 2010).

In addition to ecological relevance, the use of behaviour as a toxicity endpoint has the advantage of being a sensitive indicator of toxicity at ecologically-relevant concentrations (Peakall, 1996; Lovern *et al*., 2007). Behavioural endpoints are more sensitive than the traditional endpoint of lethality in terms of dose and response time (Little and Finger, 1990; Grue *et al*., 2002; Hellou, 2011). Fish behaviour endpoints such as swimming, ventilation and foraging behaviours have been shown to be more sensitive than lethality (Beitinger, 1990; Beitinger and McCauley, 1990; Gerhardt, 1994). Concentrations of toxicants that elicit a behavioural response are often fractions of lethal concentrations (Beitinger, 1990; Little and Finger, 1990). This increased sensitivity has led to the use of behavioural responses in early-warning biomonitoring systems (EWBS), as discussed in section 1.5.2.

The behaviour of many model organisms, including those studied in these experiments, can be easily observed in the laboratory with relatively inexpensive tools (Clotfelter *et al*., 2004). While many automated technologies have been developed in the past two decades for quantitative behavioural response testing, these instruments are usually complicated, suffer from background noise, high variance and large standard deviations, and are prohibitively expensive

for many small-scale research facilities (Fleet, 2010; Solnik, 2011). Alternatively, behavioural responses can be directly visually observed by well-trained personnel. Intervals of video capture may be employed for record-keeping and verification purposes (Marshall, 2009).

Lastly, behavioural bioassays allow for non-destructive testing (Scherer, 1992; Peakall, 1996). Organisms can be continually monitored at intervals for stress behaviour throughout a long-term test, such as a reproduction test. They can also be monitored for adaptations during, and recovery following a toxicity test (Scherer, 1992). Also, the same sample of animals used for a behaviour test can then be subjected to biochemical, physiological and genetics investigations to further understand the mechanism of toxicity (Scherer, 1992).

Criticism of Behaviour as an Endpoint

While the use of behaviour as a toxicity endpoint has many inherent advantages, it also suffers from criticism regarding its biological and ecological significance and its role in regulatory decision-making (Peakall, 1996; Grue *et al*., 2002). Little (1990) identifies the two major challenges for the discipline of behavioural ecotoxicology: (1) field verification of behavioural responses and (2) standardization of methods. With regard to field verification, behavioural responses are likely not readily verified in the field due to the inherent difficulty and cost associated with field experiments (Little, 1990). While behaviour can be documented in the laboratory, observing an organism in its natural environment is made difficult by the complexity of biological systems; social, predatory or any number of other intricate factors may be at play, confounding the behavioural response that may be elicited solely due to the pollutant (Little, 1990). Few studies exist that verify the behaviours observed under laboratory conditions to those seen in the animal's natural environment (Grue *et al*., 2002). However, even more importantly, is the lack of connection between behavioural responses observed in the laboratory and their relevance to ecological effects seen in the field. With the exception of avoidance responses, few behavioural responses have been linked to population, community or ecosystem-level changes, and this relationship remains poorly understood (Kane *et al*., 2005). Some examples include a study by Krebs and Burns (1977) that linked impaired locomotor behaviour of the fiddler crab caused by fuel oil exposure to reductions in the population. Also, a study by Weis *et al*. (2000) linked impaired foraging behaviour of the mummichog (*Fundulus heteroclitus*) caused by a number of contaminants to a decline in their growth and longevity coupled with the population

increase of its prey, the grass shrimp. The lack of studies demonstrating the ecological relevance of behavioural endpoints is undoubtedly the most ardent criticism of behavioural ecotoxicology (Heinz, 1989; Little, 1990; Calow, 1994; Clements and Kiffney, 1994; Peakall, 1996; Grue *et al*., 2002; Kane *et al*., 2005).

Very few standardized methods exist for assessing behavioural toxicity. Standardized methods published by national or international regulatory bodies only include ASTM Standard Guides for the measurement of behaviour (ASTM, 2013a) and ventilatory behaviour (ASTM, 2008) during fish toxicity tests as well as a generic guide to behavioural testing in aquatic toxicology (ASTM, 2013b). Other regulatory bodies (Environment Canada, EPA, OECD, etc.) only mention that abnormal behaviours during the test should be noted, and do not require behavioural data to be measured or incorporated into the final report (see Appendix II). Little (1990) offers that the lack of standard methods is not surprising because there are many different biological taxa and methods currently being researched that have yet to be brought together. Little (1990) suggests that broad generic protocols that are appropriate for numerous species and exposure conditions are required. The ASTM standard guide for behavioural testing in aquatic toxicology, first published in 1994 and re-evaluated in 2013, begins to fill this void; however, the guide is more geared towards the more common fish behavioural toxicology than invertebrate research.

Introduction to Behavioural Ecotoxicology

The study of behaviour in the field of ecotoxicology, or behavioural ecotoxicology, is at the junction of three disciplines: ethology, the study of behaviour (see section 1.5.4); ecology and toxicology (Figure 4) (Dell'Omo, 2002). Behavioural ecotoxicology

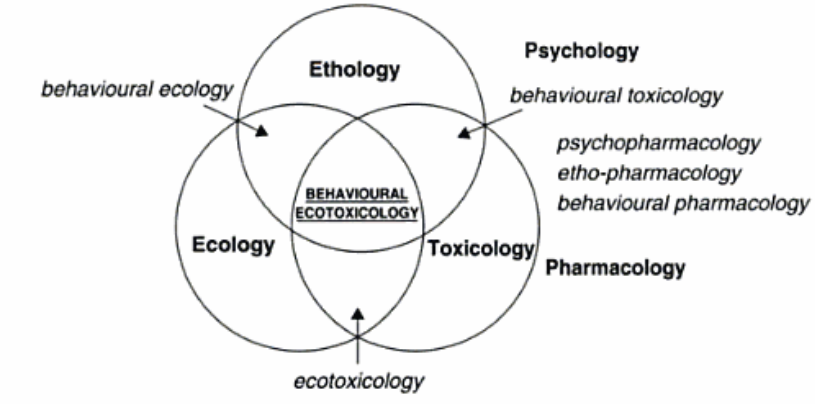

Figure 4 Behavioural ecotoxicology and other disciplines (Dell'Omo, 2002)

draws on the observational nature of ethology, the study of relationships from ecology, and the study of toxic agents from toxicology (Dell'Omo, 2002). Pioneers in the field of aquatic behavioural ecotoxicology worked primarily with small freshwater fish and observed the behaviour of individual fish when exposed to water containing the chemical of interest. Early examples include the work of Shelford and Allee who designed experiments studying fish avoidance behaviour to gases (1913) and effluents (1914). Abramson and Evans (1954) looked at the behaviour of Siamese Fighting Fish (*Betta splendens*) when exposed to the drug LSD. Weiss and Botts (1957) observed hyperexcitability, tremors, and rigid pectoral fins in response to exposure to a nerve gas. Beginning in 1964, Canadian researcher J. B. Sprague published a series of papers on the avoidance reactions of freshwater fish to various pollutants, including copper and zinc (1964), zinc sulphate (1968a) and phenol, chlorine, detergent and bleached kraft pulp mill effluent (1968b). Parallel to Sprague's work, Japanese researcher S. Ishio was also investigating the behaviour of fish exposed to toxic substances (1965). Although certainly not the first to use animal behaviour in response to a stressor, Warner *et al*. (1966) were the first to suggest quantitative measurement of behaviours to assess the impact of toxicants on the environment. In the 1960s and 1970s, behavioural abnormalities of nesting Lake Ontario herring gulls began to be observed, which were eventually tied to organochlorines such as DDT (Fry, 1995). Changes in the parental behaviour led directly to the death of the offspring and subsequent population decline (Fry, 1995). Fish behaviour research exploded in the 1970s and 1980s, with increasing complexity in the behavioural endpoints, test conditions and data gathering (Robinson, 2009). Many behavioural analysis systems that make use of different exposure factors and tank designs, dependent on the study objective, have been described (see Kane *et al*., 2005; Delcourt *et al*., 2012 for reviews). A number of papers have reviewed fish behaviour in response to contaminants in detail (e.g. Giattina and Garton, 1983; Beitinger, 1990; Little and Finger, 1990; Sandheinrich and Atchinson, 1990; Scott and Sloman, 2004; Kane *et al*., 2005).

Aquatic invertebrate behaviour studies are comparatively less common than fish studies. Early research on invertebrates included studies on amphipod mate guarding behaviour (Wildish, 1972; Linden, 1976; Davis, 1978) and bivalve mollusc valve closure (Davenport and Manley, 1978). Although a young field compared to fish behaviour, invertebrate behaviour studies quickly focused on the use of video digital analysis and automated monitoring systems to record

and track behaviours. For example, through the 1980s to 2000s, research on changes in *Daphnia magna* swimming behaviour in response to contaminants made use of various video digital analysis systems (e.g. Meador, 1986; Dojmi Di Delupis and Rotondo, 1988; Dodson and Hanazato, 1995; Dodson *et al*., 1995; Baillieul and Scheunders, 1998; Shimizu *et al*., 2002; Lovern *et al*., 2007), including automated systems such as the BehavioQuant (Schmidt *et al*., 2005, 2006) and Multispecies Freshwater Biomonitor (Gerhardt *et al*., 1994) (see section 1.5.5). A review by Boyd *et al*. (2002) discusses invertebrate behaviour testing in detail.

Several conferences and special issue journals have been published in the field of behavioural ecotoxicology. The earliest major conference focusing specifically on behaviour was the Marine Technology Society's Workshop on Marine Bioassays in 1974. The aim of this workshop was to explore various aspects of applying behavioural measures to bioassays (Workshop on Marine Bioassays, 1974). The workshop discussed species to study as well as the need for field observations and standardized protocols (Workshop on Marine Bioassays, 1974). Two years later, the 3rd Aquatic Toxicity Workshop, held in Halifax, Nova Scotia, included sessions on behavioural assays (Scherer, 1977), invertebrate avoidance and preference assays (Maciorowski *et al*., 1977), *Gammarus* sp. behavioural assays (Wallace, 1977) and fish avoidance field studies (Birtwell, 1977). In 1987, the Society of Environmental Toxicology and Chemistry (SETAC) held a Behavioural Toxicology Symposium in Florida, USA (Little, 1990). Twelve of the studies presented were subsequently published together in a special issue of *Environmental Toxicology and Chemistry* in 1990 (see Little, 1990 for series introduction). Twenty years later, in 2007, the journal *Human and Ecological Risk Assessment* published a series of Perspectives articles focusing on key developments in aquatic behavioural ecotoxicology (see Chapman, 2007 for series introduction).

Behavioural toxicity testing first achieved North American legal status when, in 1986, the United States' Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) of 1980 allowed animal behaviour as irrefutable evidence of injury under Section 301 of the Clean Water Act (Little, 1990). Admissible behaviours included qualitative observations of lethargy or tremors in birds and mammals, as well as avoidance reactions of fish (Little, 1990). However, not all behavioural endpoints were considered admissible, and

locomotory activity in fish and migratory behaviour in birds and mammals failed to meet criteria due to the lack of field verification or standardized methods (Little, 1990).

Although in recent decades behavioural endpoints have gained acceptance as a more sensitive endpoint than lethality, limited field experiments and standard methodologies have made behavioural bioassays more of a complementary test to lethality or chronic bioassays than a stand-alone test method. This is seen in the 2013 ASTM "Standard guide for measurement of behaviour during fish toxicity tests," the only published behaviour protocol by a recognized regulatory agency. This fish behaviour test is meant to be adjunct to other toxicity tests and not used alone to assess toxicity (ASTM, 2013). The future of behavioural ecotoxicology lies in strengthening the case for behaviour as an endpoint through field experiments and the creation and testing of standard protocols.

The Role of Ethology in Behavioural Ecotoxicology

Ethology, or the comparative study of behaviour (Eibl-Eibesfeldt, 1970), is an observational science that will be used in this research to compare reference and stress behaviours. Ethology is a pure science that seeks to understand behaviour rather than control it (Silverman, 1988). Ethology emerged out of the field of zoology (Eibl-Eibesfeldt, 1970). Early studies in the late 1800s and early 1900s described animal behaviours; however, it was von Frisch, Lorenz and Tinbergen in the 1930s who formed the systematic basis of the ethological study as we know it today (Eibl-Eibesfeldt, 1970; Silverman, 1988). The use of ethology in ecotoxicology is not new with multiple approaches examined and reviews published (Silverman, 1988; Scherer, 1992; Cohn and MacPhail, 1996; Zala and Penn, 2004). A new term, ethotoxicology, combining ethology and environmental toxicology, was coined by Parmigiana *et al*. (1998).

Ethologists identify behaviours and situations where they occur reliably enough for experimental use (Silverman, 1988). Such behaviours are innate to the species and are the result of movement coordination (Eibl-Eibesfeldt, 1970). These identified behaviours are called fixed action patterns (FAPs), and can be described in a physical or functional sense (Eibl-Eibesfeldt, 1970). FAPs are identified only after careful observation of the animal in its natural, or as close to natural as possible, setting (Eibl-Eibesfeldt, 1970). FAPs are basic activities that surround survival such as reproduction, defense, foraging, and in some animals parental behaviours (Cohn

and MacPhail, 1996). Fixed action patterns are then combined into the basis of the ethological study: the ethogram. An ethogram is a "precise catalogue of all the behaviour patterns of an animal" (Eibl-Eibesfeldt, 1970, p. 10).

The study of animal behaviour can utilize two approaches: the ethological and experimental approach (Cohn and MacPhail, 1996). The ethological approach utilizes observational techniques to study behaviour in the natural environment (Cohn and MacPhail, 1996). The experimental approach is manipulative, and studies the behaviour of an individual and the conditions under which the behaviour is acquired and maintained (Cohn and MacPhail, 1996). This research combines both ethological and experimental approaches. First, the ethological approach is used to observe the animals' behaviour, although in a laboratory setting and not the natural environment. Silverman (1988) calls for the laboratory behavioural testing method to begin with undirected observation of the animal for long periods. Silverman states that "eventually, from the apparent chaos of the behaviour, regularities gradually emerge" and that actions, once observed, can be identified, named, and observed again (Silverman, 1988). This approach is used to identify the animals' reference and stress behaviours, or FAPs, from which an ethogram is constructed. Then this ethogram is used in the experimental approach, where the animals' environment is manipulated through the addition or non-addition of a stressor, and stress behaviours that are acquired and maintained will be documented.

Automated Behaviour-Monitoring Technologies

Quantitative behavioural ecotoxicology studies have made use of a multitude of automated behaviour-monitoring technologies, some of which have been developed as early-warning biomonitoring systems (EWBS) or biological early-warning systems (BEWS). These systems utilize the behavioural response of an organism to estimate water quality (Kramer and Botterweg, 1991). The goal is for the automated system to detect contaminants and alarm operators in real-time (Lechelt *et al*., 2000). The fundamentals of aquatic biomonitoring systems have been reviewed extensively (Cairns and van der Schalie, 1980, Kramer and Botterweg, 1991; Gerhardt *et al*., 2006). In recent decades, several biological monitoring systems have been developed that make use of either fish or aquatic invertebrate species. Early fish biomonitoring systems include the work of Cairns and colleagues (Shirer *et al*., 1968; Waller and Cairns, 1972) and Fisher *et al*. (1982; 1983; 1984). In Cairns' system, fish activity was detected by

interruptions in a light beam (Shirer *et al*., 1968). A decade later, Fisher developed a biomonitoring system that detected Bluegill fish (*Lepomis macrochirus*) activity; the activity created disturbances in the vessel's water column, which was then detected by the immersed paddles and measured using strain gages (Fisher *et al*., 1982). This system was the precursor to the US Army's 1990 Intelligent Aquatic Biomonitoring system, which is now implemented to monitor water quality in cities around the United States including New York City, San Francisco and Washington, D.C. (Mott, 2006). Fish biomonitoring systems have been extensively reviewed by Kane *et al*. (2005).

Digital image analysis *Daphnia* spp. biomonitoring systems include the BBE *Daphnia* toximeter, developed by BBE Moldaenke and described by Lechelt *et al*. (2000), which uses trajectory analysis to track swimming velocity and activity. The BehavioQuant® system tracks *Daphnia* spp. motility using digital image analysis converted to pixels and counted (Schmidt *et al*., 2005, 2006). An automated grid counter, developed by Jeon *et al*. in 2008, uses multiple channels, each containing a daphnid; the movements are recorded by video and plotted on a grid to assess swimming activity (Jeon *et al*., 2008). Conversely, the "Dynamic *Daphnia* Test," developed by Knie (1978), as described in Hendriks and Stouten (1993), assesses *D. magna* swimming activity using multiple infrared beams.

One of the most popular automated systems in literature is the Multispecies Freshwater Biomonitor (MFB) developed by Gerhardt *et al*. (1994). The MFB differs from other automated systems in that it is a non-optical system. Instead, the MFB measures the changes in an electrical field that is passed through chambers housing individual aquatic organisms. While the MFB has been extensively demonstrated in literature (e.g. Gerhardt *et al*., 1994; Gerhardt, 1995; Gerhardt and Schmidt, 2002; Craig and Laming, 2004; Kirkpatrick *et al*., 2006; Ren *et al*., 2007; Gerhardt, 2009; Ren *et al*., 2009; Sardo and Soares, 2010; Mohti *et al*., 2012), studies at Ryerson University found that the system was unable to consistently detect behavioural deviations (Fleet, 2010; Solnik, 2011). Results from the MFB suffered from high variance and large standard deviations and required complex statistical analysis (Fleet, 2010; Solnik, 2011). Further study is therefore needed before the system can be implemented.

1.5.3 Reproduction

Historically, assessing toxicity to biota has relied principally on traditional acute, and to a lesser extent, chronic methods (Cooper, 1995). Acute bioassays tend to cost less than their chronic counterparts and consequently the majority of the toxicological database is skewed in favour of acute response data, such as LC_{50} values (Birge et al., 1985; Cooper, 1995). However, sub-lethal endpoints such as reproductive impairment have been found to be a more sensitive measure of toxicity than survival (Beisinger and Christensen, 1972). Many contaminants in our environment are persistent and non-degradable, are found at low levels, and their exposure time to biota is long. Assessing a toxicant through an entire or partial reproductive period (i.e. egg, juvenile, adult, and reproduction) is a more accurate representation of the potential hazard of contaminants in our natural systems (Cooper, 1995). Impairment of reproduction, measured by endpoints such as delayed reproduction time and smaller numbers of offspring, is a key parameter for assessing toxicity at concentrations that are encountered in our environment (Neilson et al., 1994). As an endpoint, reproduction is ecologically-relevant as impairment can disturb the balance and diversity of the biological community drastically (Schober and Lampert, 1977; Gourmelon and Ahtiainen, 2007). This point becomes even more significant when the reproductive impairment is seen in at low trophic levels, such as phytoplankton and benthic invertebrates (Gourmelon and Ahtiainen, 2007).

Life cycle testing first started in the mid-1960s as methods for culturing indigenous fish and invertebrates improved (Cooper, 1995). Full fish life cycle tests were first conducted, but their extreme cost and length caused many researchers to turn to invertebrate life cycle tests as well as early life stage and partial life cycle testing (Cooper, 1995). Today many published methods by regulatory agencies such as Environment Canada, the EPA, OECD and ASTM include reproductive endpoints as measures of toxicity.

1.6 Bioassay Organisms

1.6.1 Rationale for Selection of Bioassay Organisms

Daphnia magna, *Hyalella azteca*, *Lumbriculus variegatus* and *Lemna minor* were chosen on the basis of ecological relevance, ability to easily observe stress behaviours and the availability of previously established stress behaviour libraries and existing culturing and toxicity testing protocols. The species are ecologically-relevant as each is commonly found in many

natural systems including the Great Lakes, with the exception of *Daphnia magna*, which although not native to the Great Lakes, is commonly used as a surrogate for native *Daphnia* species (Kaiser, 1984). In addition, each species is an important member of the freshwater aquatic food chain and thus represents a sensitive indicator for the surrounding ecosystem. If the introduction of a toxicant resulted in modification of that species' population to the point of population collapse, it could have serious ecological effects (Hallam *et al*., 1983). Further, it is recommended that species from multiple trophic levels be included in the battery of bioassays to ensure that the test is reliable, as different species are sensitive to different toxicants (Gerhardt *et al*., 1994; Blaise, 2000). In the 1950s, prominent ecotoxicologist J. Cairns, Jr. stated "that picking representative organisms from different levels of the food chain would more faithfully display the range of response to toxicants than fish alone" (Cairns, 1956).

The species chosen represent both broad areas of the aquatic environment, the water column and the sediment, and therefore interact with contaminants found in each area. As both hydrophilic and hydrophobic contaminants, such as triclocarban, exist in the environment it is important to have methods suited to each. Hydrophobic contaminants may adsorb to particles and settle in the sediment or remain suspended in the water column (Servos *et al*., 2001). Two of the organisms, *Hyalella azteca* and *Lumbriculus variegatus*, reside on and in the sediment and therefore interact with hydrophobic contaminants. The remaining two organisms, *Daphnia magna* and *Lemna minor*, are found in the water column and therefore interact primarily with hydrophilic contaminants. By conducting bioassays with these four organisms, this study was able to present methods to assess the toxicity of all contaminants, whether they are hydrophobic or hydrophilic.

1.6.2 *Daphnia magna*

Background and Use in Toxicity Testing

Daphnia magna Straus (Figure 5) are a species of freshwater crustaceans that are routinely used in freshwater toxicity testing due to their high sensitivity to toxic substances, ecological relevance and ease of culturing (Mount and Norberg, 1984; Environment Canada, 1990b; Persoone and Janssen, 1993; Environment Canada, 2000). As filter feeders, the *D. magna* digestive tract and overall body surface are constantly exposed to the aquatic environment, including any dissolved or suspended contaminants (Green *et al*., 2003). *D. magna* are therefore

sensitive to a broad range of aquatic contaminants and are often considered to be excellent model organisms for predicting the impact of contaminants on biota in the environment (Dodson and Hanazato, 1995; Kiss *et al*., 2003; Schmidt *et al*., 2005; Ren *et al*., 2007).

Figure 5 Stereoscope image of *Daphnia magna* (Puddephatt, 2013)

Their use as a model toxicity test organism dates to 1900, when Warren described lethality in varying concentrations of sodium chloride (Warren, 1900). Other early toxicity tests include tests with mercuric chloride (Breukelman, 1932), copper (Riley, 1939), acetylcholine (Baylor, 1942), and industrial waste effluents (Anderson, 1944). Although now considered a model organism, criticism of its widespread use exists. It has been suggested that *D. magna* is not a representative zooplankton due to its large size and restricted habitat (Koivisto, 1995) and that other cladoceran species such as *Ceriodaphnia reticulata* be employed (Mount and Norberg, 1984). *D. magna* are relatively large (5-6 mm adult females) compared to other cladocerans such as *D. pulex* (2.5-3.5 mm adult females) or *Ceriodaphnia* sp. (<1.5 mm adult females) (Koivisto, 1995). This large body size has been related to increased tolerance of toxic substances, compared to other, smaller zooplankton (Koivisto *et al*., 1992) and increased predation by visually feeding fish (Lynch, 1980). In further criticism, *D. magna* are not native to the Great Lakes; however, the species is commonly used as a surrogate for native *Daphnia* species (Kaiser, 1984). The choice to use *D. magna* over other, native Great Lakes cladoceran species in this study was due to the larger size, both at neonate and adult stages, and subsequent ease of observing behaviour, as well as its extensive use in literature showing its sensitivity as a bioassay organism.

Under optimal conditions, females reproduce by parthenogenesis. Large species such as *D. magna* can have broods of up to 100 neonates, making them relatively easy to culture under laboratory settings and ideal for laboratory toxicity tests (Koivisto, 1995). Although they are the

largest of the cladocerans, they still require minimal space and water requirements (Environment Canada, 1990b; Dodson and Hanazato, 1995; Ren *et al*., 2007). Many culture protocols exist (Leonhard and Lawrence, 1981; Environment Canada, 1990b; APHA, 1998; EPA/USACE, 1998; EPA, 2002a; ASTM, 2004; MOE, 2012a).

Ecological Relevance

Daphnia magna are a key species in the aquatic food web (Dodson and Hanazato, 1995). Often the dominant herbivore, *D. magna* have the ability to affect water quality through their select consumption of algae (Luecke *et al*., 1992), and are an important link between trophic levels as predatory zooplankton and prey for fish (Duquesne and Kuster, 2010). Therefore, a change in *D. magna* population dynamics is likely to affect the entire aquatic community (Jones *et al*., 1991). Figure 6 illustrates *D. magna*'s position in the food web. Notice their key position with other zooplankton between algae and bacteria and fish (Dodson and Hanazato, 1995).

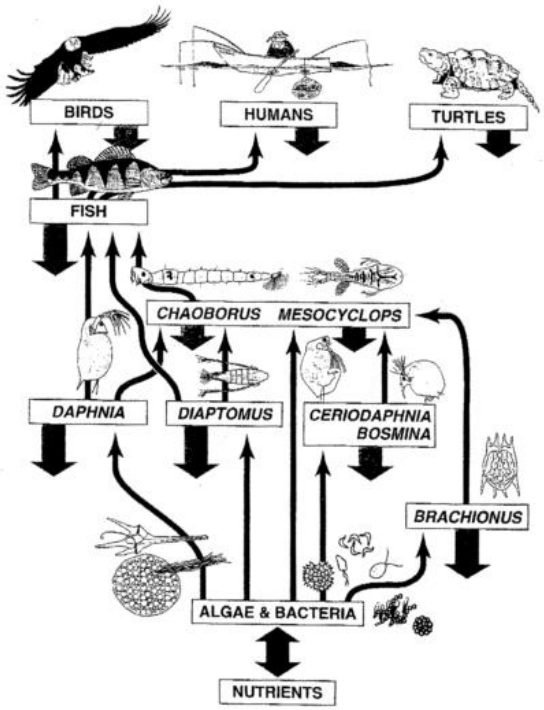

Figure 6 An idealized freshwater food web (Dodson and Hanazato, 1995)

Anatomy and Physiology

The internal structures of *D. magna* are protected by the carapace, a double-walled polysaccharide chitin shell between which the hemolymph flows (Ebert, 2005). Outside the carapace, *D. magna* have two sets of antennae; the first are used as a sensory organ and the second, much larger set are for locomotion (Ebert, 2005). *D. magna* also possess a compound eye for basic vision and orientation (Ebert, 2005). Inside the carapace, the heart, gut, ovaries, abdominal legs, claw and postabdomen are contained (Ebert, 2005). Separate from this part of the carapace chamber is the dorsal brood pouch, which houses the eggs and embryos, (Fryer,

1991). Figure 7 shows the basic anatomy of a female *Daphnia* spp. For a detailed review of anatomy and functional morphology of *D. magna*, see Fryer (1991) and Ebert (2005).

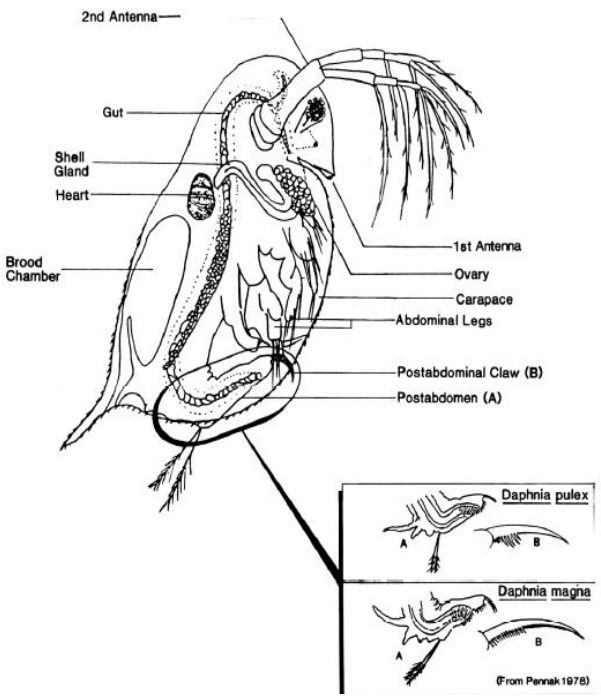

Figure 7 Anatomy of female *Daphnia* spp. (Environment Canada, 1990)

Life Cycle and Reproduction

The *D. magna* life cycle consists of four main stages: egg, juvenile, adolescence and adult (Pennak, 1989). The eggs hatch in the brood chamber and are released as juveniles when the female *D. magna* molts (EPA, 2002a). Juvenile *D. magna* females have three to five instars in which they grow rapidly (EPA, 2002a). During the one instar adolescent stage, females produce their first clutch of eggs (EPA, 2002a). If feeding conditions permit, the subsequent 6-22 adult instars each correspond with a molt, a period of growth, the release of young from the brood chamber as well as the release of a new clutch of eggs from the ovary into the brood chamber (EPA, 2002a). Adult instars can last between two and seven days, depending on environmental conditions (EPA, 2002a).

Under optimal growing conditions, *D. magna* reproduce asexually or parthenogenically (Dodson and Hanazato, 1995; EPA, 2002a; Ebert, 2005; Schmidt *et al*., 2005). Female *D. magna* produce diploid eggs that can mature into males under harsh environmental conditions, but more often mature into females. When environmental conditions deteriorate, such as under cooler temperatures or a decreased food supply, females produce diploid males followed by resting eggs (Ebert, 2005). Resting eggs are encapsulated in a saddle-shaped, heavily pigmented protective coating called a ephippium which is cast off together with the eggs at the next molt (Ebert, 2005). The resting eggs can be haploid, which require fertilization by the males produced prior to the resting eggs, or they can be diploid. Figure 8 illustrates both the sexual and asexual, or parthenogenic, life cycle of *Daphnia* spp.

Under laboratory conditions, *D. magna* may live up to approximately 2 months; however, for unknown reasons, lifespan appears to decrease with optimal feeding conditions (Ebert, 2005). This lifespan has been seen in the laboratory, with well-fed *D. magna* living in culture conditions 1.5 to 2 months.

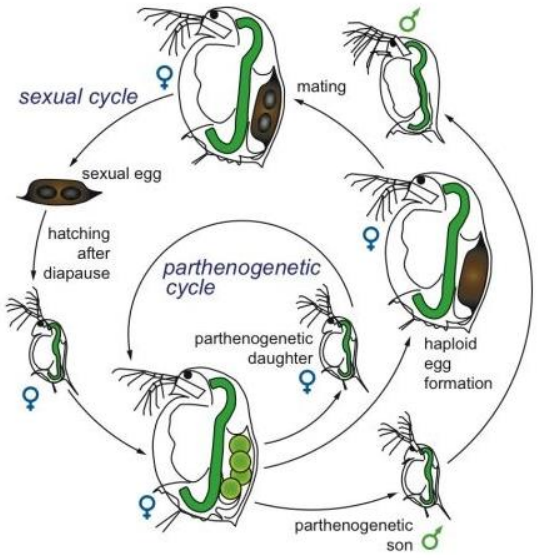

Figure 8 Sexual and asexual (parthenogenic) life cycle of *Daphnia* spp. (Ebert, 2005)

Feeding and Behaviour

To feed, *Daphnia magna* filter suspended particles out of the water column using filter plates on their antennae (Fryer, 1991). They then pass these filter plates into the median food groove of the mouth, where the food is swept forward into the mouthparts and ingested (Fryer, 1991). Another method of feeding that is unique to *D. magna* and *D. obtusa* is the ability to feed on settled organic matter on surfaces (Fryer, 1991). These species are able to settle on their ventral carapace margins and glide forward over a surface, collecting food material with scraper-like spines on the second trunk limbs (Fryer, 1991). Food particles are then swept into the median food groove and mouthparts and ingested (Fryer, 1991). These secondary feeding behaviours have been observed extensively in the laboratory, especially within smooth-bottomed glass vessels with a build-up of detritus.

Daphnia spp. reference and stress behaviours have been studied extensively (Fox and Mitchell, 1953; Flickinger *et al*., 1982; Meador, 1986; Dojmi Di Delupis and Rotondo, 1988; Goodrich and Lech, 1990; Fryer, 1991; Gerhardt *et al*., 1994; Dodson and Hanazato, 1995; Dodson *et al*., 1995; Baillieul and Scheunders, 1998; Ryan and Dodson, 1998; Kieu *et al*., 2001; McWilliam and Baird *et al*., 2002; Shimizu *et al*., 2002; Untersteiner *et al*., 2003; Christensen *et al*., 2005; Schmidt *et al*., 2005; Reynaldi *et al*., 2006; Schmidt *et al*., 2006; Lovern *et al*., 2007; Schafers *et al*., 2007; Stanley *et al*., 2007; Marshall, 2009; Ren *et al*., 2009; Duquesne and Kuster, 2010). Specifically, swimming behaviour is most studied. During normal swimming the secondary antennae work simultaneously in a regular sweeping motion punctuated by brief rests

to drive water backwards and give the characteristic "jumping" saltatory behaviour (Fryer, 1991). The sweeping motion is followed by a period of brief rest, to either maintain position in the water column or to drift downwards (Fryer, 1991). This swimming behaviour is important to allow *D. magna* to locate food and to maintain their position in food patches (Ryan and Dodson, 1998). When in an area of high food concentration, *D. magna* have been observed to reduce vertical swimming and turning (Young and Getty, 1987).

Abnormal swimming behaviours could be the result of the *D. magna* attempting to avoid a substance in the water or the result of metabolic impairment due to a toxic substance (Green *et al*., 2003). Abnormal swimming behaviours include escape swimming behaviours such as short, rapid bursts of swimming or spinning (Dodson *et al*., 1995). Also, changes in phototactic response have been seen following exposure to chemical contamination (Michels *et al*., 1999; Kieu *et al*., 2001; Martins *et al*., 2007). Phototaxis is an oriented reaction to a light stimuli (Kieu *et al*., 2001). Most *Daphnia* species strains are negatively phototactic, meaning they migrate away from the light and therefore group near the bottom of the water column during the day to avoid being seen by predators from below (Cushing, 1951; Kieu *et al*., 2001). Positive phototactic strains, that move towards light sources, have also been used in ecotoxicological studies (Kieu *et al*., 2001).

Toxicity Studies Utilizing *Daphnia* spp.

Daphnia spp. have been used in aquatic toxicity testing for more than a century (Warren, 1900; Brueukelman, 1932; Riley, 1939; Baylor, 1942; Anderson, 1944; Anderson, 1950; Freeman and Fowler, 1953; Crosby and Tucker, 1966; Winner *et al*., 1977; Adema, 1978; Maki and Bishop, 1979; LeBlanc, 1980; Berglind and Dave, 1984; MacIsaac *et al*., 1985; Nebeker *et al*., 1986; Borgmann *et al*., 1989; Kohn *et al*., 1989; Munzinger and Monicelli, 1991; Janssen and Persoone, 1993; Baun and Nyholm, 1996; Stanley *et al*., 2007; Ra *et al*., 2007; Coors and Meester, 2008; Ra *et al*., 2008; Lee *et al*., 2010; Zhu *et al*., 2010; Zhao and Wang, 2011). The most common endpoint observed has historically been mortality; however, sub-lethal endpoints such as changes in growth and development, behaviour (see section 1.8 Feeding and Behaviour), and reproduction are now frequently utilized.

More specifically related to this study is the use of *Daphnia* spp. to evaluate toxicity of WWTP effluent since it is the primary source of triclocarban to the environment. Studies have

assessed chronic toxicity utilizing endpoints such as growth and reproduction (Schroder *et al*., 1991; Rutherford *et al*., 1994; Manusadzianas *et al*., 2003), as well as acute toxicity testing with the endpoint of lethality (Ra *et al*., 2007; Ra *et al*., 2008; Maltby *et al*., 2000; Pessala *et al*., 2004) and immobilization (Hernando *et al*., 2005; Pignata *et al*., 2012). This study contributes to the use of *D. magna* to evaluate the toxicity of triclocarban by evaluating toxicity though the use of behaviour and reproduction endpoints.

Water-Only *Daphnia* sp. Protocols

Daphnia spp. have been widely used for water-only and effluent toxicity testing in Canada, North America, and internationally. Within Canada, Environment Canada has developed standard biological test methods for assessing the acute lethality of effluents using either *Daphnia magna* or a related species, *Daphnia pulex* (Environment Canada, 2000). In the United States, the EPA has developed standard test procedures for determining the acute toxicity of effluents using *Daphnia magna* or *Daphnia pulex* (EPA 2002a). Also, the EPA has published a series of methods for estimated chronic toxicity of effluents and their receiving waters using the Daphniidae *Ceriodaphnia dubia* (EPA, 2002b). Internationally, no recognized methods for assessing toxicity of effluents exist; however, the Organisation for Economic Co-operation and Development (OECD) has developed acute (OECD, 2004) and chronic (OECD, 2008) reproduction tests for determining toxicity of chemicals to *Daphnia* spp. that could be adopted for effluent toxicity testing. Similarly, the International Organization for Standardization (ISO) has developed an acute toxicity test based on inhibition of mobility (ISO, 1996) and a long-term toxicity test using *Daphnia magna* (ISO, 2000) and ASTM International has developed a *Daphnia magna* life-cycle toxicity test (ASTM, 2004a).

1.6.3 *Hyalella azteca*

Background and Use in Toxicity Testing

Hyalella azteca (Figure 9) are a species of freshwater amphipods that are commonly used in measuring the toxicity of freshwater sediments-associated contaminants, and more recently waterborne contaminants, due to their sensitivity, ecological relevance and ease of culturing (Borgmann *et al*., 1989; Phipps *et al*., 1995; Borgmann *et al*., 1996; Environment Canada, 1997; EPA/USACE, 1998). *Hyalella azteca* are sensitive to a wide range of pollutants as they live at the sediment-water interface and are therefore exposed to contaminants in both the water column and sediment pore-water (Borgmann and Munawar, 1989; Burton, 1991; Collyard *et al*., 1994; Phipps *et al*., 1995; Borgmann *et al*., 1996; Hatch and Burton, 1999; Wang *et al*., 2004). Due to their small size, with maximum length for males at 8 mm, and females at 6 mm and easily supplied food source, *H. azteca* are considered to be easily cultured in the laboratory (Borgmann *et al*., 1996; Environment Canada, 1997; Wang *et al*., 2004). Many culture protocols exist (de March, 1981; Borgmann *et al*., 1989b; Nelson and Brunson, 1995; Environment Canada, 1997; EPA/USACE, 1998; EPA, 2000; Othman and Pascoe, 2001; ASTM, 2010a; MOE, 2012b).

Figure 9 Stereoscope image of *Hyalella azteca* (Puddephatt, 2013)

The advantages of easy culturing are offset by the increased reproduction and whole life-cycle test difficulty (Borgmann *et al*., 1989). Reproduction in *Hyalella azteca* occurs sexually, and sufficient numbers of male and female *H. azteca* individuals must be in the same test container for mating to occur (Borgmann *et al*., 1989; Environment Canada, 1997). Sexual maturity is reached in 28 to 33 days, and broods are at least three weeks apart (Geisler, 1944). Therefore, reproduction and whole-life cycle tests employing *H. azteca* are much longer and more difficult to execute than those utilizing *Daphnia magna*, and will not be utilized in this study.

Ecological Relevance

As amphipods, *Hyalella azteca* are an abundant and ecologically important part of the food web of freshwater ecosystems (de March, 1981; Borgmann and Munawar, 1989; Blockwell *et al*., 1998). They are a dominant food source for fish and waterfowl and are therefore a link between top carnivores that prey on fish and waterfowl and the detrital energy stores (Cooper, 1965; de March, 1981). They can be found in a variety of freshwater habitats throughout North and South America, including the Great Lakes (Bousfield, 1958; Borgmann *et al*., 1989) and are the most widely distributed North American freshwater crustacean (Bousfield, 1958). They can also be found in ponds, pools, marshes, rivers, ditches and streams (Pennak, 1989) that support aquatic plants and associated periphyton for food. *H. azteca* and several species of *Gammarus*, another amphipod, often dominate the biomass of nearshore or shallow areas of the Great Lakes (Borgmann and Munawar, 1989). Densities of *H. azteca* have exceeded more than 10 000 animals per square metre in preferred habitats (de March, 1981).

Anatomy and Physiology

The body of an adult *Hyalella azteca* is 4 to 6 mm long and consists of a 7-segmented thorax, fused to the head, and a 6-segmented abdomen (Geisler, 1944). Two round, sessile, compound eyes are found on the head along with two jointed antennae (Geisler, 1944). Seven pairs of legs extend from the thorax, each of which with the exception of the first and last pairs, have gills on the inner side of the first leg joint (Geisler, 1944). Movement of the legs pushes water past the gills, which have a thin cuticle and a large surface area to facilitate oxygen exchange (Rinderhagen *et al*., 1999). The first two

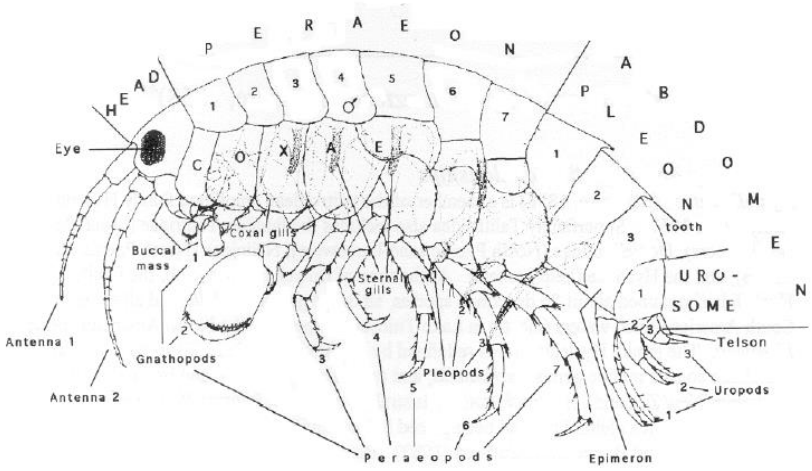

Figure 10 Anatomy of *Hyalella azteca* (Environment Canada, 1997)

pairs of the thoracic legs, closest to the head, are termed gnathopods while the other five pairs are peraeopods (Geisler, 1944). The first three abdominal segments each have three pairs of limbs called pleopods that aid in swimming and direct water toward the gills (Geisler, 1944). In addition, the first two abdominal segments each have a dorsal tooth projecting from the posterior edge (Geisler, 1944). The posterior three abdominal segments each have a pair of uropods which are directed backward and are functional for springing (Geisler, 1944). The *H. azteca* circulatory system is open, with blood and coelomic fluid flowing around the organs (Rinderhagen *et al*., 1999).

Males can be relatively easily differentiated from females based on their larger size and their larger secondary gnathopods (Geisler, 1944). Depending on environmental conditions, gender can be determined based on morphology of the secondary ghathopod after the 6th instar, or at approximately 19-21 days (Geisler, 1944; Pennak, 1989). Alternatively, mature females can be distinguished based on the presence of ova in the ovaries which are ventral to the heart (Geisler, 1944). *H. azteca* are typically coloured light brown to green; however, bluish, purple, dark brown and reddish populations have been found (Pennak, 1989).

Life Cycle and Reproduction

Although this study does not make use of *Hyalella azteca* life cycle tests (see section 3.2.2), minimal background on life cycle and reproduction is described here.

Hyalella azteca reproduces sexually, with a pairing of the genders, called amplexus, initiating reproduction (EPA/USACE, 1998). During pairing the female's eggs move from the oviducts into the marsupium where they are fertilized (EPA/USACE, 1998). The embryos hatch and are released at the next molting (EPA/USACE, 1998). Geisler (1944) found that the period from fertilization to hatching, in which the eggs were developing in the female's brood pouch, to be approximately 21 days. Each brood numbers between 1 and 50 (Environment Canada, 1997), with 18 being the average (Embody, 1911; Cooper, 1965; Pennak, 1989). Broods are approximately 3 weeks apart (Geisler, 1944).

The life cycle of *H. azteca* follows a minimum of nine instars, with a moulting period between each (Geisler, 1944). Instars 1 through 5 constitute the juvenile stage of development, followed by the adolescent stage of instars 6 and 7 (Geisler, 1944; Pennak, 1989). Instar 8 is the

nupital stage of the animal, when the animals usually pair and reproduce for the first time and is reached in 28-33 days (Geisler, 1944; Othman and Pascoe, 2001). All subsequent instars are adult stages, where continued growth and mating for reproduction occurs (Geisler, 1944; Pennak, 1989). Under laboratory culturing conditions, newly born animals were found to be approximately 1 mm in length, and grew to a maximum of 7 mm after 120 days, with weight continually increasing (Othman and Pascoe, 2001). Males are usually larger than females (Geisler, 1944; Othman and Pascoe, 2001). In northern areas, *H. azteca* mainly reproduce in the early summer months, and have a life span of about one year (Pennak, 1989).

Feeding and Natural Behaviour

Hyalella azteca are strongly photophobic and negatively phototaxic and are consequently much more active at night (Bethel and Holmes, 1973; de March, 1981). During the day, *H. azteca* hide under emergent vegetation and other aquatic litter where they can feed (de March, 1981; Pennak, 1989; EPA/USACE, 1998). They can also be found occasionally at the sides and undersurfaces of floating plant material or even dead waterfowl (Bethel and Holmes, 1972). When disturbed, the animals quickly dive and vanish into turbid water or sediment (Bethel and Holmes, 1972). When cultured in the same vessel as *Lemna minor*, *H. azteca* can often be found clinging to the underside of the floating plant, and when disturbed, quickly release and dive to the bottom of the vessel. *H. azteca* also readily burrow into aquatic sediments (Cairns *et al*., 1984; Borgmann *et al*., 2005; Doig and Liber, 2010) and beds of vegetation and organic debris (Cooper, 1965). These behaviours have been observed in our laboratory under our culturing conditions. Animals have also been found to burrow more rapidly into fine, organic-rich sediments than sandy sediments (Doig and Liber, 2010). Borgmann and colleagues (2005) observed during field collection that the majority of *H. azteca* were found just below the sediment surface, and did not burrow deeply into the anoxic sediment layers.

Hyalella azteca behaviour has been extensively described by Kruschwitz (1972), who observed *H. azteca* in both field and laboratory settings to describe behaviour and functional morphology. For locomotion on substrates, *H. azteca* utilize the segmented pereiopods to crawl, or walk while clinging (Kruschwitz, 1972). Individuals also scoot along and plow through the substratum (Kruschwitz, 1972). Occasionally *H. azteca* can walk outside of the water (Bousfield, 1958; Kruschwitz, 1972). *H. azteca* swim using their pleopods, which are in constant motion

even when not swimming (Kruschwitz, 1972). When stationary, *H. azteca* either cling to substrata using their pereiopods or sit on bottom substratum using their body and appendages for support (Kruschwitz, 1972). *H. azteca* also clean or groom themselves using their smaller gnathopods and mouthparts (Kruschwitz, 1972). Females were observed cleaning eggs in the marsupium with their flexible, small gnathopods (Kruschwitz, 1972).

A few behaviours that could be classified as aggressive have been observed in *H. azteca*. Males have been observed pushing other males attempting to carry the same female (Kruschwitz, 1972). Thrashing, by thrusting of the pereipods against another individual was seen in crowded situations with competition for food (Kruschwitz, 1972).

H. azteca is omnivorous, eating both animal and plant matter but preferring food high in protein (Kruschwitz, 1972; de March, 1981; EPA/USACE, 1998). *H. azteca* feeds on bacteria and other microorganisms and organic debris that is found on substrates such as leaves and stems in their habitat (Pennak, 1989). In field collection, *H. azteca* are commonly found associated with algae and vascular plants and decaying vegetation (Kruschwitz, 1972) *H. azteca* often hold macroscopic food by their gnathopods and anterior pereiopods for tearing, ripping and chewing (Kruschwitz, 1972; Pennak, 1989). While in a precopula, males cannot use their gnathopods for feeding as they are involved in holding of the female (Kruschwitz, 1972). Instead, males hold food with their maxillipeds and feed on smaller food such as algae (Kruschwitz, 1972). Kruschwitz (1972) found that male digestive tracts were full over extended periods of precopula, indicating that feeding still occurred without the use of the gnathopods.

Reproduction behaviour is described in section 1.6.3 *Hyalella azteca* Life Cycle and Reproduction. Moore and Farrar (1996) showed that reduced growth, forced by food rationing, delays amplexus and therefore reproduction. It is therefore important in life-cycle toxicity tests that the food level be sufficient enough not to delay reproduction.

Toxicity Studies Utilizing *Hyalella azteca*

The use of *Hyalella azteca* as a bioassay organism dates to 1955, when Clemens and Jones used the species to assess the toxicity of brine water from oil wells. In 1984, Cairns and colleagues conducted copper-spiked sediment toxicity bioassays with *H. azteca* (Cairns *et al*., 1984). A similar study looking at the cadmium-spiked sediment toxicity was completed in 1986

(Nebeker *et al*., 1986). These studies showed the use of *H. azteca* as a sensitive sediment toxicity testing organism. Building on this work, Borgmann and Munawar published their standardized sediment toxicity protocol in 1989, which led to more sediment toxicity studies in the 1990s (e.g. Ingersoll and Nelson, 1990; Ankley *et al*., 1991; Hoke *et al*., 1995; Ingersoll *et al*., 1998; Munawar *et al*., 1999; McCarthy *et al*., 2004). The use of *H. azteca* in water-only aquatic toxicity tests followed (e.g. Nebeker *et al*., 1989; Borgmann *et al*., 1990; Schubauer-Berigan *et al*., 1993; Borgmann, 1994; Phipps *et al*., 1995; Borgmann *et al*., 1996; Blockwell *et al*., 1998; Call *et al*., 2001; Othman and Pascoe, 2002; Burton *et al*., 2005; Borgmann *et al*., 2007; Pandey *et al*., 2011).

In acute sediment and aqueous studies with *H. azteca*, lethality was the most commonly used endpoint (e.g. Clemens and Jones, 1955; Cairns *et al*., 1984; Nebeker *et al*., 1986; Borgmann *et al*., 1989; Borgmann and Munawar, 1989; Nebeker *et al*., 1989; Borgmann *et al*., 1990; Ingersoll and Nelson, 1990; Ankley *et al*., 1991; Schubauer-Berigan *et al*., 1993; Borgmann, 1994; Hoke *et al*., 1995; Phipps *et al*., 1995; Borgmann *et al*., 1996; Blockwell *et al*., 1998; Ingersoll *et al*., 1998; Munawar *et al*., 1999; Call *et al*., 2001; McCarthy *et al*., 2004; Burton *et al*., 2005; Borgmann *et al*., 2007; Norwood *et al*., 2007; Pandey *et al*., 2011). In sub-lethal studies, growth (Borgmann and Munawar, 1989; Nebeker *et al*., 1989; Borgmann *et al*., 1990; Ingersoll and Nelson, 1990; Borgmann, 1994; Nelson and Brunson, 1995; Ingersoll *et al*., 1998; Othman and Pascoe, 2002; Borgmann *et al*., 2007; Norwood *et al*., 2007) and reproduction (Nebeker *et al*., 1989; Borgmann *et al*., 1990; Ingersoll and Nelson, 1990; Ingersoll *et al*., 1998; Othman and Pascoe, 2002; Borgmann *et al*., 2007) are commonly studied. Bioaccumulation of metals, PCBs and complex mixtures of contaminants under water-only (Borgmann *et al*., 1989; Nebeker *et al*., 1989; Borgmann *et al*., 1990; Borgmann *et al*., 1993; Borgmann *et al*., 1996; Norwood *et al*., 2007; Shuhaimi-Othman and Pascoe, 2007) and with-sediment conditions (Borgmann *et al*., 1989; Ingersoll *et al*., 1994) has also been extensively studied.

H. azteca behaviour, including burrowing, swimming, precopulatory guarding and grouping has been studied and shown to be a quantifiable endpoint for measuring chronic toxicity of aquatic contaminants (Blockwell *et al*., 1998; Hatch and Burton, 1999).

Water-Only *Hyalella azteca* Protocols

Several published protocols exist for sediment, but not for water-only toxicity testing with *Hyalella azteca*. However, an unpublished but available protocol from the Ontario Ministry of the Environment describes a 96-hour acute, water-only test for chemicals (MOE, 2012b). Environment Canada, the EPA and ASTM have standard test methods for assessing the survival and growth in sediment using *H. azteca* (Environment Canada, 1997; EPA, 2000; ASTM, 2010a), which could be adopted for effluent samples. The EPA method (2000) also employs reproduction as an endpoint. OECD nor ISO has published protocols for sediment or aquatic toxicity testing with *H. azteca*.

1.6.4 *Lumbriculus variegatus*

Background and Use in Toxicity Testing

Lumbriculus variegatus (Figure 11) are a species of freshwater oligochaete worms that are commonly used in assessing the toxicity of freshwater sediments due to their ecological relevance and ease of culturing (Drewes and Cain, 1999; O'Gara *et al*., 2004; Gerhardt, 2007). *L. variegatus* are considered excellent test organisms for studying the chronic bioaccumulation of hydrophobic sediment-bound contaminants because they live in close contact with the sediment and feed on subsurface sediment material (Leppanen and Kukkonen, 1998). They can also be considered for studying aquatic contaminants, as they burrow headfirst into sediment to feed, but leave their caudal, or posterior end above the sediment-water interface to facilitate gas exchange via the dorsal blood vessel (Drewes and Fourtner, 1989; Phipps *et al*., 1993; Penttinen *et al*., 1996). They are therefore exposed to contaminants in both the sediment and water compartments of the aquatic environment (Gerhardt, 2007). It has been reported that *L. variegatus* has exhibited different responses when exposed to industrial effluents collected on different dates from the same outlet,

Figure 11 Stereoscope image of *Lumbriculus variegatus* (M. Raby, 2013)

therefore indicating their sensitivity and usefulness for detecting changes in complex wastewaters (Hornig, 1980).

Within the laboratory, *Lumbriculus variegatus* are relatively easy to culture and test due to their small size (20-90 mm in length, 0.8 to 1.5 mm in diameter), easy handling and asexual reproduction (Drewes and Fourtner, 1989; Phipps *et al.*, 1993; Leppanen and Kukkonen, 1998; Ding *et al*., 2001; Gerhardt, 2007). In the laboratory, only asexual reproduction occurs, allowing for easy reproduction tests as mating pairs are not required. Many culture protocols exist (Dermott and Munawar, 1992; Phipps *et al*., 1993; USEPA/USACE, 1998; ASTM, 2010b; EPA, 2000; OECD, 2007; MOE, 2012c).

Ecological Relevance

The species is ecologically-relevant for Canadian freshwater toxicity studies as they are commonly found in the shallows of freshwater ponds, lakes and wetlands of North America and Europe where they freely crawl on sediment and submerged, decaying vegetation (Putzer *et al*., 1990; Phipps *et al*., 1993; Drewes and Cain, 1999; O'Gara *et al*., 2004). *Lumbriculus variegatus* are important members of the freshwater aquatic community, where they aid in the decomposition of organic materials in the sediment and serve as food for higher trophic organisms (O'Gara *et al*., 2004). As oligochaetes, *L. variegatus* also acts to bioturbate or mix aquatic sediments by feeding at depths and then deposits fecal materials at the sediment-water interface (Landrum *et al*., 2002). Densities of *L. variegatus* have been recorded at up to approximately 11 000 individuals per square metre in European streams by Cook (1969). In addition, *L. variegatus* are subject to contamination from all routes, including ingestion of contaminated particles (Phipps *et al*., 1993) and through water and sediment body contact (Gerhardt, 2007) and are therefore good bioindicators of an area's whole toxicity.

Anatomy and Physiology

Lumbriculus variegatus are typically 20-90 mm in length, with a diameter of 0.8 to 1.5 mm and are a reddish brown colour (Cook, 1967; Phipps *et al*., 1993). The prostomium, or first segment, is conical in shape, while the peristomium, or second segment, is considered to be more long than broad in shape (Cook, 1967). Each segment is markedly biannulate, or has two rings (Cook, 1967). The blood circulatory system consists of two central vessels, dorsal and ventral,

connected by circumintestinal commissures, or a bundle of nerve fibers, as well as branched, posterial lateral blood vessels (Cook, 1967).

Life Cycle and Reproduction

Lumbriculus variegatus are capable of reproduction through sexual and asexual means; however, individuals with sexual organs are extremely rare (Cook. 1967; Phipps *et al*., 1993) and have yet to be seen in laboratory cultures. In laboratory cultures, reproduction occurs via architomy or morpholaxis, in which new worms bud off the anterior end of a parent worm (Phipps *et al*., 1993). The parent worm then regrows up to eight new segments (Phipps *et al*., 1993).

Sexually mature adult worm morphology has been described by Cook (1967). Sexual reproduction yields cocoons, such as those gathered from submerged, decaying leaves in the shallow water of a marsh by Drewes (1999). Each cocoon contained 4-12 orange embryos, and when hatched, the juvenile worms were 5-6 mm long.

Feeding and Behaviour

Lumbriculus variegatus usually position the anterior, or head end of its body, into the sediment where it feeds on organic matter (Phipps *et al*., 1993), as seen in Figure 12. The worm often tunnels into the aerobic zone of the sediment (Phipps *et al*., 1993), a behaviour that has been observed in the laboratory through the glass walls of the aquarium. The posterior end of the worm undulates in the water column to facilitate gas exchange (Phipps *et al*., 1993). Again, these behaviours, along with clumping of the worms into colonies, have been observed in the laboratory. Swimming behaviour through the water column has been observed in the laboratory with tactile stimulation, and may be an important means of predator avoidance (Drewes and Fourtner, 1989; Drewes,

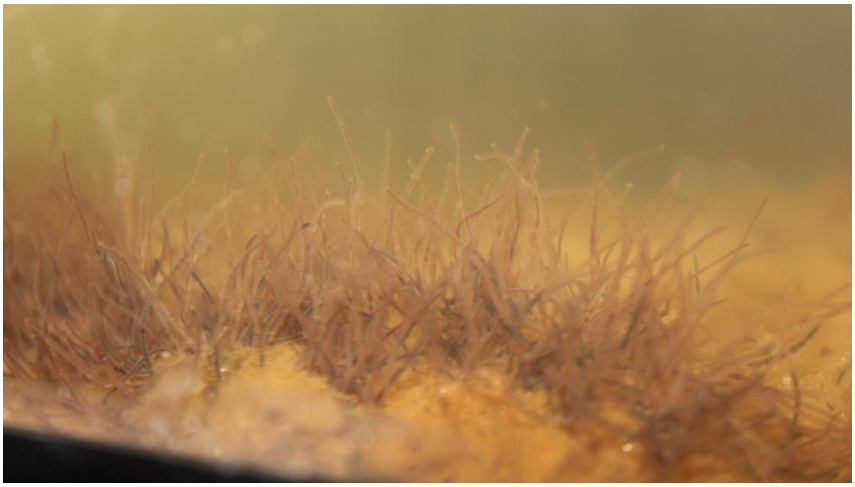

Figure 12 *Lumbriculus variegatus* feeding position in paper towel substrate (M. Raby, 2013)

1999). When touched on the anterior segments, the worm reverses its body in a fast (0.4 second) movement thought to prepare the worm for an escape swimming response (Drewes, 1999). When touched on the posterior segments, the worm swims in a rapid, rhythmic sequence of helical body waves that alternate between clockwise and counter clockwise helical rotations for thrust (Drewes, 1999). These characteristic escape responses have been used as endpoints of behaviour change in response to stressors (Drewes, 1999; Ding *et al*., 2001). Other behavioural responses that have been investigated include sediment re-working or biological burying rate in which the feeding activity of the worm buries a radiolabeled marker layer in the sediment, thus allowing measurement of burial rate in response to exposure to contaminants (Landrum *et al*., 2002; 2004).

Toxicity Studies Utilizing *Lumbriculus variegatus*

The use of *Lumbriculus variegatus* as a toxicity testing organism is relatively new compared to other bioassay organisms. Bailey and Liu (1980) were the first to suggest the use *L. variegatus* as a representative oligochaete for toxicity testing. Interestingly, they also noted that *L. variegatus* exhibited several sub-lethal responses, including colour changes, swelling and fragmentation under stress (Bailey and Liu, 1980). Bailey and Liu tested various metals and organic compounds in water-only tests. Closely following Bailey and Liu, Hornig (1980) utilized *L. variegatus* as an industrial effluent biomonitoring organism. In the following decades, *L. variegatus* has been used as a toxicity testing organism in both sediment (Ankley *et al*., 1991; Dermott and Munawar, 1992; Schubauer-Berigan *et al*., 1993; Leppanen and Kukkonen, 1998; West and Ankley, 1998; Landrum *et al*., 2002; Landrum *et al*., 2004; Oetken *et al*., 2005; Gerhardt, 2007; Paumen *et al*., 2008; Higgins *et al*., 2009; Sardo and Soares, 2010) and water-only tests (Ewell *et al*., 1986; Nebeker *et al*., 1989; Schubauer-Berigan *et al*., 1993; Phipps *et al*., 1993; Phipps *et al*., 1995; Drewes, 1999; Call *et al*., 2001; Ding *et al*., 2001; O'Gara *et al*., 2004). Acute endpoints such as lethality (Hornig, 1980; Ewell *et al*., 1986; Nebeker *et al*., 1989; Schubauer-Berigan *et al*., 1993; Phipps *et al*., 1995; Call *et al*., 2001; Ding *et al*., 2001; O'Gara *et al*., 2004) as well as sub-acute, endpoints such as a change in locomotor behaviour (Drewes, 1999; Ding *et al*., 2001), growth or reproduction (Phipps *et al*., 1993; Hickey and Martin, 1995; Leppanen and Kukkonen, 1998) or a change in feeding rate (Leppanen and Kukkonen, 1998b) have been studied. Also, studies have utilized bioaccumulation of sediment-associated

contaminants as a chronic endpoint (Schuytema *et al*., 1988; Phipps *et al*., 1993; Higgins *et al*., 2009).

Water-Only *Lumbriculus variegatus* Protocols

The only established *Lumbriculus variegatus* toxicity protocols are for testing sediment. OECD has published a sediment-water *L. variegatus* toxicity test using whole or spiked sediment (OECD, 2007), and the EPA has published a bioaccumulation test for contaminated sediments (EPA, 2000). Environment Canada, ASTM and ISO have not published guidelines for sediment toxicity testing with *L. variegatus* and no major environmental or standard organizations have published aquatic or effluent toxicity protocols for *L. variegatus*.

While *Lumbriculus variegatus* has been widely used in sediment-associated contaminant toxicity tests, comparatively few toxicity tests have evaluated aqueous contaminants in water-only tests (Bailey and Liu, 1980; Hornig, 1980; Ewell *et al*., 1986 ; Nebeker *et al*., 1989; Schubauer-Berigan *et al*., 1993; Phipps *et al*., 1995; Call *et al*., 2001; Ding *et al*., 2001; O'Gara *et al*., 2004). To date, the only water-only toxicity test employing *L. variegatus* was an acute lethality test evaluating the toxicity of an industrial wastestream (Hornig, 1980).

1.6.5 *Lemna minor*

Background and Use in Toxicity Testing

Lemna minor (Figure 13) are a species of freshwater and estuarine vascular macrophytes commonly used for aquatic toxicity tests due to their sensitivity, ecological relevance and ease of culturing (OECD, 2006; Environment Canada, 2007). The species has been studied since the 1920s and used as a test organism for assessing phytotoxicity since the 1930s (Hillman, 1961; Environment Canada, 2007). *L. minor* are considered to be suitable test organisms for aquatic contaminants and are especially sensitive to substances that concentrate at the air-water interface, where they are found floating (Taraldsen and Norberg-

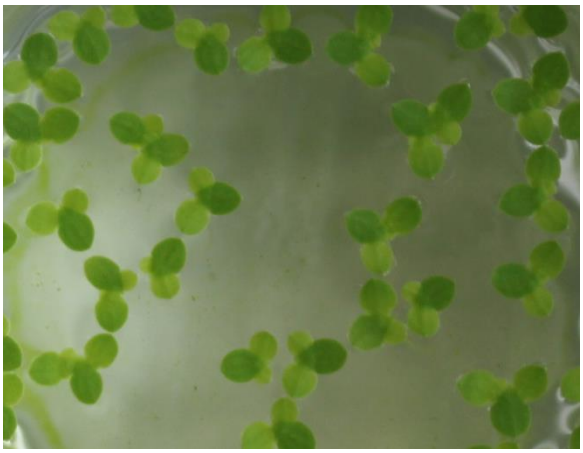

Figure 13 Image of *Lemna minor* (M. Raby, 2013)

King, 1990; Wang, 1990). In addition, *L. minor* are preferable for testing turbid waters such as wastewater and receiving water samples over other primary producers such as algae because the samples can be tested "as is," or without filtration that could compromise the sample's toxicity (Taraldsen and Norberg-King, 1990; Wang, 1990; Environment Canada, 2007). Individuals are small and therefore little space is needed for culturing and testing (Correll and Correll, 1972; Wang, 1990). Their size and simple structure is sufficient for easy visual observation of toxicity effects (Wang, 1990). Lastly, *L. minor* are relatively easy to culture and test under laboratory conditions (Hillman, 1961; Wang, 1986; Wang, 1990; Environment Canada, 2007; Naumann *et al*., 2007). *L. minor* propagation is rapid and exponential, with populations doubling approximately every two days, thereby producing unlimited test specimens if sufficient nutrients are provided (Hillman, 1961; Smith and Kwan, 1989; Wang, 1990; Naumann *et al*., 2007). Because propagation is clonal and the resultant population is genetically homogenous, the factor of genetic variability is eliminated in life cycle toxicity tests (Hillman, 1961; Smith and Kwan, 1989; Naumann *et al*., 2007).

Numerous culture protocols exist (Taraldsen and Norberg-King, 1990; EPA, 1996; OECD, 2006; Environment Canada, 2007; APHA, 1998).

Ecological Relevance

Lemna minor are important components of aquatic ecosystems as they are often a major fraction of the total photosynthetic biomass (Wang, 1990; Greenberg *et al*., 1992). *L. minor* are ecologically-relevant for Canadian freshwater toxicity studies as they are widely found in quiescent water bodies including ponds, lakes and quiet streams across North America as well as other tropical to temperate zones (Godfrey and Wooten, 1979; Wang, 1990; Mohan and Hosetti, 1999). Since *L. minor* have a high bioconcentration capacity, they could represent an important entry point of aquatic contaminants into the food web (Rodgers *et al*., 1978; Greenberg *et al*., 1992). Finally, *L. minor* play a role in hosting small invertebrates such as flies and beetles and are part of the diet of a number of vertebrates including water birds such as ducks and fish (Hillman, 1961).

Anatomy

The *Lemna minor* plant usually consists of a frond and a single root. The frond floats on the water surface while the root extends downwards into the water column. The fronds are flat,

2-4 mm in size, oval in outline and leaf-like (Hillman, 1961; Correll and Correll, 1972; Wang, 1990). A root is attached to each frond, which is less than 0.5 mm in diameter and with a length that varies with environmental conditions (Hillman, 1961). The plant can grow without roots as the fronds can absorb nutrients directly from the medium (Hillman, 1961).

Life Cycle and Reproduction

Groups of attached fronds are called colonies, and are produced when "daughter" fronds grow out of "mother" fronds and remain attached (Hillman, 1961). Daughter fronds are produced alternately from side to side of the mother frond from two pockets on the narrow end, near the node where the roots begin (Hillman, 1961). *L. minor* are extremely fast growing, with the doubling time ranging from 1.3 to 2.8 days (Wang, 1990). Population growth is exponential and is only limited by nutrients and space.

Toxicity Studies Utilizing L*emna minor*

Lemna minor have been used as toxicity test organisms since the 1930s, and were among the first to show the effects of herbicides on plants (Blackman and Robertson-Cumminghame, 1955; Environment Canada, 2007). Since then, *L. minor* have continued to be used as a bioassay organism for herbicides as well as other organic and inorganics such as metals. Studies have made use of the acute endpoint lethality, which was measured as a reduction of the increase in the number of fronds compared to the control (Bishop and Perry, 1981; Wang, 1986; Wang and Williams, 1988; Lockhart, 1989; Smith and Kwan, 1989; Taraldsen and Norberg-King, 1990; Wang and Williams, 1990; Clement and Bouvet, 1993; Baun and Nyholm, 1996; Tong and Hongjun, 1997; Kiss *et al*., 2003; Environment Canada, 2007; Naumann *et al*., 2007; Radic *et al*., 2010; Radic *et al*., 2011). An reduction in an increase in frond number is, however, considered to be the least reliable toxicity test endpoint because frond number is irrelevant to frond size, biomass or whether the frond is alive or dead (Wang, 1990; Mohan and Hosetti, 1999; Radic *et al*., 2011). Other common growth endpoints include a change in biomass (Bishop and Perry, 1981; Lockhart, 1989; Smith and Kwan, 1989; Wang and Williams, 1990; Clement and Bouvet, 1993; Environment Canada, 2007; Naumann *et al*., 2007; Radic *et al*., 2010; Radic *et al*., 2011) and chlorophyll content (Smith and Kwan, 1989; Taraldsen and Norberg-King, 1990; Tong and Hongjun, 1997; Naumann *et al*., 2007; Radic *et al*., 2010). Sub-acute, or chronic endpoints such as chlorosis (loss of green pigment), necrosis (localized tissue death), colony

breakup, root destruction, loss of buoyancy, gibbosity (swelling) and biochemical endpoints are also used (Wang, 1986; Wang and Williams, 1988; Wang, 1990; Clement and Bouvet, 1993; Radic *et al*., 2010).

Several studies have utilized *Lemna minor* to assess the toxicity of complex waters or effluents, such as WWTP effluent which is the primary source of triclocarban to the environment. Studies have evaluated the acute and chronic toxicity of industrial effluents (Wang and Williams, 1988; Wang and Williams, 1990), landfill leachate (Clement and Bouvet, 1993), WWTP effluent (Taraldsen and Norberg-King, 1990) and surface waters, including receiving waters of WWTP effluent (Radic *et al*., 2011). This study will contribute to the use of *L. minor* to evaluate sub-lethal toxicity of triclocarban, by evaluating chronic toxicity though the use of frond quality and reproduction endpoints.

Established Toxicity Protocols for *Lemna minor*

There are several established aquatic toxicity protocols for *Lemna* sp. which can adapted for or are specifically designed for assessing the toxicity of a water-only chemical exposure or effluent. Within Canada, Environment Canada has developed a 7-day chronic exposure test for measuring the inhibition of growth, measured by the number of fronds, when *Lemna minor* is exposed to chemicals, effluents, leachates, elutriates and receiving waters (Environment Canada, 2007). In the United States, the EPA has developed a similar 7-day chronic exposure test for chemicals that could be adopted to WWTP effluent (EPA, 1996). Internationally, the OECD, ASTM and ISO have all published 7-day chronic exposure tests for assessing toxicity of aqueous contaminants using *Lemna* sp. with frond number being the primary endpoint (ASTM, 2004b; ISO, 2005; OECD, 2006).

1.7 Triclocarban

Triclocarban (3, 4, 4'trichlorocarbanilide; *N*-(4-chlorophenyl)-*N'*-(3,4-dichlorophenyl)-urea; TCC; Figure 14) is a popular antimicrobial compound added to a variety of personal care products including antimicrobial soaps, detergents, cosmetics, deodorants, shampoos and shaving creams (European Commission, 2005; Halden and Paull, 2005). An estimated 227–454 metric tonnes of TCC are used annually in the United States (TCC Consortium, 2002). TCC is a "down the drain" contaminant, and is transported in domestic sewage and wastewater to municipal WWTPs where it is eventually discharged in biosolids, and to a lesser degree in effluent, to the environment (Chu and Metcalfe, 2007). Due to its low water solubility and moderate K_{ow} values, TCC persists through various WWTP processes and is concentrated in the biosolids sludge, or solid fraction of the wastewater (Snyder *et al*., 2010a). Throughout North America, biosolids are land-applied to agricultural fields as an cost-effective source of fertilizer to promote plant growth and to maintain soil structure (O'Connor *et al*., 2005). Thus, TCC can be introduced to the environment through land application of biosolids and associated runoff, as well as WWTP effluent discharge. For the purposes of this study, we will only be considering TCC input through WWTP effluent discharge.

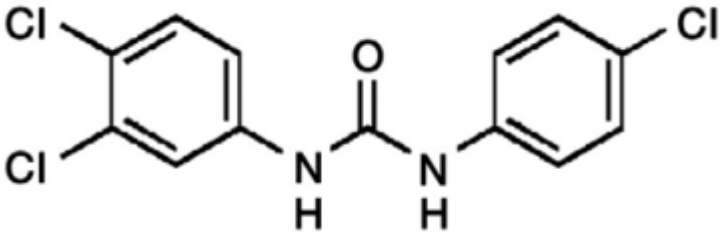

Figure 14 Chemical structure of triclocarban (TCC) (Snyder *et al*., 2010b)

In use since 1957 (Halden and Paull, 2005), TCC has been discharged to the environment for decades and yet only recently has its environmental fate and impact started to be studied. Best stated by Halden and Paull in their 2005 paper, "TCC has been an overlooked and under-reported toxic contaminant of U.S. water resources for a number of years and possibly as long as half a century." A recent risk assessment of land-applied biosolids-borne TCC found no significant risk to exposed aquatic organisms even under worst-case land application scenarios, except for a single aquatic species, the shrimp *Mysidopsis bahia*; however, TCC concentrations employed in the risk assessment were extremely high, and likely represent contamination by sewage overflow (Snyder and O'Connor, 2013). It should be noted that only three organism endpoints were

investigated for the aquatic pathway: a daphnid (*Ceriodaphnia* sp.), fathead minnow (*Pimphelas. promelas),* and a shrimp (*Mysidopsis bahia).* Considering the TCC Consortium (2002) found aquatic invertebrates to be the most sensitive to TCC, this risk assessment may not have taken into account more sensitive species, for example, those species at the sediment-water interface where TCC would partition.

While a predominance of literature in the 2000s did not show impact, a recent review of emerging organic contaminants in biosolids by UK researchers Clarke and Smith ranked TCC third highest in research priority (Clarke and Smith, 2001). The need for TCC research was ranked at the same level as triclosan and polybrominated diethyl ethers and just behind perfluorochemicals and polychlorinated alkanes and napthalenes. TCC was also listed as a high priority contaminant of concern in a Water Environment Research Foundation State-of the-Science Review (Higgins *et al*., 2010 in Snyder and O'Connor, 2013).

1.7.1 Environmental Impact of Triclocarban

Mobility and persistence studies suggest that the potential for TCC transport from biosolids-amended soils via leaching or runoff is low (Edwards *et al*., 2009; Cha and Cupples, 2010; Xia *et al*., 2010. However, TCC has been detected in wastewater and surface waters in ng/L to μg/L concentrations with high frequency, as shown in Table 1 (Halden and Paull, 2004; 2005; Coogan *et al*., 2007; Sapkota *et al*., 2007; Coogan and La Point, 2008; Young *et al*., 2008; Shen *et al*., 2012; Lozano *et al*., 2013), suggesting either transport from biosolids or input from WWTP effluent.

Table 1 Recently measured TCC concentrations in surface and wastewaters

Study Region	Water Type	[TCC] (μg/L)	Reference
Baltimore-area, United States	River water	<5.600	Halden and Paull, 2004
Baltimore-area, United States	Wastewater	<6.750	Halden and Paull, 2004
United States	Downstream of a WWTP	0.084 ± 0.110	Sapkota *et al*., 2007
United States	Upstream of a WWTP	0.012 ± 0.015	Sapkota *et al*., 2007
Texas, United States	Effluent-receiving stream	0.191	Coogan and La Point, 2008
Baltimore-area, United States	Surface water	2.230	Young *et al*., 2008
Danshuei River, Taiwan	River water	0.567 – 0.610	Shen *et al*., 2012
Mid-Atlantic United States	Final effluent	0.12 ± 0.2	Lozano *et al*., 2013

TCC is an anilide, a class of compounds shown to induce cell death by adsorbing and destroying the cytoplasmic membrane (McDonnell, 2007 in Snyder and O'Connor, 2013). Limited toxicity data exist for TCC. Few peer-reviewed TCC toxicity studies exist. The majority of data, as shown in Table 2, is a result of collection by the EPA, autonomously and through the TCC Consortium. In the TCC Consortium data, aquatic invertebrates were found to be the most sensitive taxa to TCC exposure. Acutely lethal concentrations of TCC ranged from 0.25–20 μg/L for aquatic invertebrates, and 49–120 μg/L for fish.

TCC has been reported to bioaccumulate in algae (Coogan *et al*., 2007), snails (Coogan and La Point, 2008) and the aquatic worm *L. variegatus* (Higgins *et al*., 2009), as well as terrestrial organisms such as the worm *Eisenia fetida* (Snyder *et al*., 2011), Bahia grass or *Paspalum notatum* (Snyder *et al*., 2011) and the soybean *Glycine max* (Wu *et al*., 2010). TCC has also shown a potential for endocrine disruption in *in vitro* mammalian cell research (Chen *et al*., 2008; Giudice and Young, 2010). TCC was found to stimulate embryo production in a freshwater mudsnail (Giudice and Young, 2010) and was found to enhance induced tubercle formation in female fathead minnows (Ankley *et al*., 2010). To date, the only behavioural toxicity study of TCC utilized fish. In 2012, Schultz and colleagues observed that TCC decreased aggression in adult male fathead minnows exposed at 16 ug/L TCC; however, they also found substantial variability in the severity of the observed effect within treatments, suggesting that these concentrations may only affect very sensitive individuals.

Table 2 Select TCC toxicity data for freshwater organisms

Indicator Organism	Assay	Assay Parameter, Effect	Exposure Time	[TCC] (ug/L)	Reference
Pseudokirchneriella subcapitata	IC_{50}	Acute toxicity, population growth (biomass)	3 d	17	Yang *et al*., 2008
	LOEC	Acute toxicity, population growth (biomass)	3 d	10	Yang *et al*., 2008
	NOEC	Acute toxicity, population growth (biomass)	3 d	<10	Yang *et al*., 2008
Ceriodaphnia dubia	EC_{50}	Acute toxicity, immobilization	2 d	3.1	EPA, 1992a*; TCC Consortium, 2002
	NOEC	Acute toxicity, immobilization	2 d	1.9	EPA, 1992a*
	NOEC	Chronic toxicity, mortality and reproduction	7 d	1.46	TCC Consortium, 2002
Daphnia magna	LC_{50}	Acute toxicity, lethality	2 d	10 – 20	EPA, 1992b*; TCC Consortium, 2002
	LOEC	Chronic toxicity	7 d	0.5	EPA, 1992c*
	LOEC	Chronic toxicity, lethality	14 d	0.5	EPA, 1992c*
	LOEC	Chronic toxicity, lethality and reproduction	21 d	4.7	TCC Consortium, 2002
	LOEC	Chronic toxicity, lethality	21 d	0.5	EPA, 1992c*
	NOEC	Chronic toxicity	14 d	0.25	EPA, 1992c*
	NOEC	Chronic toxicity, lethality and reproduction	21 d	2.9	TCC Consortium, 2002
Gammarus fasciatus§	LC_{50}	Acute toxicity, lethality	3 d	13	EPA, 1992d*
	LOEC	Acute toxicity, lethality	4 d	14	EPA, 1992d*
Pimephales promelas	LC_{50}	Acute toxicity, lethality	4 d	92	EPA, 1992e*
	NOEC	Acute toxicity, lethality	4 d	54	EPA, 1992e*
Oncorhynchus mykiss	LC_{50}	Acute toxicity, lethality	4 d	120	EPA, 1992f*; TCC Consortium, 2002
	LOEC	Acute toxicity, lethality	4 d	49	EPA, 1992f*

* Data obtained from ECOTOX database

§ Sensitivity of organism unknown

2.0 METHODS

Prior to beginning behaviour or life cycle toxicity bioassays, it was critical that healthy, stable cultures be established. Preliminary experiments with *Daphnia magna* and to some extent *Hyalella azteca* found low survival in reference water. Reference water was City of Toronto municipal drinking water (MDW) that was aerated for >24 hours with an aquarium bubbler. After a water quality analysis and research into Toronto's water treatment (Basrur, 2001), high copper levels as well as chloramines were found in the MDW. This led to a thorough investigation of two complementary problems. The first problem was that the MDW was not suitable for the bioassay organisms for either culturing or toxicity bioassays; and the second, that the culturing procedures were not providing healthy enough organisms to survive reference conditions. A simple activated carbon filtration system was set-up to provide water adequate for culturing and toxicity bioassays, and culturing procedures were adopted that provided healthy and age-synchronized organisms for bioassays.

2.1 General

2.1.1 Dechlorination of Municipal Drinking Water

An activated carbon filter and vigorous aeration were used to remove trace metals, including copper, and dechlorinate MDW. MDW was poured through a layer of activated carbon (Marineland brand) and collected in a 20-L Nalgene carboy. The filtered water was aerated with an aquarium bubbler for at least 24 hours or until the dissolved oxygen was ≥ 8 ppm, prior to use for culturing or toxicity bioassays.

Chlorine and copper levels of select batches of water were measured before and after filtration as well as after aeration to ensure water quality. Chlorine and copper levels were measured using Hach Permachem™ Reagents and a Hach Dr/820 Colorimeter. Total chlorine was measured quantitatively using the DPD Method with DPD total chlorine reagent powder pillows. The limit of detection was 0.02 mg/L total Cl_2. After aeration, all filtered water batches tested had total chlorine levels ≤ 0.02 mg/L. Copper was measured qualitatively using CuVer® 1 Copper Reagent Powder Pillows. A pink colour development was compared to a deionized water control. All filtered water batches tested had no colour development observable by the naked eye.

Select dechlorinated water batches were tested in a 48-hour *D. magna* acute lethality bioassay. Three <24 hour old neonate *D. magna* were transferred to 50 mL samples of filtered, aerated water in individual beakers (N=5). The vessels were placed in a paper-covered aquarium on the bench top. They were neither fed nor aerated. Survival was noted at 24 and 48 hour intervals.

2.1.2 Glassware Washing Procedure

All glassware, including aquaria, were washed thoroughly prior to use to remove traces of organic matter and chemicals. Washing procedures were based on Environment Canada (1996). Glassware was soaked in a 0.5% solution of Extran soap for at least 15 minutes, then finger scrubbed to remove any residue. The item was rinsed three times with MDW to remove the Extran soap. Finally, the item was rinsed in 10% v/v hydrochloric acid and rinsed three times with deionized water before being placed in an inverted position on a drying rack to dry.

2.1.3 General Bioassay Conditions

With the exception of *Lemna minor* life cycle tests, all bioassays were conducted on the bench top at ambient lighting (8-12 umol/m^2/s) and room temperature (22 ± 2°C) conditions. All vessels were randomized in their placement at the beginning of each test, as well once daily at time of data recording.

2.2 Culturing and Age Synchronization

Rigorous culturing of the bioassay organisms is absolutely necessary to ensure a steady supply of healthy, age-synchronized organisms for toxicity bioassays. While daily culturing activities for *Daphnia magna*, *Hyalella azteca*, *Lumbriculus variegatus* and *Lemna minor* may take only approximately thirty minutes daily plus an additional two to three hours weekly, these activities must be carried out with rigour and regularity or else the cultures may fail. The amount of onerous effort and detail presented below is not readily apparent in academic research or in most government protocols; however, it must be followed to ensure healthy organisms.

2.2.1 *Daphnia magna*

Daphnia magna culturing procedures were based on the Ontario Ministry of Environment (2012a) SOP DM1.v8 protocol, which in turn was partly based on the Environment Canada protocol (1990). The following procedure, outlined in Figure 15, kept a stock of adults in brood stocks that produced a <24 hour old neonates for toxicity bioassays.

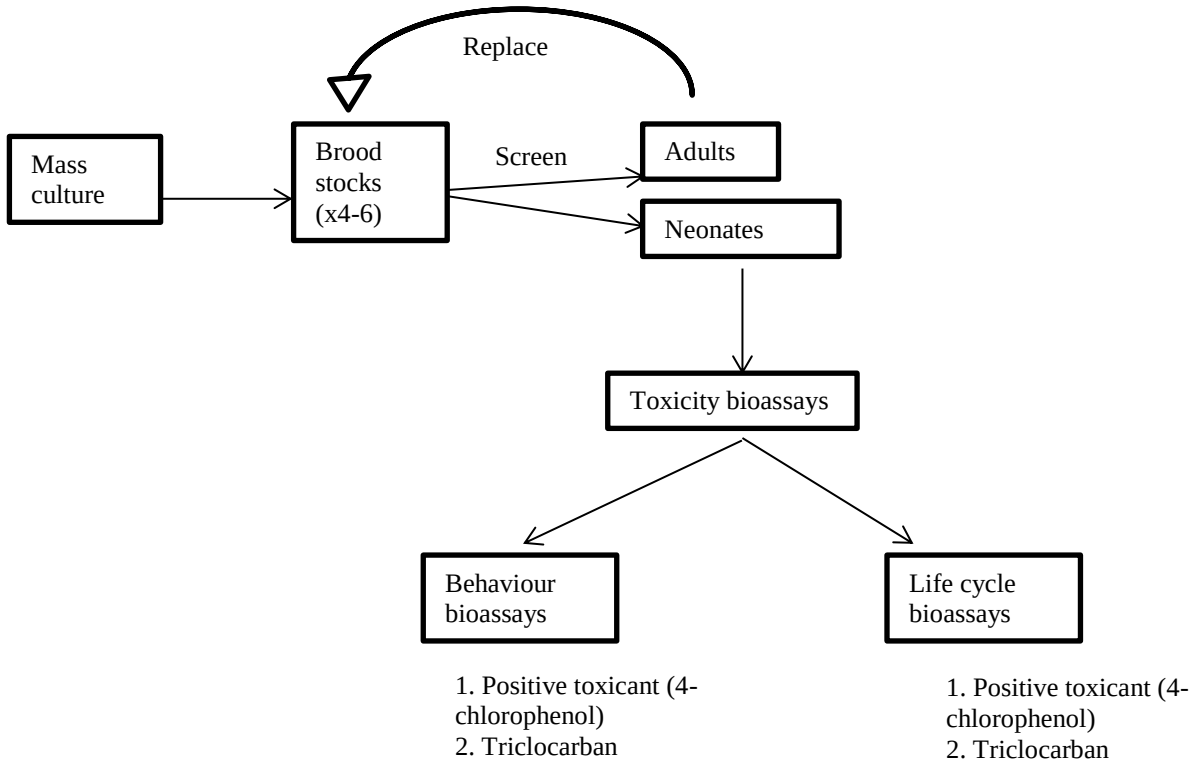

Figure 15 *Daphnia magna* culturing and toxicity bioassay flow chart

The *D. magna* mass culture consisted of mixed age animals and was the source of neonates to start each brood stock. The brood stocks provided neonates for toxicity bioassays. Each brood stock also had a matching health jar. The health jar consisted of a representative animal that indicated the health of the brood. Only the neonates produced from the animal in the health jar were counted and compared to the Ontario Ministry of Environment health criteria to ensure the health of the entire brood stock.

Health of *D. magna*

The Ontario Ministry of Environment has several health criteria that must be satisfied before a brood stock can be "passed" and its neonates can be used for toxicity bioassays. A brood

stock was considered healthy if the first brood was produced within 12 days of the individual's date of birth. If on the 12^{th} day a brood was still not produced, the entire brood stock was discarded by removing the animals to a special "retirement" tank. A new brood stock was then initiated as soon as possible.

Brood stock health continued to be monitored throughout the lifespan of the *Daphnia magna*, and the brood stock was discarded if more than 25% of the animals died or if the average number of neonates per adult for brood two or more, as measured using the health jar (described in section 2.2.1 *Daphnia magna* Brood Stock Initiation), dropped below 15 neonates. Again, animals were retired and new brood stocks were initiated as soon as possible. No health jar adults died during culturing; however, if one did, it would have simply been replaced with an adult from the same brood stock, and this replacement noted.

The general health of the organisms was also monitored. For example, *D. magna* fed with the green algae *P. subcapitata* and *C. fusca*, took on a cream or light brown colour and the digestive tract was a vivid, dark green. Brood stocks containing animals that appeared bright white, transparent, did not appear to be feeding, or showed decreased reproduction were supplemented with vitamin B12 and selenium and extra algae. If, after one week, the animals did not regain vigour, the brood stock was discarded. Also, any production of ephippa, or resting eggs, indicated a decline in health and that brood stock would have been discarded. No ephippa were seen while culturing.

***Daphnia magna* Culture Medium**

Daphnia magna were cultured in a semi-defined medium that consisted of:

1. concentrated algae cultures of *Pseudokirschneriella subcapitata* and *Chlorella fusca*, for food (10^6 cells/mL each)
2. aerated, dechlorinated MDW
3. selenium and vitamin B12 supplementation (added if *D. magna* show decreased reproduction or white or clear colouring)

To create a batch of *D. magna* culture medium, 25 mL each of *P. subcapitata* (10^6 cells/mL) and *C. fusca* (10^6 cells/mL) per 1 L MDW were added to a large glass vessel. If selenium and B12 were being added, these supplements were added to the algae at this time. The

algae mixture was then topped up with aerated, dechlorinated MDW which also thoroughly mixed the medium. This culture medium was then used in the daily culturing of the brood stocks. New culture medium was created daily.

Growth of Algae for *Daphnia magna* Food

Both *P. subcapitata* and *C. fusca* were grown in a modified Bristol's Medium, as per Ontario Ministry of the Environment protocol AL1.v6 (2012b), and given in Appendix III. Batches of 3.5 L of modified Bristol's Medium were made in 4 L glass jars. The medium was inoculated with a loopful of algae from a *P. subcapitata* agar slant (Ward's Science) or approximately 25 mL of a concentrated axenic *C. fusca* culture from the Ontario Ministry of the Environment (D. Poirier, 2013) and covered with tin foil through which aeration, consisting of aquarium tubing with a glass Pasteur pipette fitted onto the end, was placed. The vessel was placed under continuous, 24-hour lighting provided by five cool-white fluorescent bulbs with a total intensity of 85-94 μmol/m^2/s, or approximately 5000 lux. In 6-10 days the algae were a dark green colour, with a jade (*P. subcapitata*) or bright green (*C. fusca*) tint. The aerator was then removed and the entire vessel was placed in a refrigerator between 2-8°C to settle. The algae were concentrated by carefully removing the top half (1.5-2 L) of medium via siphoning with effort not to disturb the settled algae on the bottom of the vessel. The remaining algae were mixed thoroughly and transferred into glass media bottles for storage. Samples of concentrated algae were removed and microscopically examined to confirm that there was no contamination by fungi or filamentous algae. Also, the concentration of the algae was determined using a hemocytometer. While algae could be kept in the dark between 2-8°C for up to two months, they were always used for *D. magna* feeding before expiry could occur.

Daphnia magna Mass Culturing and Brood Stock Initiation

Two types of *Daphnia magna* cultures were kept: (1) a mass culture, which served as a backup in case of population crashes, and (2) brood stocks, which served as the source of neonates for toxicity bioassays.

Mass Cultures

Two mass cultures were kept, each in a 2.5 L glass aquarium; however, cultures could be kept in any large (> 2L) glass container. The mass cultures were initiated with a mixed age population of *D. magna* obtained from Ward's Science. Once per week the mass culture was fed

by replacing half of the tank water with fresh culture medium. An aquarium bubbler fitted with a glass pipette provided aeration.

Brood Stock Initiation

Brood stocks consisted of multiple separate vessels of adult *Daphnia magna* that provided neonates for toxicity bioassays . To initiate a brood stock from the mass culture stock, gravid adult female *D. magna* were transferred into separate 100 mL glass beakers with 75 mL culture medium. Half of the culture medium was changed daily, at which point the vessels were checked for neonates. Adults that had broods of more than twenty-one neonates were selected for brood stock initiation. Twenty neonates, all from one brood, were transferred into a glass vessel (20 cm tall, 9.5 cm diameter) containing 1.5 L of culture medium. One neonate from this same brood was transferred into a separate 100 mL glass beaker containing 75 mL of culture medium. This jar was the "health jar" and acted as a representative animal for the brood to evaluate reproduction and overall health. During the first week of a new brood stock the *D. magna* were not fed any additional algae beyond what was present in the culture medium when the brood stock was started. On the seventh day, and six to seven times weekly after production of the first brood, the brood stocks were checked for neonates and half of the culture medium was replaced, as outlined below in daily maintenance.

The brood and health jars were labelled with the birth date of the neonates. All neonates were transferred with a 2 mL plastic transfer pipette with the end cut to create an appropriately sized bore. Four to six brood stocks from neonates born on varying dates from varying adult *D. magna* were maintained. All brood stocks and health jars were kept within a large aquarium that was covered on the sides with neutral coloured paper to reduce moving shadows and sunlight from a nearby window. The top of the aquarium was covered with a piece of glass to prevent debris from falling into the brood stocks. All vessels were kept at room temperature, 22 ± 2°C, and ambient room lighting of 8-12 uE/m^2/s at an approximately 16h:8h light:dark cycle.

Daily Maintenance of *Daphnia magna* Brood Stocks

Brood stocks were fed and neonates were removed six to seven times weekly, approximately 24 hours apart. This ensured a constant supply of known age (<24 hour old) neonates for toxicity bioassays. A screen was used to separate and remove the neonates from the

brood stock. Once the culture medium was replenished, the adults were replaced back into the brood stock vessel.

First, a fresh batch of culture medium was mixed (see below for culture medium protocol). A 1 L beaker was filled with approximately 800 mL of aerated dechlorinated MDW and was used to hold the neonates collected from the brood stocks. Neonates were separated from adults with the use of a two-mesh screen. Pieces of interlocking black PVC piping (inner diameters of 5 cm and 6 cm; Canadian Tire, Canada) were fitted with two sizes of Nitex screening and glued in place with aquarium glue (LePage Extreme Repair Adhesive, Canadian Tire, Canada), as seen in Figure 16. The inner filter was a coarse Nitex screen of approximately 1 mm and was used to catch the adults. The outer filter was a finer Nitex screen of approximately 0.25 mm and was used to catch the neonates. To filter the brood stocks, the screens were placed one on top of another and the contents of the vessel were slowly poured through the screens into an empty glass jar of the same dimensions as the brood stock jars. Half (0.75 L) of the old culture medium was then poured back into the brood stock vessel, and the remaining old culture medium was disposed of. The brood stock vessel was then topped up with 0.75 L of fresh culture medium. The adult *D. magna* caught on the coarse screen were counted and replaced into the brood stock vessel by carefully submersing the filter into the culture medium and waiting for the *D. magna* to move away from the screen and into the water. Similarly, the number of neonate *D. magna* caught on the fine screen were approximated and carefully placed into the 1 L beaker containing aerated dechlorinated MDW. In the same manner the health jar was fed and counted; however, it was found to be easier to simply count and remove any neonates using a transfer pipette, then slowly pour half of the old culture medium from the 150 mL beaker into a waste beaker and top up with fresh culture medium to the 75 mL mark. This method was easier because the coarse screen was too large to fit into the beaker to replace the adult after separation by filtering. Also, it enabled more careful counting by pipette removal rather than counting on the screen. Once fed

Figure 16 Interlocking screens constructed of PVC piping, Nitex screening, and aquarium glue (M. Raby, 2013)

and counted, the brood stock and health jar were placed back into the aquarium and the process was continued for subsequent broods. The date and time of feeding, number of adults and estimated number of neonates in the brood stocks as well as the number of neonates in the health jar were recorded on a data sheet in a laboratory notebook.

Weekly Maintenance of *Daphnia magna* Brood Stocks

Weekly maintenance of the brood stocks was to clean the vessels of debris such as carapaces, dead *Daphnia magna* and algae and detritus build-up. Once filtered, the inside of the empty brood stock jar was wiped with a brown paper towel and rinsed with dechlorinated MDW.

The brood stocks were supplemented with selenium and vitamin B12 weekly. A 2.5 mg/L stock of Se was prepared from sodium selenate decahydrate (Sigma-Aldrich) and added to the prepared culture medium at a rate of 3 µg/L, or 1.2 mL stock per 1 L of culture medium, as outlined in Appendix IV. A 10 mg/L stock of vitamin B12 (Sigma-Aldrich) was prepared and added to the prepared culture medium at a rate of 1 µg/L, or 0.1 mL stock per 1 L of culture medium, as outlined in Appendix IV. The stocks were kept in amber bottles, in the dark between 2-8°C. The selenium stock expired after 1 year, and the vitamin B12 stock after 4 weeks.

2.2.2 *Hyalella azteca*

Hyalella azteca culturing procedures were based on Borgmann *et al*. (1989), Borgmann and Norwood (2009, unpublished) and Environment Canada (1997). The following procedure, outlined in Figure 17, kept a stock of 1-7 day old juveniles for toxicity bioassays.

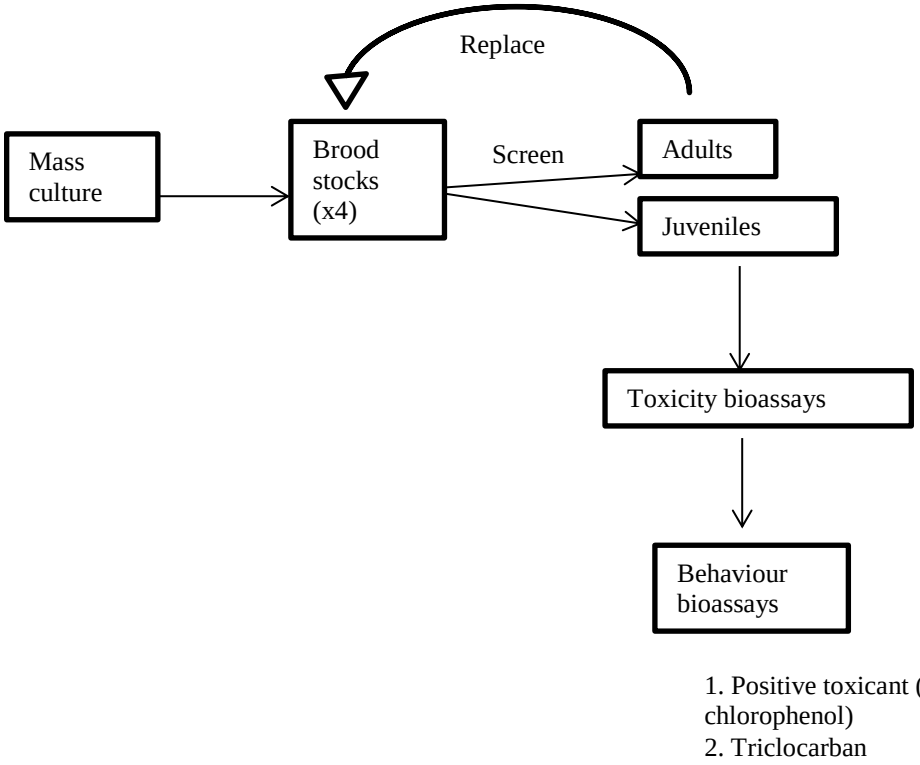

Figure 17 *Hyalella azteca* culturing and toxicity bioassays flow chart

The *H. azteca* mass culture consisted of mixed age animals and was the source of animals to start each brood stock. The brood stocks provided juveniles for toxicity bioassays.

Health of *Hyalella azteca*

According to Borgmann and Norwood (2009), each adult *Hyalella azteca* in a brood stock should produce 1-3 juveniles weekly, so 20 adult *H. azteca* per brood stock should produce 20 to 60 juveniles weekly. If the ratio of adults to juveniles fell below 1:1 after good reproduction (≥1:1 ratio) the adults were checked for illness or inactivity. If the number of adults fell to below 20 due to mortalities, more breeding pairs or individual adults were added from the mass culture tanks. Only juveniles produced from brood stocks with reproduction ratios of greater than 1:1 were used in toxicity bioassays.

***Hyalella azteca* Mass Culturing and Brood Stock Initiation**

Two types of *Hyalella azteca* cultures were kept: a mass culture, which served as a backup in case of population crashes, and brood stocks, which served as the source of juveniles for toxicity bioassays.

Mass Cultures

Two mass cultures were kept, each in a 38 L glass aquarium; however, mass cultures could be kept in any large (> 8 L) aquarium. The *H. azteca* culture consisted of multiple populations ordered from Ward's Science over the past decade. The mass culture aquariums contained 30 L of aerated, dechlorinated MDW and 3 to 5 pieces of 5x5 cm 3-layer cotton cheesecloth (Loblaws, Canada). The cheesecloth was first washed three times with deionized water, and then soaked in acetone and left to dry overnight before being placed in the aquaria. Borgmann *et al*. (1989) used one 5x10 cm piece of surgical bandage type cotton gauze; however, cotton cheesecloth was found to be a suitable alternative. The mass cultures were fed two to three times weekly with a slurry of 0.5 g finely ground Tetramin fish food and 20 mL dechlorinated MDW. Once per month half of the culture water was siphoned off and replaced with fresh aerated dechlorinated MDW. An aquarium bubbler fitted with a glass pipette was used to provide aeration. The *H. azteca* were also mass cultured with *D. magna*, snails (*Helisoma* spp.) and ostracods that functioned to keep algae buildup to a minimum.

Brood Stock Initiation

To initiate a brood stock from the mass culture, 20-30 adult (>0.37 cm in length (Othman and Pascoe, 2001) and medium to dark brown or grey coloured) *H. azteca* were transferred into a 1 L glass jar with 1 L of aerated, dechlorinated MDW. It was preferable that the adults be transferred as 15 breeding pairs, thus ensuring a 50:50 ratio of males to females and increasing the chance of reproduction. In some vessels this was achieved; however, in others, the most number of breeding pairs that could be found were transferred into the brood stock, and the remaining were individual animals taken at random. To remove the organisms from the mass culture a piece of the cheesecloth that was covered in clinging *H. azteca* was removed by hand from the aquarium and placed in a 1 L beaker or other appropriately sized glass vessel half filled with aerated, dechlorinated MDW. The cheesecloth was gently shaken to remove the organisms and placed back in the mass culture aquarium. Then the breeding pairs were removed with a plastic transfer pipette with the end cut to create a suitably sized bore and placed in the 1 L brood stock jars. Also placed in the brood stock jars was one 5x5 cm piece of 3 layer cotton cheesecloth, washed as outlined above. A minimum of four brood jars, each containing 20-30 adults, were maintained at all times. All brood stocks were kept within a large aquarium that was covered on the sides with neutral coloured paper to reduce shadows and sunlight from a nearby

window. The top of the aquarium was covered with a piece of glass to prevent debris from falling into the brood stocks. All vessels were kept at room temperature, 22 ± 2°C, and ambient room lighting of 8-12 uE/m^2/s at an approximately 16h:8h light:dark cycle.

Weekly Maintenance of *Hyalella azteca* Brood Stocks

Once weekly the juvenile *Hyalella azteca* were removed from the brood stocks, as per Borgmann and Norwood (2009). First, the gauze was grasped with a set of clean forceps and gently shaken to remove clinging *H. azteca*, removed from the vessel and placed into a glass Petri dish half filled with aerated, dechlorinated MDW. Then the entire contents of the brood stock were poured through two interlocking screens. The screens were the same used for *D. magna* culturing, and are described above. With the animals retained on the screens, the brood stock vessel was wiped with a brown paper towel to remove built-up detritus and re-filled with 1 L of aerated, dechlorinated MDW. The first screen caught the adult *H. azteca*, that were then washed with the use of a wash bottle filled with dechlorinated MDW into a second glass Petri dish half filled with water. The second screen caught the juvenile *H. azteca*. The juveniles were washed into a third petri dish filled with water. The adults were counted and placed back into the now filled 1 L brood stock jar along with the old cheesecloth. The juveniles were counted and placed into a 1 L jar filled with aerated, dechlorinated MDW and one 5x5 cm piece of cotton cheesecloth. Sometimes juvenile *H. azteca* would fall through the second screen and into the old culture water, and so it was carefully inspected before it was discarded. If the cheesecloth was too degraded to move back into the vessel in one or two pieces it was replaced, along with 1 mL of old culture water to enhance periphyton growth. This procedure was repeated for all brood stocks, with juveniles from all brood stocks being transferred into one 1 L vessel termed the "juvenile jar." Up to 500 juvenile *H. azteca* can be maintained in a 1 L jar (Borgmann and Norwood, 2009). Each brood stock was then fed with a slurry of 0.05 g of finely crushed Tetramin fish food in a 5 mL of aerated, dechlorinated MDW. Juvenile jars were fed a slurry of 0.02 g of Tetramin fish food. This counted as the first weekly feeding. Brood stocks were fed two to three times weekly. If a build-up on Tetramin fish food or detritus was observed on the bottom of the vessel, the third weekly feeding was omitted.

Since the juvenile *Hyalella azteca* were collected once per week they could be assumed to be <1-7 days old at time of collection. The *H. azteca* were then kept in the juvenile jar for 2-3

days until they were needed for a toxicity test. Thus, animals used in toxicity tests were 3-10 days old at the time of the test.

2.2.3 *Lumbriculus variegatus*

Lumbriculus variegatus culturing procedures were based on the OECD (1997), which is in turn based on Phipps *et al*. (1993), Burnson *et al*. (1998), ASTM (2000) and US EPA (2000), and the Ontario Ministry of Environment (2012b) SOP LV1.v4 protocol, which in turn was partly based on the US EPA protocol (1997). The following procedure, outlined in Figure 18, kept a mass stock of organisms as well as separate age-synchronized cultures for attempted life cycle toxicity bioassays.

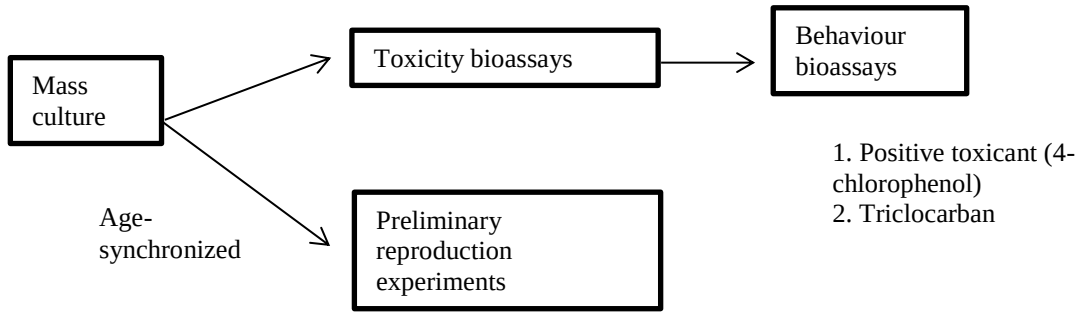

Figure 18 *Lumbriculus variegatus* culturing and toxicity bioassay flow chart

Health of *Lumbriculus variegatus*

OECD (2007) recommends that after regeneration, only worms that are intact and which actively swim or crawl when gently prodded should be used for the test. Worms that were active and did not appear to have any injuries were used for toxicity tests.

***Lumbriculus variegatus* Mass Culturing**

A mass culture was initiated in a 38 L aquarium filled with 25 L of aerated, dechlorinated MDW and a 1-3 cm layer of shredded, unbleached paper towel (Classic Brand) (OECD, 1997; OMOE, 2012b). A paper shredder was used to shred the paper towel into small (approximately 0.5-1 cm wide, 2-15 cm long) strips which were then soaked in dechlorinated MDW to aid in sinking for >24 hours prior to addition to the aquarium. The paper towel was allowed to settle to the bottom of the tank before addition of approximately 500 worms. Also, a small population of 15-30 adult snails (*Helisoma* sp.) were added and maintained in the tank to reduce algae and fungal growth and to help break down the paper towel substrate (MOE, 2012b). The worm culture was a mixed culture from Ward's Science and the Ontario Ministry of the

Environment. The snail cultures were obtained from the Ontario Ministry of the Environment. New paper towel was added to the tank every few weeks as needed. An aquarium filter fitted with a biological filter sponge, activated carbon, and ammonia remover pouch was attached to the aquarium to act as a flow-through filter and reduce the need for water changes. Also, an aquarium bubbler fitted with a glass pipette was added as back-up aeration in case the filter failed.

Worms were fed crushed trout chow (Martin's Feed Mill, Pet Menagerie, Toronto, Canada) (OECD, 1997; OMOE, 2012b) once weekly. The MOE protocol gives a feeding rate of 6 g/tank; however, the MOE tanks are connected to a flow-through system that replenishes the tank with fresh, dechlorinated MDW at a rate of 200 mL/min. As the water in the mass culture tank in our lab was not changed as frequently and the population of worms was considerably smaller, the feeding rate had to be adjusted. Varying feeding rates were tried to find the highest rate that did not produce excessive algal or fungal growth or cloudy water. A rate of 6 pellets (0.48 ± 0.034 g, N=9) per 25 L tank was used. The pellets were blended with 50 mL of aerated, dechlorinated MDW in a small Magic Bullet blender for 2 minutes or until no trout chow pieces were visible and the mixture was a cloudy, light brown liquid. The food was then poured evenly throughout the tank.

Once weekly, or more frequently if algal or fungal buildup was present or the water column was excessively cloudy, two-thirds of the water in the *L. variegatus* mass culture tank was changed. The old culture water was siphoned out of the tank with careful attention not to disturb the worms or paper towel substrate. The tank was then topped up to the 25 L mark with aerated, dechlorinated MDW. This was achieved by siphoning freshwater into the tank or by carefully pouring it from a 1 L beaker, with attention to avoid disturbing the worms or sediment. However, if the worms or sediment did become churned up from the incoming flow of water, they settled out in 1-2 hours with no observable effect on the worms. The worms re-clumped together in colonies within 1-2 hours.

Lumbriculus variegatus Age Synchronization

Age synchronization methods were based on OECD (2007). *L. variegatus* reproduce asexually via morphallaxis that results in fragments that regenerate either a head or a tail. Worms were artificially fragmented to obtain a population of the same developmental stage or age. First,

the worms were removed from the mass culture tank by gently scooping a clump of worms from below by hand and gently shaking in the water column to discard as much substrate as possible. Then the clump of worms was placed in 1 L beaker filled with approximately 500 mL of aerated, dechlorinated MDW. The number of age-synchronized worms needed were removed from the beaker into a shallow glass dish (35 x 25 x 4 cm) half filled with 1 cm of aerated, dechlorinated MDW with the use of a plastic transfer pipette. Only large worms (> 1 cm long) with no visible signs of morphallaxis were selected. Recent morphallaxis has occurred when one half of the worm's body is a much lighter colour than the other half. With the use of a razor blade, each worm was individually dissected as close to the middle of the worm as possible. The head and tail sections were transferred using a transfer pipette into two separate circular glass dishes (14 cm diameter, 4 cm tall) containing paper towel substrate and half filled with aerated, dechlorinated MDW. This procedure was repeated for each worm. The method is most efficient when the razor blade is held in one hand and the pipette in the other to transfer the worms. The head is wider and darker than the tail, and continues to move after dissection. The heads and tails were left to regenerate their tails and heads, respectively, in the circular glass dishes without feeding. Each day a few worms were observed under a dissecting microscope for signs of regeneration. Head regeneration was observed when the worms began to bury their heads in the substrate. Once regeneration was complete, or after 7 days, whichever came first, the worms were fed a slurry of 1 pellet (approximately 0.08 g) in 10 mL of water. Only the tail, or posterior end of the worm with a regenerated head was used in toxicity bioassays. Worms with a regenerated tail were placed back in the mass culture tank. Worms were used in toxicity bioassays 1-7 days after head regeneration, at 7-14 days of age.

2.2.4 *Lemna minor*

Lemna minor culturing procedures were based on OECD (2006) and Environment Canada (2007), with modifications. The following procedure kept several non-axenic cultures for toxicity bioassays. Fronds were taken directly from the mass cultures for use in appearance and life cycle bioassays, as shown in Figure 19.

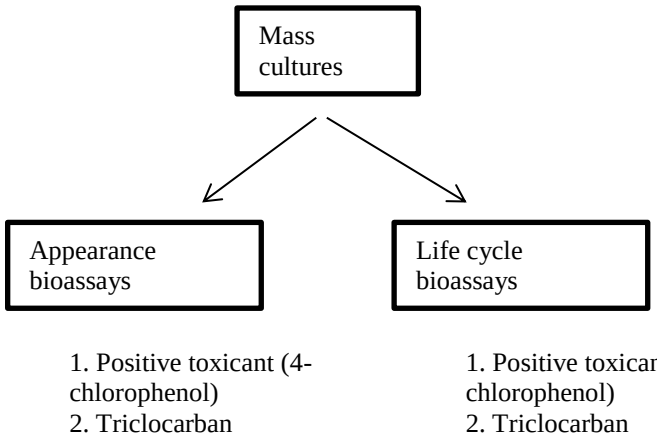

Figure 19 *Lemna minor* culturing and toxicity bioassay flow chart

Lemna minor Health Criteria

The culture of *Lemna minor* used in toxicity bioassays was first tested for health criteria. The health criteria test was carried out in cylindrical glass vessels (8 cm tall, 6.5 cm opening diameter) with 100 mL SIS medium. One three-frond plant was transferred from the culture to each of six test vessels and incubated under continuous light, as described above, for 7 days. To pass the health criteria test, the mean number of fronds in each vessel must have increased to ≥ 8 times the original number of fronds, or ≥24 fronds per vessel (Environment Canada, 2007). Only fronds that were bright green with no discoloured areas and that were from the culture that had passed the health criteria test were used in toxicity tests (Environment Canada, 2007).

Lemna minor Mass Cultures

Several mass cultures were kept at a time, each in circular glass vessels (14 cm diameter, 4 cm tall) or large glass dishes (20 x 33 x 5 cm) half filled with SIS media (see growth medium). Each mass culture was started as a sub-culture with one 3-frond colony from another mass culture. Transfer was made using a metal inoculating loop, acid-washed and rinsed with deionized water. Cultures were kept under 24-hour light given by five cool-white fluorescent lights (85-94 μmol/m^2/s), as per Environment Canada (2007) and at room temperature, 22 ± 2°C. During times when no toxicity tests were being conducted, the mass cultures were sub-cultured when the fronds covered approximately two-thirds of the surface area of the growth medium as

per Environment Canada (2007). The mass cultures were also subcultured at least once per week in the 14 days prior to a toxicity test (Environment Canada, 2007).

Lemna minor Growth Medium

Environment Canada (2007) recommends the use of Hoagland's E+ medium for toxicity bioassays involving wastewater. All attempts to culture *Lemna minor* in Hoagland's E+ medium resulted in fungal growth in the media and around the plant fronds, even in sterilized media. Instead, SIS (Swedish Standard) media was used. SIS media is recommended for culturing and bioassays with *L. minor* by OECD (2006). SIS media was prepared as per OECD (2006), with modifications. Stock solutions I, II, III, IV and VI were prepared as 10x stocks and the amounts added to deionized water changed accordingly (Appendix V). A fresh batch of SIS media was prepared when sub-culturing or for toxicity test set-up.

2.3 Positive Toxicant

While a reference (i.e. dechlorinated MDW) is deemed necessary by government protocols, the use of positive toxicants is only occasionally suggested. It was thought in the current study that it was absolutely essential to use a positive toxicant even though it was time consuming.

2.3.1 Behaviour Development

Behaviour bioassay protocols were developed for three organisms: *Daphnia magna*, *Hyalella azteca*, and *Lumbriculus variegatus*. An appearance-based bioassay protocol was developed for *Lemna minor*. In developing behaviour bioassays, observations made under positive toxicant conditions come crucial. Therefore, much time was spent on this aspect. Protocol development for all four organisms followed this design:

1. Extensive general observations of organism behaviour (or appearance) under non-stress, or control conditions. Behaviour (or appearance) was observed in mass cultures and in individual vessels at various times of day and under different lighting conditions. Observations were ongoing for ten months while culturing methods were tested and refined.
2. Observations of organism stress behaviour (or appearance) after addition of multiple positive toxicants, including drop wise addition of ethanol and a concentrated salt (NaCl)

solution into individual vessels containing 1-3 organisms of the same species. Observations were also made under a dissecting microscope. Behaviour (or appearance) under stress conditions was noted.

3. Observations of organism reference and stress behaviour (or appearance) in a positive reference toxicant dilution series. The positive toxicant was 4-chlorophenol. Dilution series were started at approximately twice the known 48-hour LC_{50} for the toxicant and organism, and reduced in a 0.5 geometric sequence for a total of five concentrations. One to three organisms were placed in each concentration, depending on the species, and observed at time points t = 0, 10 minutes, 1 hour and 24 hours after exposure.
4. Identification of organism stress behaviours (or appearance), as compared to reference behaviour (or appearance). Ethograms, or collections of a specific animal's behaviours, were constructed for each organism based on reference and stress behaviours seen in a variety of positive toxicants. Three behaviours (or appearances) were chosen to evaluate for each organism and were partially based on work by Marshall (2009). These behaviours are shown in Figure 20.

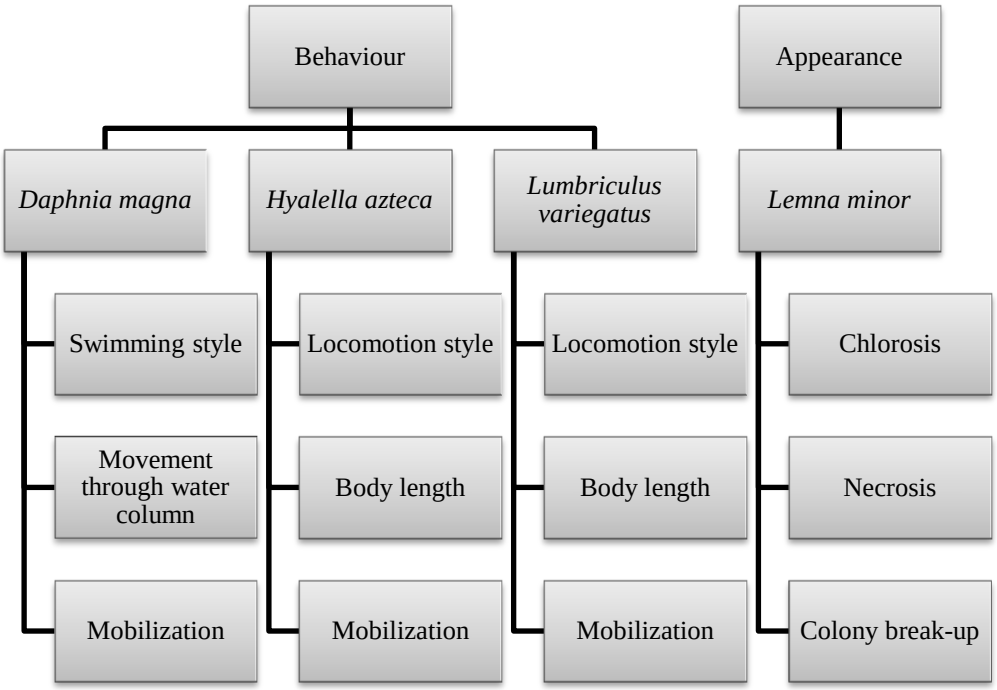

Figure 20 Selection of behaviours and appearances

5. A scoring regime was devised for each of the organism stress behaviours (or appearance). The regimes included graded scales as well as counting of specific behaviours and animals immobilized.
6. The positive reference toxicant dilution series test was repeated blind (N≥5), to ensure ranking behaviour methodology was sound.

2.3.2 Behaviour

Daphnia magna* & *Hyalella azteca

Daphnia magna and *Hyalella azteca* positive toxicant behaviour bioassays were performed similarly. A 200 ppm stock solution of reagent grade 4-chlorophenol was prepared in aerated de-chlorinated MDW. A dilution of the 4-chlorophenol along a 0.5 geometric dilution series were prepared with aerated, dechlorinated MDW. Five dilutions, or treatments, were prepared. All solutions were prepared in volumetric flasks. The control was aerated, dechlorinated MDW. 150 mL of each of the five treatments and one control were transferred into cylindrical glass vessels (8 cm tall, 6.5 cm opening diameter). Three age-synchronized organisms (*D. magna* <24 hours old, *H. azteca* 7-10 days old) were transferred into the vessel under the water surface using a plastic transfer pipette. The pipette was acid-washed and rinsed well with deionized water between transfers. First, a preliminary test with n = 1 was conducted, where behaviour was described at t = 0, 1 hour and 24 hours. A secondary test was conducted blind with n = 6. Behaviour was observed for 30 seconds at t = 10 minutes, 1 hour and behaviours were scored or counted according to the appropriate scoring regime.

Lumbriculus variegatus

Lumbriculus variegatus positive toxicant behaviour bioassays were performed similarly to *D. magna* and *H. azteca*, with some modifications. *L. variegatus* tests were performed in a 6-well plate, with each well containing 15 mL of treatment. In the preliminary test, four concentrations of the 4-chlorophenol positive toxicant plus two controls were tested, instead of five concentrations and one control. One age-synchronized worm was transferred into each well instead of three. It was decided to observe individual, rather than group behaviour as *L. variegatus* forms colonies that may shield the inner worms from the positive toxicant, thus affecting behaviour. Behaviour was observed for 1 minute and described. A secondary test was conducted blind with n = 5, under the same conditions. Behaviour was observed for 1 minute at t

= 10 minutes, 1 hour and 24 hours after exposure and scored according to the appropriate scoring regime.

Lemna minor

Lemna minor positive toxicant appearance bioassays were performed similarly to *D. magna* and *H. azteca*, with some modifications. Preliminary bioassays with *L. minor* established very limited growth in aerated, dechlorinated MDW. Therefore, the same media used in culturing (SIS media) was used in the appearance bioassay. The stock solution and dilutions of 4-chlorophenol were prepared in SIS media. 100 mL of the treatments were transferred to the same cylindrical glass vessels as used in the *D. magna* and *H. azteca* bioassays. Two three-frond colonies from a culture that had previously passed a health criteria test were transferred into each vessel using an inoculating loop. The plants were placed under the same conditions as used for culturing *L. minor*. The bioassay was run for 7 days, and photographs were taken at t = 0, 3 and 7 days. The *L. minor* appearance bioassay was run simultaneously with the life cycle bioassay (see section 4.2.1). The test was not run blind.

2.3.3 Life Cycle

Daphnia magna

Daphnia magna positive toxicant life cycle bioassays were based on OECD (2008), with modifications. A geometric dilution series of the positive toxicant was prepared in *D. magna* culture medium using a 200 ppm 4-chlorophenol stock solution. Three dilutions of the positive toxicant and a control of *D. magna* culture medium were prepared for a total of 4 treatments. Five replicates were conducted for each positive toxicant treatment and 10 replicates for the control, for a total of 25 vessels. OECD (2008) recommended a minimum of five dilutions plus a control with 10 replicates; however, that was a necessary step for constructing an EC_{50} curve, which was not the purpose of the life cycle bioassays. Two hundred and twenty five mL of each treatment was transferred into 400 mL beakers to give a ratio of 1 neonate:75 mL culture medium, or the same ratio used in *D. magna* culturing. Three <24 hour old *D. magna* neonates were transferred into each vessel. All neonates were from one brood stock that was made up of daughters from a single *D. magna* and was between 2 and 5 weeks old to ensure healthy animals. Feeding and test medium renewal were modeled after culturing methods. On day 7 of the bioassay, and every day thereafter, half of the culture medium containing the positive toxicant

dilution was renewed and the neonates were counted and removed. Dissolved oxygen was measured at the end of the test, and found to be >8 ppm and sufficient to support *D. magna*. The test continued until the first brood was produced with one additional day to ensure the entire brood had been released and the *D. magna* were not counted mid-way through the brood. Vessels were kept on the bench top and were surrounded by a paper screen to minimize shadows. Vessels were randomized in their placement daily after counting.

Lemna minor

Lemna minor positive toxicant life cycle bioassays were based on Environment Canada (2007), with modifications. Five dilutions of the positive toxicant 4-chlorophenol was prepared in SIS media, and the control was SIS media, for a total of six treatments. Three replicates were conducted for each treatment, for a total of 18 vessels. Environment Canada (2007) recommends a minimum of seven chemical dilutions in a dilution series; however, this was for the purpose of constructing an EC_{50} curve, which was not the purpose of the life cycle bioassays. One hundred mL of each treatment was transferred into cylindrical glass vessels (8 cm tall, 6.5 cm opening diameter). One three-frond colony from a culture that had previously passed a health criteria test was transferred into each vessel using an inoculating loop. The plants were placed under the same conditions as used for culturing *L. minor*. The bioassay was run for 7 days and the number of fronds were counted each day. In addition to the number of fronds, the appearance of the fronds were noted and photographs were taken at t = 0, 2 and 7 days for the positive toxicant appearance bioassay (see section 4.1.2).

Hyalella azteca* & *Lumbriculus variegatus

Hyalella azteca life cycle bioassays were not conducted due to the nature of the *H. azteca* life cycle. Under our laboratory conditions it took 6-8 weeks for *H. azteca* to mature, mate and produce a brood, which was too lengthy of a bioassay for this study.

Lumbriculus variegatus positive toxicant life cycle bioassays were not conducted after repeated attempts to induce *L. variegatus* to reproduce in laboratory conditions were unsuccessful.

2.4 Triclocarban

2.4.1 Behaviour

Daphnia magna, *Hyalella azteca*, *Lumbriculus variegatus* and *Lemna minor* behaviour/appearance bioassay methods with triclocarban (TCC) were performed under the same methodology used for the positive toxicant behaviour bioassays. A concentrated TCC stock solution was prepared in DMSO solvent (1 023 mg/L). A first dilution was prepared in aerated, dechlorinated MDW (512 μg/L) from which the TCC treatment solutions were prepared. For *Daphnia magna*, *Hyalella azteca*, *Lumbriculus variegatus*, four concentrations with six replicates each were tested in a 0.5 geometric dilution series: 10.0, 5.00, 2.50 and 1.25 μg/L. Concentrations used were within the range of environmentally-relevant concentrations (see Table 1), noting that 10 ppb is above the highest found. TCC is a hydrophobic substance (see section 1.7) and therefore a solvent was needed to dissolve the TCC in dechlorinated MDW. Dimethyl sulfoxide (DMSO) was chosen based on its use in previous studies (Marshall, 2009) and its lack of noted toxicity. A reference of aerated, dechlorinated MDW and a DMSO reference, corresponding to the maximum DMSO concentration (11 mg/L or 0.001%) was also tested. For *Lemna minor* the concentration series consisted of: 5.00, 10.0, 20.0 and 40.0 μg/L and a reference of SIS media and a DMSO reference (0.01% in SIS media). Each treatment was tested with three replicates.

2.4.2 Life Cycle

Triclocarban life cycle bioassays for *Daphnia magna* and *Lemna minor* were performed under the same methodology as for the positive toxicant life cycle methods. *Hyalella azteca* and *Lumbriculus variegatus* life cycle bioassays were not conducted. All TCC treatments were prepared from the same TCC stock used in the behaviour bioassays. For both *D. magna* and *L. minor*, the concentrations tested were 5.00, 10.0, 20.0 and 40.0 μg/L with five and three replicates, respectively. A reference of aerated, dechlorinated MDW and a DMSO reference, bioassays the maximum DMSO concentration (0.01%) was also tested.

2.5 Statistical Analysis

All raw data were discrete and therefore non-parametric by nature. Wilcoxon Mann-Whitney tests were used to analyze differences between reference and treatment conditions (Pers. Comm. A. Laursen, Ryerson University, 24 June 2013). Data were not analyzed between

treatment concentrations or temporally, as the analysis objective was to discern differences only between reference and treatment conditions. Data were analyzed in SAS Enterprise 5.1.

3.0 RESULTS AND DISCUSSION

3.1 Culturing

There are many abiotic and biotic factors that may affect toxicity within a bioassay. These factors should be controlled as much as possible to ensure the most accurate assessment of toxicity. Abiotic factors include temperature, pH, water hardness and light (Cooney, 1995). Abiotic factors can be controlled to a high degree by controlling the bioassay environment (i.e. the laboratory). Biotic factors, which are much more difficult to control, include organism life stage, size, age, disease and nutritional status (Cooney, 1995). Deviations within a population of these biotic factors may have a modifying effect on the test results. Therefore, it is important to ensure that all organisms used in a bioassay come from the same healthy cohort, or brood, and are impartially assigned to each replicate test vessel (Cooper, 1995). The culturing procedures implemented provided a steady supply of healthy, age synchronized organisms.

3.1.1 Dechlorination of Municipal Drinking Water

There is very little guidance in government protocols regarding the use of water for bioassays. A major protocol is developing reconstituted water which we have tried several times in the McCarthy laboratory but with no success. In addition, unfortunately our initial bioassays with *Daphnia magna* and *Hyalella azteca* found low reference survival in aerated Toronto MDW (intake source Lake Ontario (Basrur, 2001; Toronto Water, 2013)) and it required time consuming methodological improvement to develop appropriate reference water. After a red-brown particulate matter was found in the MDW after building repairs, a water quality analysis was ordered by Ryerson University. The analysis showed high copper levels. Also, after discussion with D. Poirier (personal communication, 10 October 2012) and research into Toronto's water (Basrur, 2001) it was found that the City of Toronto uses chloramine as a residual disinfection agent. Chloramines are used for residual disinfection as they do not decompose spontaneously and are therefore available for continuous disinfection (Coventry *et al*., 1935). The difficulty in culturing aquatic animals, including fish and invertebrates, in municipal water treated with chloramines was first described by Coventry *et al*. in 1935. Coventry and colleagues tested multiple ways to de-chlorinate their municipal drinking water, including with the use of chemicals, boiling, aeration by atomizing and by porous artificial stone blocks, and finally by adsorption to activated carbon. They found adsorption to activated carbon

to be the most efficient method. One disadvantage of using activated carbon is that it also removes the oxygen; the water must be re-oxygenated before use in aquaria or tests (Coventry *et al*., 1935).

Coventry and colleagues (1935) suggested the construction of a simple filter out of a 5-gallon glass aspirator jar, filled with a fine layer of gravel, followed by a two inch layer of sand and a 6-7 inch layer of activated carbon. Municipal drinking water was connected with an intake valve at the top, and flowed through the filter into a corrugated aeration trough (Coventry *et al*., 1935). Using this idea, municipal drinking water was poured through a layer of activated carbon. All batches of filtered water tested showed minimal chlorine levels after filtration, and no chlorine, or levels below the limit of detection after aeration. Filtered batches also showed no visible colour change for the qualitative copper test. Municipal drinking water filtered in this manner followed by >24 hours of aeration or until the measured dissolved oxygen content was ≥ 8 ppm, was used for subsequent culturing and toxicity testing with all organisms and gave excellent reference survival for *D. magna*, *H. azteca* and *L. variegatus*.

Select batches of aerated, dechlorinated MDW were tested in a 48-hour *D. magna* survival bioassay. *D. magna* were chosen for testing the water quality because they were the most sensitive of the organisms cultured in the laboratory. *D. magna* survival after 24 and 48 hours was significantly different (chi-square approximation = 39, $p < 0.0001$ and chi-square approximation = 38.1, $p < 0.0001$ respectively) in filtered versus unfiltered MDW, as shown in Figure 21.

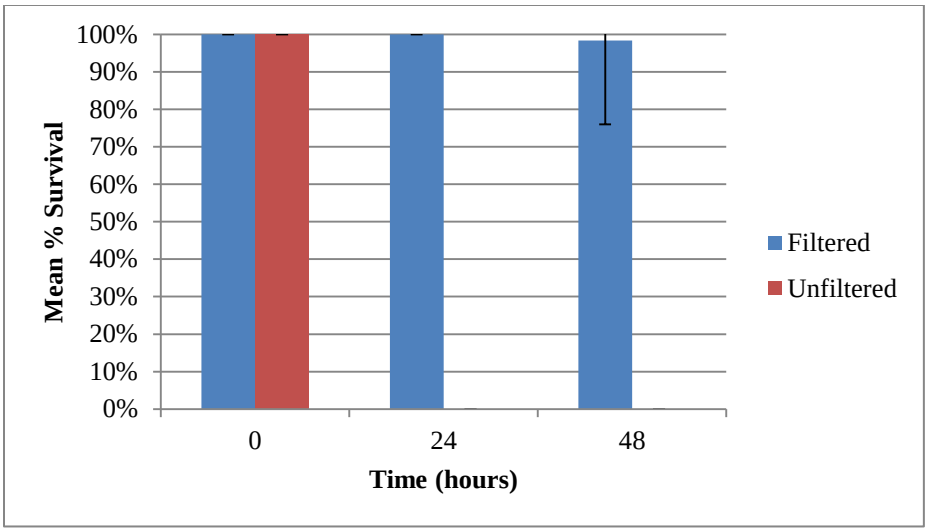

Figure 21 *Daphnia magna* survival at 0, 24 & 48 hours in aerated, filtered & aerated, unfiltered water

3.1.2 *Daphnia magna*

After examination of multiple culturing and age synchronization methods (Nebeker *et al*., 1984; Environment Canada, 1990; Lazorchak *et al*., 2009; MOE, 2012a), culturing of *Daphnia magna* using brood stocks using the MOE (2012a) method was attempted and was successful. Daily maintenance was minimal, requiring approximately 20-30 minutes of time to prepare the culture media and to screen, count and replenish the media in the brood stocks. Four to six brood stocks were maintained at all times, which gave approximately 1000-2000 neonates per week provided all *D. magna* were healthy. Only a small proportion of these *D. magna* were actually used in toxicity testing. The remaining animals were either placed in a "retirement" tank or in mass culture tanks with *Hyalella azteca*, where they functioned to restrict the algae blooms that often occurred due to overfeeding of the *H. azteca* with TetraMin fish food.

Weekly maintenance was also minimal and required an additional few minutes to add the selenium and vitamin B12 solutions to the culture medium and to wipe out the brood stock vessels. Selenium and vitamin B12 have been identified as required micronutrients for successful culturing of *D. magna* and other *Daphnia* species (Keating and Dagbusan, 1984; Winner, 1984; Keating, 1985; Elendt, 1990; Elendt and Bias, 1990). Once per week the brood stocks were supplemented with selenium (Se) and vitamin B12 to increase vigour, as per MOE (2012a). Symptoms of selenium deficiency in D. magna include increased mortality, reduced reproductive success and the incomplete development of secondary antennae after molting (Keating and

Dagbusan, 1984; Winner, 1984; Elendt, 1990; Elendt and Bias, 1990). A deficiency in vitamin B12 also leads to reduced reproductive success (Keating, 1985). Keating (1985) found *D. pulex* reproduction to be the highest when supplemented with 0.75–1.0 μg/L B12. Prior to the weekly addition of selenium and vitamin B12, many of the adult *D. magna* in the brood stocks were a white to clear colour, indicating a selenium deficiency (D. Poirier, personal communication, 19 February 2013). After regular selenium addition, very few white *D. magna* were seen. Instead, the *D. magna* took on a cream to light brown colouring, with vivid dark green digestive tracts. Whether the addition of selenium and vitamin B12 had any impact on reproduction cannot be elucidated from the culturing data because there were no non-supplemented brood stocks. However, increased reproduction with supplementation has been well-documented in literature (Keating and Dagbusan, 1984; Keating, 1985; Winner, 1984; Elendt, 1990; Elendt and Bias, 1990).

The date and size of each brood produced by the *Daphnia magna* in each brood stock health jar was recorded. The time-to-first brood was 10.3 ± 0.6 days (N = 12). Mean first brood size was 12 ± 4.5 neonates (N = 12). Mean brood size generally increased over time, as seen in Figure 22, with the greatest number of neonates produced around the 7^{th} brood. Except for the first brood, the average number of neonates produced did not fall below the MOE health criteria of 15 neonates produced per adult *D. magna* per brood. The first brood was not used in any toxicity testing. Only neonates from mothers aged 2 to 5 weeks (approximately 3^{rd}–8^{th} brood) were used in toxicity testing. The time-to-first brood agrees with literature values of 9–12 days at room temperature (Leonhard and Lawrence, 1981; Goss and Bunting, 1983; Keating and Dagusan, 1984; De Schamphelaere *et al*., 2004; Lazorchak *et al*., 2009). Goss and Bunting (1983) cultured *D. magna* at temperatures varying from 5-30°C, and found *D. magna* development and young production to be dependent upon temperature. The shortest time-to-first brood, 6.5 days, occurred at 25°C; however, this shorter time-to-first brood resulted in fewer young produced (Goss and Bunting, 1983). The greatest young production occurred at 15 and 20°C, with first broods measuring 14.3 and 14.4 neonates, respectively (Goss and Bunting, 1983). Culturing was performed at 20 ± 2°C, and the first brood size of 12 ± 4.5 neonates is in agreement with this literature value of 14.4 neonates ($X^2 = 0.6464$, $p = 0.4214$).

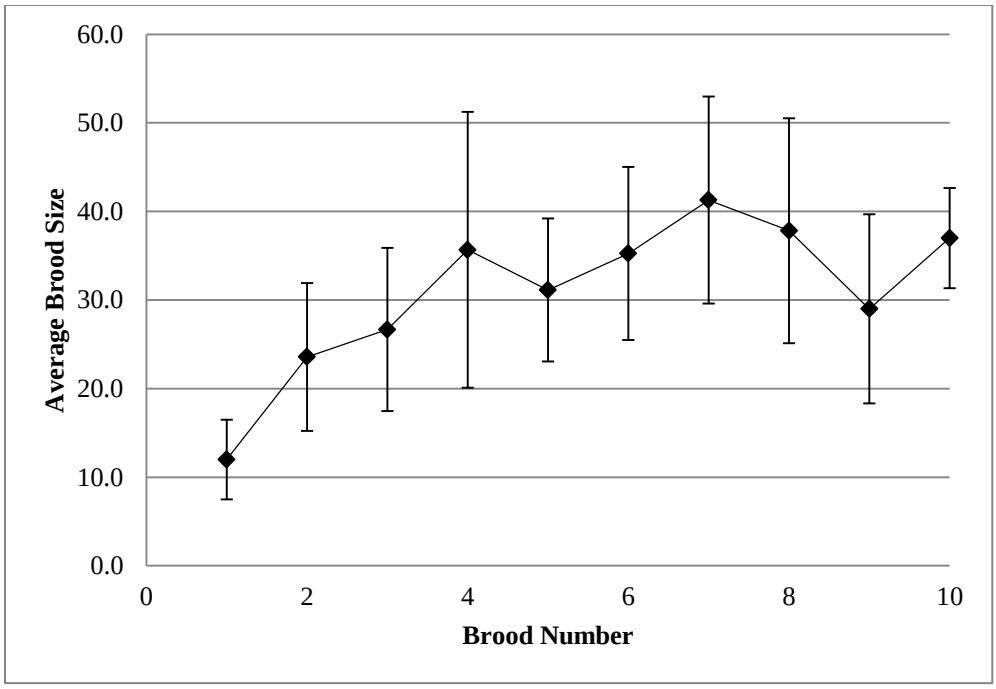

Figure 22 Average *Daphnia magna* brood size over time

Adema (1978) recommended the use of one day old *Daphnia magna* neonates for toxicity testing. One day old neonates are more sensitive than older *D. magna* and require less time to culture (Adema, 1978). Methods to obtain <24 hour old neonates by separating adults and their produced neonates daily using a screen or filter were first described by Nebeker and colleagues in 1984. This method was then subsequently used in studies such as Munawar *et al*. (1999). Food is added daily in the Nebeker *et al*. protocol, and the culture water is changed three times weekly (Nebeker *et al*., 1984). Similar to the Nebeker *et al*. (1984) protocol is the Lazorchak *et al*. (2009) protocol in which culture water is changed four times weekly, as well as 12 hours before a test, to give <24 hour old neonates for toxicity testing. While these methods, with their multiple weekly or even daily water changes can seem labour intensive, once the researcher is familiar and fast with the technique, the culturing takes only approximately 30 minutes daily. Culturing using brood stocks ensures that all adult *D. magna* within one jar are the same age, as they are all from the same brood. Also, it is easy to keep track of the mother *D. magna*'s health and age. It is recommended that toxicity tests be set up with neonates from mothers aged 2 to 5 weeks (Environment Canada, 1990). Therefore, brood stocks over 5 weeks old can be automatically discarded.

Another common method of obtaining <24 hour old neonates is to simply transfer multiple gravid females into individual vessels containing food, and to collect the neonates 24 hours (or less) later. This basic methodology has been used by Lews and Horning (1991), Kilham *et al*. (1997) and Ren *et al*. (2007; 2009). Although this method is much less labour intensive compared to the screening method, it would be difficult to assess or keep track of the mother *D. magna*'s health and age if the adults are simply kept in a mixed age culture. If the adults are kept in vessels in which they all are the same age, then neonates would be produced every 2-3 days and separating the neonates from the adults would be necessary to keep age synchronized cultures. Thus, it would simply be easier to screen the *D. magna* to separate the ages and the step of sequestering gravid females would be eliminated.

Daphnia magna Culture Medium

Non-axenic cultures of *Pseudokirschneriella subcapitata* and *Chlorella fusca* were grown in a Modified Bristol's Medium for feeding of *Daphnia magna*. The algae were concentrated for easier storage and to minimize *D. magna* exposure to the metals contained in the modified Bristol's medium when feeding (D. Poirier, personal communication, 19 February 2013). Samples of concentrated algae were often removed and microscopically examined to confirm that there was no extreme contamination, such as by bacteria, fungi or filamentous algae, and to determine the concentration of the algae using a hemocytometer. All counted concentrated algae batches had a concentration of at least 10^6 cells/mL. If the algae showed bacterial, fungal or filamentous algae contamination, had a musty smell, or appeared olive or tan in colour, they were discarded. No attempt was made to keep the cultures axenic, as these algae were only used for *D. magna* feeding. Contamination by filamentous algae was observed when algae culturing first began. These batches were discarded. All batches contaminated by filamentous algae were inoculated with previous batches of concentrated algae. This practice was stopped and all subsequent batches were inoculated from sterile, axenic slant agar or sterile liquid cultures. One new batch of *C. fusca* and one of *P. subcapitata* was initiated approximately once per week and usually took 7 to 8 days to reach a uniform dark green colour; however, this depended on whether the media was inoculated with a loopful of algae from a slant or from a liquid culture. Inoculation from a slant usually took 1 to 2 days longer to reach the desired concentration.

3.1.3 *Hyalella azteca*

Hyalella azteca was cultured using brood stocks according to methods by Borgmann *et al*. (1989), Borgmann and Norwood (2009, unpublished) and Environment Canada (1997) and was successful. There was very little maintenance aside from feeding 2–3 times weekly, and once weekly screening and separation of adults and juveniles. Weekly maintenance took approximately 1-2 hours to screen, count, replace and feed the *H. azteca*. Culturing by brood stocks produced 100-400 juveniles weekly for toxicity testing provided all animals were healthy. Only a small proportion of juveniles were used in toxicity testing; the remaining animals were placed in one of the mass culture *H. azteca* tanks.

Culturing of *Hyalella azteca* using a cotton substrate was first described by Borgmann *et al*. (1989). Borgmann *et al*. (1989) used pre-soaked sterile cotton gauze bandage. Culturing was first attempted using artificial plastic plants as substrate (Borgmann *et al*., 1989). Cotton gauze was found to improve reproduction 5-fold (Borgmann *et al*., 1989). Two types of cotton gauze were tried: a surgical bandage type and a cotton cheesecloth. In a mass culture setting in our laboratory, *H. azteca* preferred the cotton cheesecloth to squares of surgical bandage type cotton gauze, with many more *H. azteca* found clinging to the cheesecloth than the bandage. Borgmann and Norwood (2009) as well as personal communication with Dr. W. Norwood (9 July 2012) indicated that after several weeks the gauze should show periphyton growth, indicated by a light brown or green colouring, and then break down. The surgical gauze turned black, possibly indicating fungal growth, and did not break down under mass culture conditions. It is possible that the gauze was treated with an antimicrobial compound and therefore did not support a microbial community and was not degraded or consumed by the *H. azteca*. The cheesecloth did support periphyton growth and break down after several weeks. Therefore, the cheesecloth was chosen as the preferred substrate for culturing.

As stated in Borgmann and Norwood (2009) and in personal communication with Dr. W. Norwood (9 July 2012) there will usually be a lag period of several weeks once a brood stock is set up before significant reproduction occurs. This lag period was observed in the laboratory when the vessels were first set up. However, the lag period was reduced by adding new breeding pairs from the mass culture tanks to brood stocks when the population decreased instead of

starting entirely new brood stocks. In this method, at least four brood stocks with 20 to 30 adult *H. azteca* were maintained at all times.

Weekly maintenance of the brood stocks was to separate, count and record the number of adult and juvenile *Hyalella azteca*, replace the culture water and gauze (if needed), and feed the animals. The ratio of juveniles produced: surviving adult *H. azteca* was calculated per jar, and is presented compiled in Table 3and over time in Figure 23. During the gap in data from November – March, *H. azteca* brood stocks were maintained but not counted due to time constraints.

Table 3 Mean *Hyalella azteca* juvenile produced: surviving adult ratio for each brood stock, A-D

Brood Stock	Mean Juvenile: Adult Ratio	N (weeks)
A	1.9 ± 1.2	25
B	1.8 ± 1.1	23
C	2.0 ± 0.9	23
D	1.7 ± 1.8	14

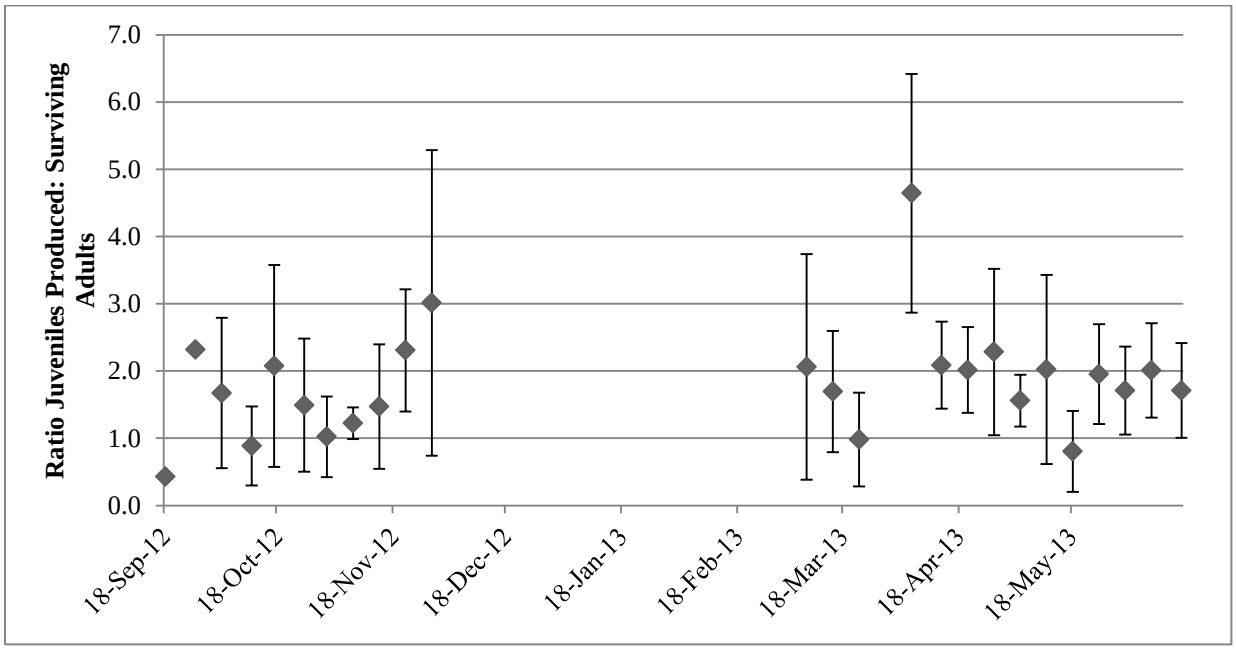

Figure 23 *Hyalella azteca* ratio juveniles produced: surviving adults in culturing brood stocks over time

All juveniles produced in a given week were combined in a 1 L vessel with cotton gauze and Tetramin food. Weekly maintenance was the same as for the brood stocks; however, the animals were not counted. Only precopulatory pairing, the presence of juveniles was noted. The time-to-first brood, or when juveniles were first produced, was at 7.2 ± 0.45 weeks old (N = 5). The first brood was not counted. After the first brood was produced the animals were placed in one of the two mass culture tanks.

3.1.4 *Lumbriculus variegatus*

Lumbriculus variegatus culturing procedures were based on OECD (2007) and MOE (2012b). One mass culture of organisms for toxicity testing was kept, as well as separate age-synchronized cultures that were used in attempted life cycle toxicity testing.

OECD (2007) suggests culturing using paper towel substrate. The OECD (2007) protocol states: "The substrate is prepared by cutting unbleached brown paper towels into strips, which may then be blended with culture water for a few seconds to result in small pieces of paper substrate." At first, multiple sizes of strips were tried in the culture tank. Strips of paper towel were cut into wide (approximately 2 cm) pieces and placed in the tank; these strips were too wide and laid flat on the tank and did not give the *Lumbriculus variegatus* a thick enough substrate to anchor their anterior ends in. As a result, they laid flat along the bottom of the tank and likely were not able to feed optimally. Blending the paper towel resulted in a glutinous mass that had too many small particulates for the *L. variegatus*, and they perished quickly. After discussion with D. Poirier (personal communication, 10 October 2012), it was found that the paper towel had to be cut into small strips, preferably with rough edges. This created a lofty mass of paper towel that was dense enough for the worms to anchor their anterior ends, but still allowed circulation of water and allowed the worms to move throughout the substrate. A paper shredder allowed for easy preparation of large volumes of substrate.

OECD (2007) and MOE (2012b) state that *Lumbriculus variegatus* cultures are expected to double every 10-14 or 11-12 days, respectively. Although not quantitatively measured, the cultures did not appear to grow as rapidly as suggested, perhaps due to water quality. The cultures were kept semi-static, with weekly water changes. OECD and MOE recommend flow-through systems, which could not be implemented in the lab. Overall, the *L. variegatus* cultures

appeared to be healthy, but the lack of reproduction could have meant the cultures' health was less than optimal.

3.1.5 *Lemna minor*

Lemna minor was cultured based on protocols by OECD (2006) and Environment Canada (2007), with modifications. Both methods recommend all cultures used for toxicity testing be axenic, and free from algal, bacterial or fungal contamination; however, this was not feasible under our laboratory conditions. Glassware, media, inoculating loops and other materials were not kept aseptic. Initially, attempts were made to culture the *L. minor* axenically with autoclave-sterilized glassware, media and flamed inoculating loops. Subcultures were started with bleach-sterilized fronds and cultures were covered with clean glass plates. However, all attempts always failed as green algae grew in the media after a few days. It was therefore decided that culturing and toxicity testing would continue with non-axenic cultures, with an emphasis on sub-culture into fresh growth medium when green algae was noticed in the cultures. Fungal or bacterial growth was only seen in cultures grown in Hoagland's E+ media and never seen in cultures grown in SIS media.

The culture of *L. minor* used for all toxicity tests was first tested for health under the same conditions used for the toxicity tests. The mean number of fronds in each vessel increased from 3 ± 0 fronds to 38.3 ± 11.0 fronds, or an increase of 12.8 times, thus satisfying the health criteria.

3.2 Bioassay Method Refinement

This study aims to build on existing knowledge regarding organism behaviour (see Introduction) and refine methods by Marshall (2009). *Daphnia magna*, *Hyalella azteca*, *Lumbriculus variegatus* behaviour and *Lemna minor* appearance were observed in varying concentrations of the positive toxicant 4-chlorophenol: (1) to aid in the refining of the behaviour/appearance bioassay criteria and scoring system through the use of an experimental control; (2) to establish a library of behaviour/appearance data; and (3) to further practice behaviour/appearance scoring by the researcher. *Daphnia magna* and *Lemna minor* life cycle tests were conducted: (1) to implement life cycle protocols in the laboratory; and (2) to observe the effect of the positive toxicant on development and reproduction.

3.2.1 Behaviour

Daphnia magna, *Hyalella azteca* and *Lumbriculus variegatus* behaviour was observed under both reference and stress conditions using a variety of positive toxicants (ethanol, sodium chloride, zinc, 4-chlorophenol) over a period of approximately one year. Ethograms, composed of all observed reference and stress behaviours, were created. The format of all ethograms was based on work by Kruschwitz (1972), with modifications.

Daphnia magna

All observed *Daphnia magna* behaviours under both reference and stress conditions were compiled into an ethogram, as seen in Figure 24.

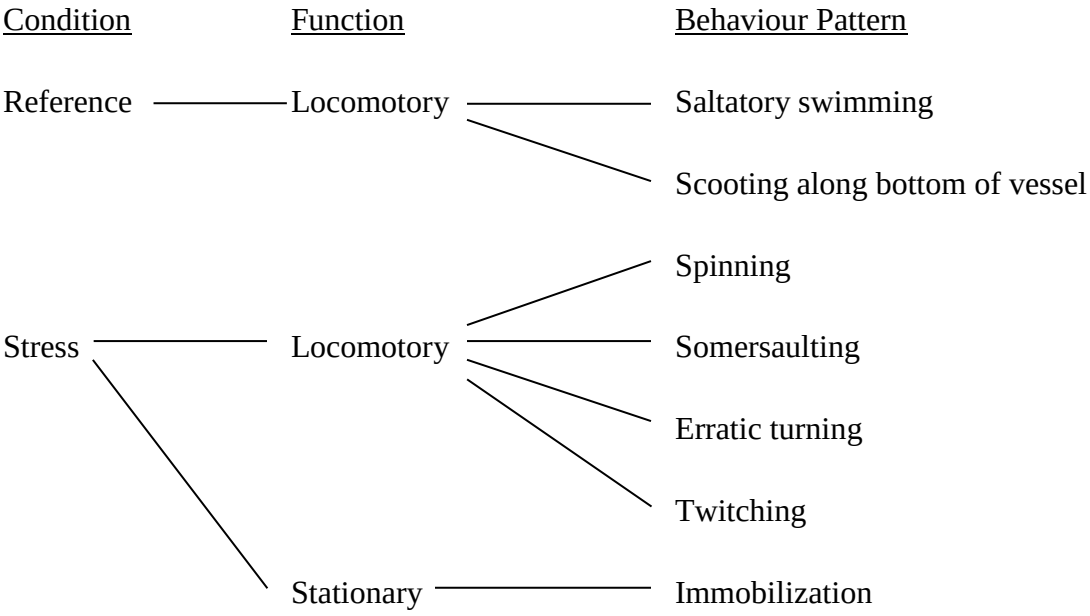

Figure 24 *Daphnia magna* ethogram

Building on the above ethogram, preliminary observations, work by Marshall (2009), and other *D. magna* behavioural studies in the literature (see Introduction), three behaviour categories were chosen: swimming style, movement through the water column, and mobilization. In the preliminary studies, these behaviours all showed a general dose-response relationship, with increasing concentrations of the positive toxicant leading to differing levels of each behaviour.

Table 4 summarizes *D. magna* reference and stress behaviours.

Table 4 *Daphnia magna* reference versus stress behaviour for three behaviour categories

Behaviour Category	Reference Conditions	Stress Conditions
1. Swimming style	• Short, saltatory yet fluid movements • Languid pace • Upright body	• Short, saltatory but jerky movements with rapid changes in direction • Rapid swimming speed compared to reference • Body position more horizontal than upright • Whole-body twitching • Somersaulting • Overall style appears erratic and frantic
2. Movement through the water column	• Change in swimming height • Animals move throughout water column	• No change in swimming height; remains in one portion of the water column, usually either the very bottom or very top
3. Mobilization	• All organisms mobile; constant swimming	• Immobilization on bottom of vessel, or caught in surface tension

D. magna behaviour was evaluated in a five concentration geometric dilution series of the positive toxicant 4-chlorophenol. Each concentration was evaluated with six replicates. Each replicate, or vessel, contained three animals. Behaviour was evaluated at three time points after initial exposure: 10 minutes, 1 hour, and 24 hours. Each vessel containing three organisms was observed for a period of 30 seconds. Swimming style, movement through the water column and mobilization were evaluated simultaneously during that 30 second period. The first behaviour evaluated, swimming style, was scored according to the following 3-rank regime:

Score 0 - majority of the animals' swimming style was that of the reference conditions; movements were saltatory, and the body was held upright.

Score 1 - majority of animals' swimming style was slightly erratic, with the swimming speed was faster than that of the reference; body held more horizontal than vertical

Score 2 - majority of animals' showed the traits characteristic to a score of 1, as well as looping, twirling or spinning behaviour; score of 2 was also assigned if the majority of animals were immobilized, either at the surface or on the bottom of the vessel, or dead.

Figures illustrating mean behaviour/appearance criteria are presented for each organism with error bars representing standard deviations. Asterisks show treatments that were statistically significantly different ($\alpha = 0.05$) from reference conditions. All statistical values are tabulated in Appendix VI.

Figure 25 illustrates the mean swimming score in the positive toxicant 4-chlorophenol over time.

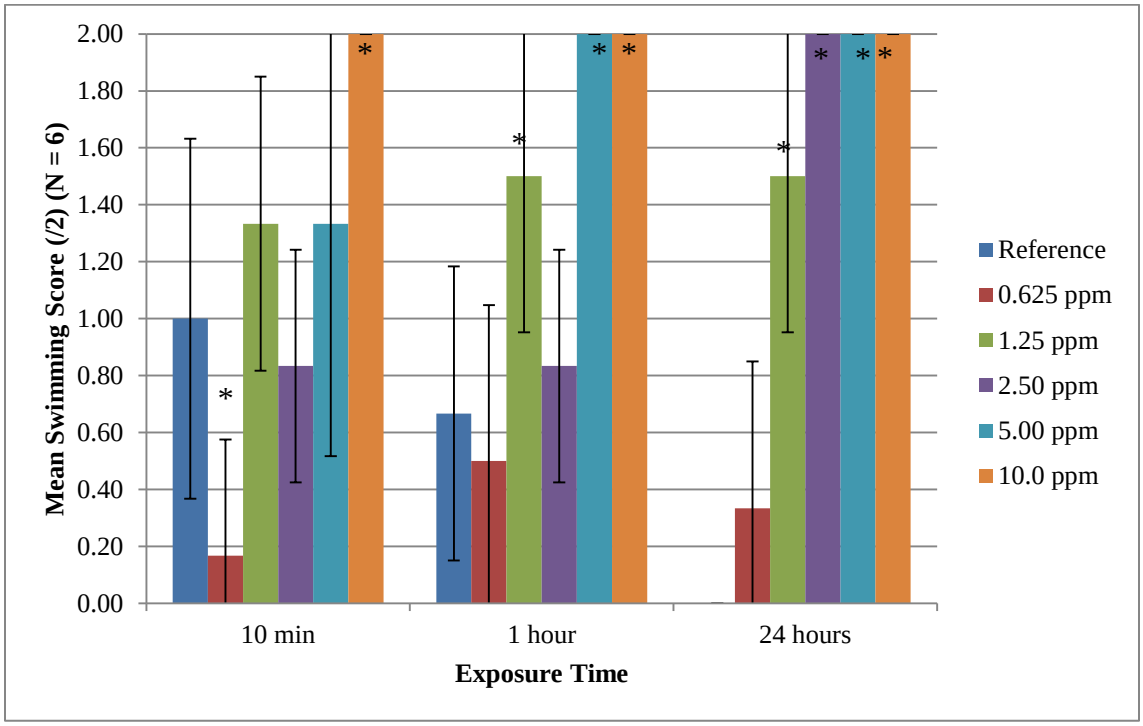

Figure 25 *Daphnia magna* mean swimming score in 4-chlorophenol over time

Ten minutes after *D. magna* exposure to 4-chlorophenol, organisms in the lowest concentration of 0.625 ppm showed a significantly lower swimming score compared to the reference ($\chi^2 = 4.96$, $p = 0.026$). Organisms in the highest concentration of 4-chlorophenol, 10.0 ppm, showed a significantly higher swimming score compared to the reference ($\chi^2 = 7.50$, $p = 0.006$). After 10 minutes of exposure it is likely that the organisms were still stressed from transfer into the vessel, and thus the reference mean swimming score of 1. After one hour of

exposure, organisms in 1.25 ppm ($\chi^2 = 4.69$, $p = 0.030$), 5.00 ppm ($\chi^2 = 9.90$, $p = 0.002$) and 10.0 ppm ($\chi^2 = 9.90$, $p = 0.002$) 4-chlorophenol showed significantly higher swimming scores when compared to the reference treatment; however, in the 5.00 and 10.0 ppm treatments an increase in swimming score was partially due to organism mortality, as shown in Figure 28. After one hour of exposure the organisms were likely more acclimated to the test vessels and recovered from the stress of transfer, and thus the decrease in the reference mean swimming score. After 24 hours, significant differences in the swimming score of organisms exposed to all 4-chlorophenol treatments but the lowest were seen when compared to the reference. Significantly higher swimming scores were seen in 1.25 ppm ($\chi^2 = 9.78$, $p = 0.002$), 2.50 ppm ($\chi^2 = 11.00$, $p = 0.001$), 5.00 ppm ($\chi^2 = 11.00$, $p = 0.001$) and 10.0 ppm ($\chi^2 = 11.00$, $p = 0.001$) 4-chlorophenol treatments when compared to the reference. In general, swimming score increased as the concentration of 4-chlorophenol increased, as well as over time in the 4-chlorophenol treatments, and decreased in the reference. In other words, *D. magna* swimming style became more stressed and erratic as 4-chlorophenol concentration increased as well as over time in the 4-chlorophenol treatments, and became less stressed and erratic in the reference.

The second behaviour evaluated was movement through the water column. This behaviour was evaluated by counting the number of times the animals crossed a threshold. On each vessel, the water column was divided evenly into three 2 cm sections with a small line of black marker. During the 30 second observation period, the total number of times the three *D. magna* completely crossed either of the thresholds was counted. Figure 26 illustrates mean number of boundary crossings in 4-chlorophenol over time.

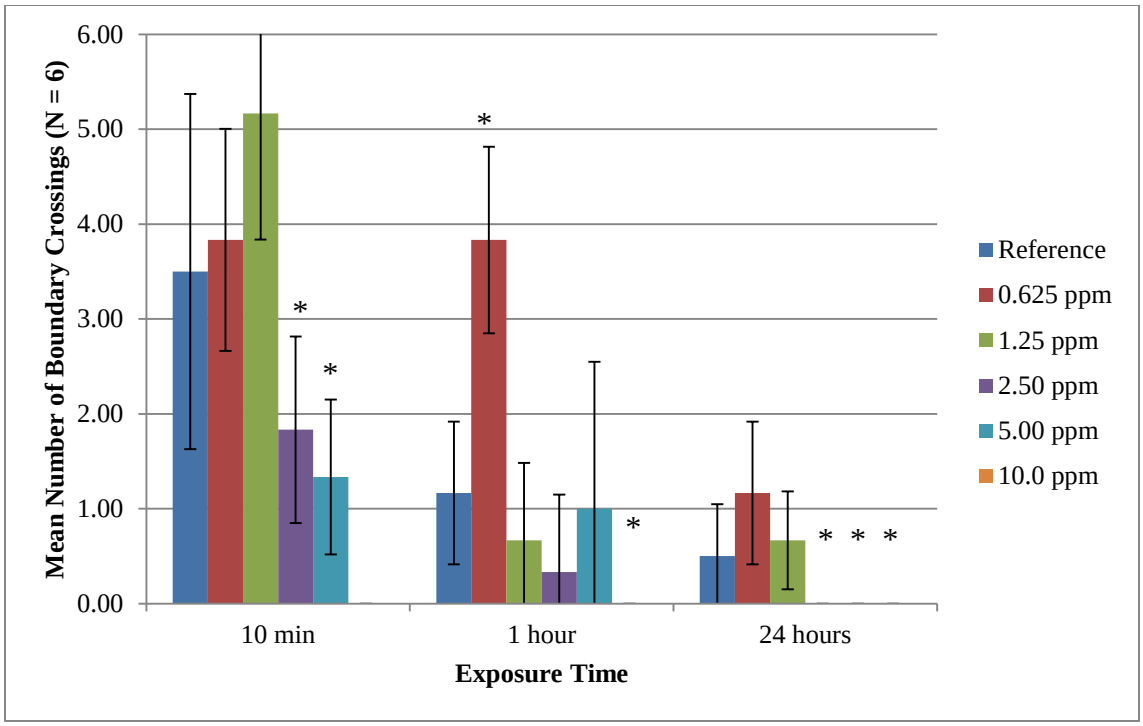

Figure 26 *Daphnia magna* mean number of threshold crossings in 4-chlorophenol over time

After ten minutes of exposure to 4-chlorophenol, organisms in the two highest concentrations showed significantly fewer boundary crossings compared to the reference. Organisms in 5.00 ppm and 10.0 ppm 4-chlorophenol showed reduced movement throughout the water column compared to the reference ($\chi^2 = 4.12$, $p = 0.042$ and $\chi^2 = 7.24$, $p = 0.007$, respectively). One hour after exposure, organisms in 0.625 ppm moved more throughout the water column compared to reference conditions ($\chi^2 = 4.69$, $p = 0.030$), while organisms in the highest 4-chlorophenol concentration of 10.0 ppm moved less ($\chi^2 = 9.90$, $p = 0.002$). After 24 hours, organisms in the three highest concentrations of 4-chlorophenol, or 2.50 ppm ($\chi^2 = 9.21$, $p = 0.002$), 5.00 ppm ($\chi^2 = 9.21$, $p = 0.002$) and 10.0 ppm ($\chi^2 = 10.3$, $p = 0.001$) showed significantly fewer boundary crossings when compared to the reference treatment; however, in the 5.00 and 10.0 ppm treatments no boundary crossings were observed partially due to organism mortality, as shown in Figure 28. In general, the number of boundary crossings decreased as 4-chlorophenol concentration increased, as well as over time in both the reference and 4-chlorophenol treatments. In other words, *D. magna* moved less throughout the water column as 4-chlorophenol concentration increased, as well as over time in all treatments.

The last behaviour evaluated was mobilization. The number of *D. magna* that were swimming using their secondary antennae and were not immobilized for ≥ 2 seconds were tallied. Immobilization was typically seen on the bottom of the vessel and when animals were caught in the surface tension of the water at the air-water interface. The secondary antennae were motionless; however, the thoracic appendages were still moving. Figure 27 illustrates mean percent mobilization in 4-chlorophenol over time.

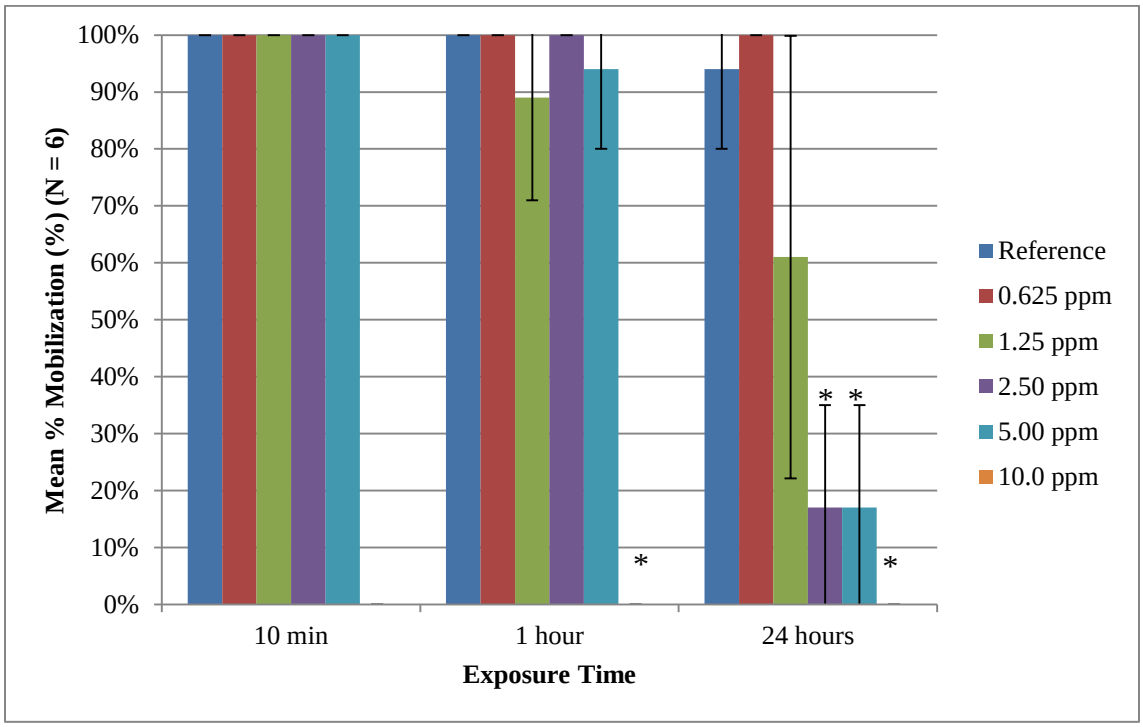

Figure 27 *Daphnia magna* mean percent mobilization in 4-chlorophenol over time

After ten minutes and one hour of exposure to 4-chlorophenol, organisms in the highest concentration showed no mobility, which was significantly lower than 100% mobility observed in the reference ($\chi^2 = 11.00$, $p = 0.001$). After 24 hours, organisms in the three highest concentrations of 4-chlorophenol, 2.50 ppm ($\chi^2 = 9.21$, $p = 0.002$), 5.00 ppm ($\chi^2 = 9.21$, $p = 0.002$) and 10.0 ppm ($\chi^2 = 10.29$, $p = 0.001$) showed significantly lower mobility when compared to the reference treatment; however, in the 5.00 and 10.0 ppm treatments a decrease in mobility was partially due to organism mortality, as shown in Figure 28. In general, mobility decreased as 4-chlorophenol concentration increased, as well as over time in both the reference and 4-chlorophenol treatments.

After 24 hours some mortality was seen. Mortality was confirmed under a dissecting microscope by ensuring all cessation of visible signs of movement or activity indicating life, including second antennae, abdominal legs, and heartbeat, as defined by Environment Canada (1990). After 24 hours of exposure the organisms in each vessel were classified as either mobile, immobile (no movement for >2 seconds) or dead. This proportion of mobile, immobile and dead organisms in each treatment is shown in Figure 28.

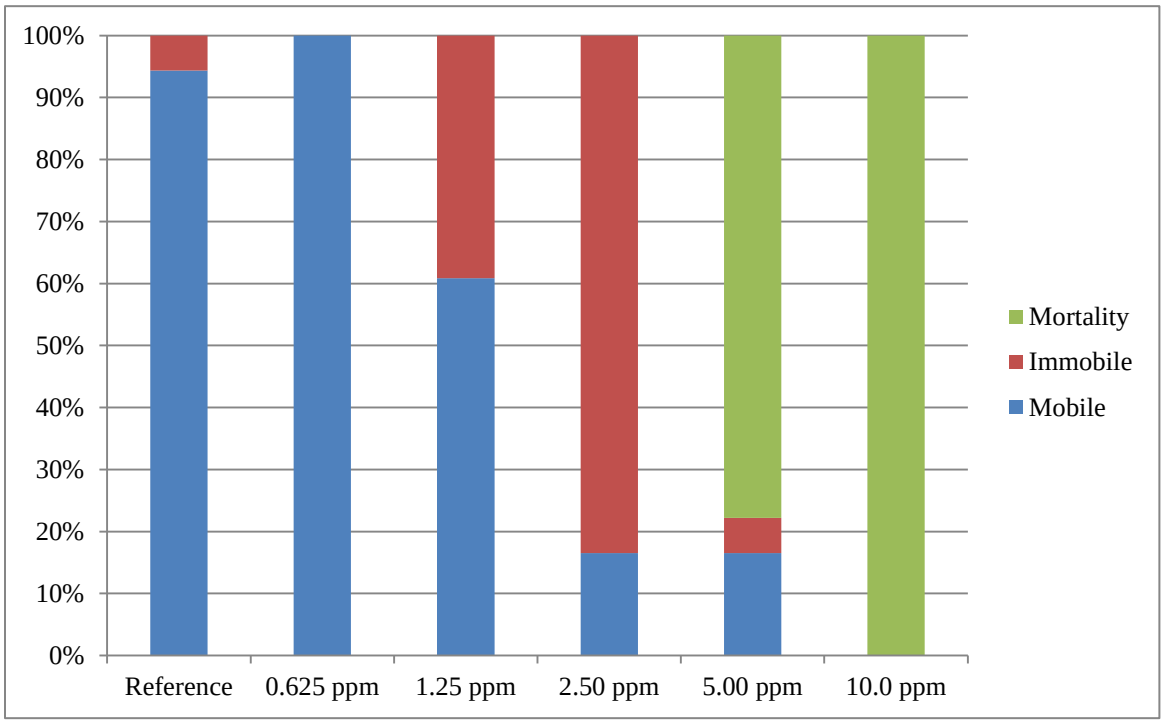

Figure 28 *Daphnia magna* mortality at 24 hours in 4-chlorophenol

No mortality was observed after ten minutes and 1 hour of *D. magna* exposure to 4-chlorophenol. After 24 hours, however, 78 ± 27% and 100% of organisms in 5.00 and 10.0 ppm of 4-chlorophenol, respectively, exhibited mortality, as shown in Figure 28. This mortality partly contributed to the increased assigned swimming score and the decrease in boundary crossings and mobilization of organisms.

Assessing *D. magna* behaviour was moderately successful. While the highest concentrations of the positive toxicant could be elucidated under blind conditions based on the difference in swimming behaviour and mobility compared to the reference, the relationship of boundary crossings appears to be skewed, with low 4-chlorophenol concentrations increasing *D. magna* swimming through the water column compared to the reference. The use of the positive

toxicant 4-chlorophenol allowed the scoring system to be tested and created a library of behaviour data that can be referenced by future ecotoxicologists undertaking *D. magna* behaviour work.

Hyalella azteca

All observed *Hyalella azteca* behaviours that were not reproduction-based (i.e. male grasping for precopulatory pairing) under both reference and stress conditions were compiled into an ethogram, as seen in Figure 29. The *H. azteca* ethogram is based on Kruschwitz (1972).

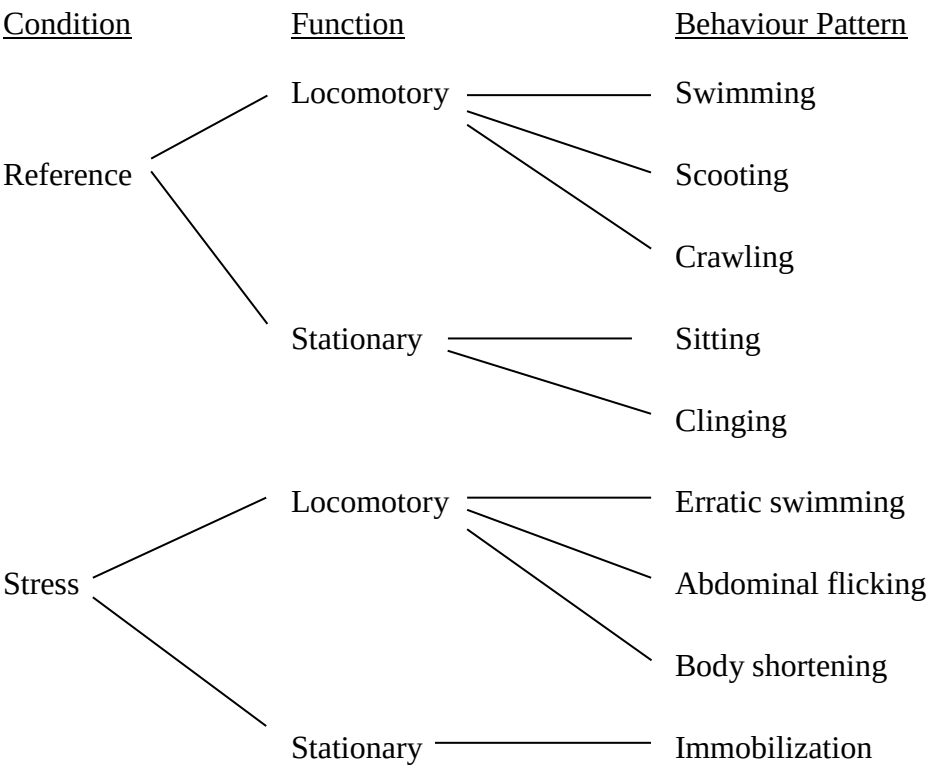

Figure 29 *Hyalella azteca* ethogram

Building on the above ethogram, preliminary observations, work by Marshall (2009), and other *H. azteca* behavioural studies in literature (see Introduction), three behaviour categories were chosen: swimming style, body length, and mobilization. In the preliminary studies, these behaviours all showed a general dose-response relationship, with increasing concentrations of the positive toxicant leading to differing levels of each behaviour. Table 5 summarizes *H. azteca* reference and stress behaviours.

Table 5 *Hyalella azteca* reference versus stress behaviour for three behaviour categories

Behaviour Category	Reference Conditions	Stress Conditions
1. Locomotion style	• Languid swimming with substrate crawling	• Erratic swimming with little substrate crawling and increased swimming events • Abdominal flicking
2. Body length	• Elongated body length	• Shortened body length; may form C-shape
3. Mobilization	• No immobilization > 10 s	• Immobilization > 10 s

In the same manner as the *D. magna* bioassay, H. *azteca* behaviour was evaluated in a 5 concentration geometric dilution series of the positive toxicant 4-chlorophenol with six replicates and three animals per replicate. Behaviour was evaluated for a 30 second interval at 10 minutes, 1 hour, and 24 hours after exposure. Swimming style, body length and mobilization were evaluated simultaneously. The first behaviour evaluated, swimming style, was scored according to the following 3-rank regime:

Score 0 - majority of the animals' swimming style was that of the reference conditions; swimming movements were languid and fluid and substrate crawling occurred

Score 1 - majority of animals' swimming style was slightly erratic and faster than reference swimming

Score 2 - majority of animals' showed the traits characteristic to a score of 1, as well as abdominal and/or whole body twitching; score of 2 was also assigned if the majority of animals were immobilized, either at the surface or on the bottom of the vessel.

Figure 30 illustrates mean locomotion score in the positive toxicant 4-chlorophenol over time.

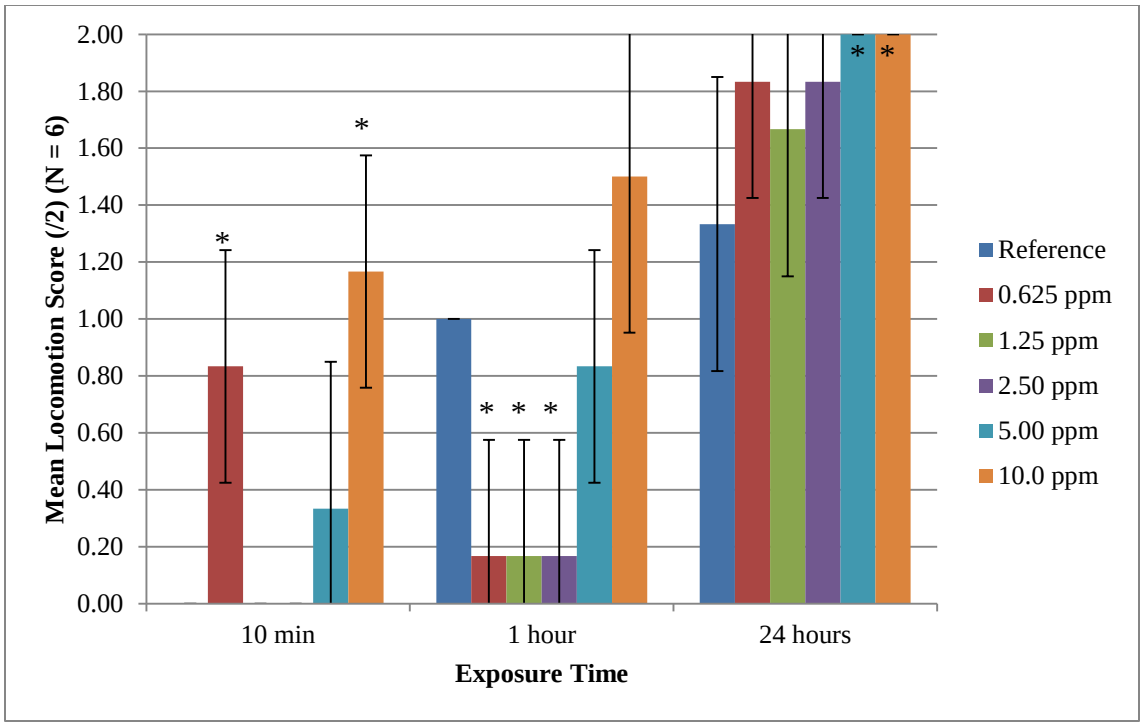

Figure 30 *Hyalella azteca* mean locomotion score (/2) in 4-chlorophenol over time

Ten minutes after *Hyalella azteca* exposure to 4-chlorophenol, organisms in 0.625 ppm (χ^2 = 7.86, p = 0.005) and 10.0 ppm (χ^2 = 10.29, p = 0.001) treatments showed significantly higher locomotion scores compared to the reference. The reference, 1.25 and 2.50 ppm 4-chlorophenol treatments showed locomotion scores of 0, indicating reference locomotion style. After one hour of exposure, organisms in 0.625 ppm (χ^2 = 7.86, p = 0.005), 1.25 ppm (χ^2 = 7.86, p = 0.005), and 2.50 ppm (χ^2 = 7.86, p = 0.005) treatments showed significantly lower locomotion scores compared to the reference. After 1 hour of exposure nearly all of the locomotion scores increased, indicating stress. It is possible that the organisms were spooked or somehow stressed between the 10 minute and 1 hour time points. After 24 hours of exposure, only organisms in the two highest concentrations of 4-chlorophenol, 5.00 (χ^2 = 5.50, p = 0.019) and 10.0 ppm (χ^2 = 5.50, p = 0.019) showed locomotion scores that were significantly different from the reference. In general, locomotion scores increased over time in the 4-chlorophenol treatments as well as the reference, indicating increasing stress for all vessels. All *H. azteca* showed increased stress behaviour over time, indicating possible external stress on all vessels.

Also, at 24 hours *H. azteca* mortality was seen in all treatments, including the reference (Figure 33), which increased the locomotion score of all treatments.

The second behaviour category evaluated was body length. Body length was evaluated qualitatively and was scored according to the following 3-rank regime:

Score 0 – majority of animals show no change in body length compared to that of the reference conditions; body is elongated and smooth

Score 1 – majority of animals exhibit some body shortening or curling

Score 2 – all animals exhibit body shortening or culturing; some or all animals' bodies form a C-shape

Figure 31 illustrates mean locomotion score in the positive toxicant 4-chlorophenol over time.

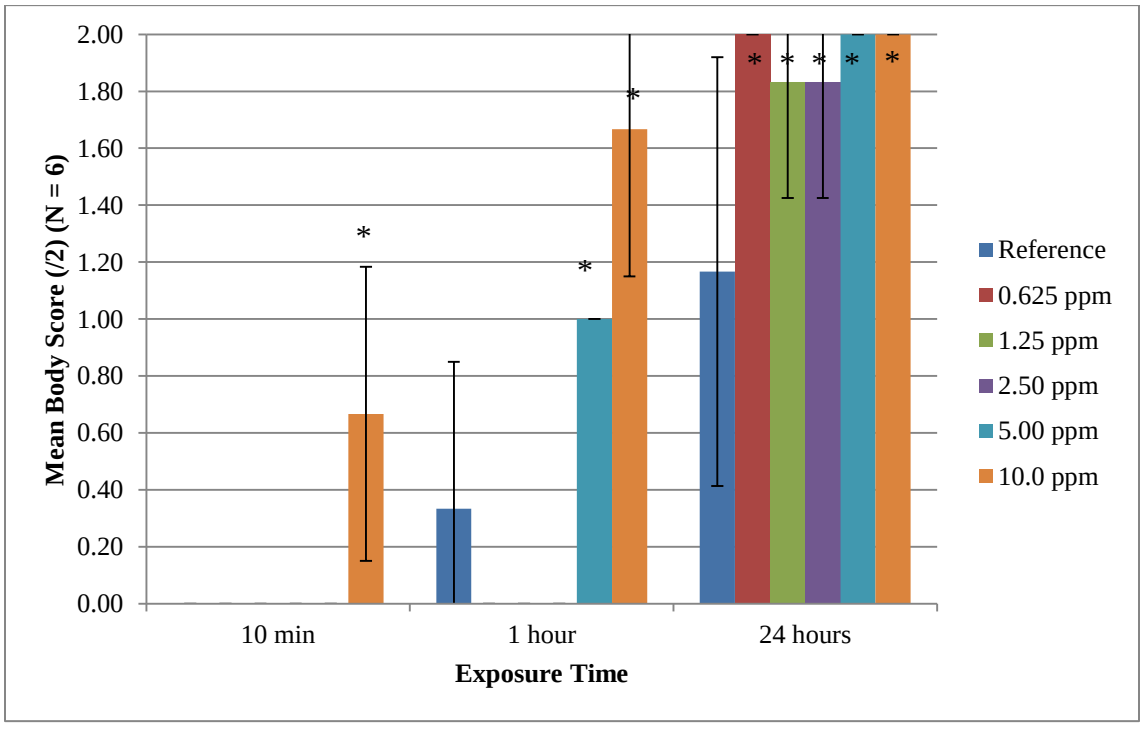

Figure 31 *Hyalella azteca* mean body score (/2) in 4-chlorophenol over time

After 10 minutes of exposure to 4-chlorophenol, only organisms in the highest concentration of 10.0 ppm showed a significantly higher body score compared to the reference ($\chi^2 = 5.50$, $p = 0.019$). After one hour of exposure, the two highest concentrations of 5.00 ppm ($\chi^2 = 5.50$, $p = 0.019$) and 10.0 ppm ($\chi^2 = 7.33$, $p = 0.007$) showed significantly higher body scores compared to the reference. After 24 hours, organisms in 0.625 ppm 4-chlorophenol ($\chi^2 = 5.33$, $p = 0.021$), 5.00 ppm ($\chi^2 = 5.33$, $p = 0.021$), and 10.0 ppm ($\chi^2 = 5.33$, $p = 0.021$) showed significantly higher body scores compared to the reference. A higher body score corresponds to increased stress as indicated by a shortened or curled body. Similar to locomotion style score, body score increased over time in the 4-chlorophenol treatments as well as the reference, indicating increasing stress for all vessels. Also, at 24 hours *H. azteca* mortality was seen in all treatments, including the reference (Figure 33), which increased the body score of all treatments as dead animals had shortened bodies. In general, body score increased over time in the 4-chlorophenol treatments as well as the reference, indicating increasing stress for all vessels over time.

The last behaviour evaluated was mobilization. The number of *Hyalella azteca* that were mobile either in swimming or substrate crawling behaviours and were not immobilized for ≥ 10 seconds were tallied. Immobilization was typically seen on the bottom of the vessel and when animals were caught in the surface tension of the water. The immobilized animal was usually in a shortened body position.

Figure 32 illustrates mean percent mobilization in 4-chlorophenol over time.

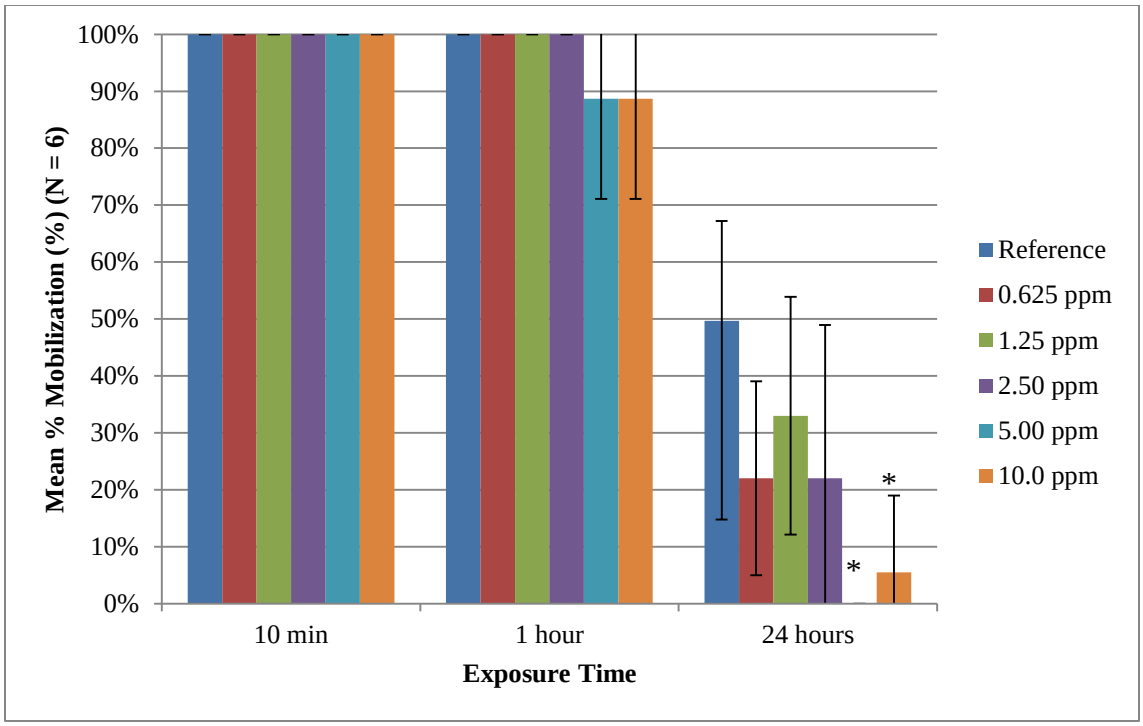

Figure 32 *Hyalella azteca* mean percent mobilization in 4-chlorophenol over time

After 10 minutes and 1 hour of *H. azteca* exposure to 4-chlorophenol no treatments showed significantly different % mobility compared to the reference. After 24 hours of exposure, organisms in the two highest concentrations of 4-chlorophenol, 5.00 ppm ($\chi^2 = 7.24$, $p = 0.007$) and 10.0 ppm ($\chi^2 = 5.43$, $p = 0.020$) showed significantly lower mobility compared to the reference. Again, loss of mobilization was partly due to death in all treatments, as shown in Figure 33. In general, mobility decreased over time in the 4-chlorophenol treatments as well as the reference, indicating increasing stress for all vessels over time.

In addition to behaviour, any mortality was noted and confirmed under a dissecting microscope by ensuring all cessation of visible signs of movement or activity, as defined by Environment Canada (1997). After 24 hours of exposure the organisms in each vessel were classified as either mobile, immobile (no movement for >10 seconds) or dead. This proportion of mobile, immobile and dead organisms in each treatment is shown in Figure 33.

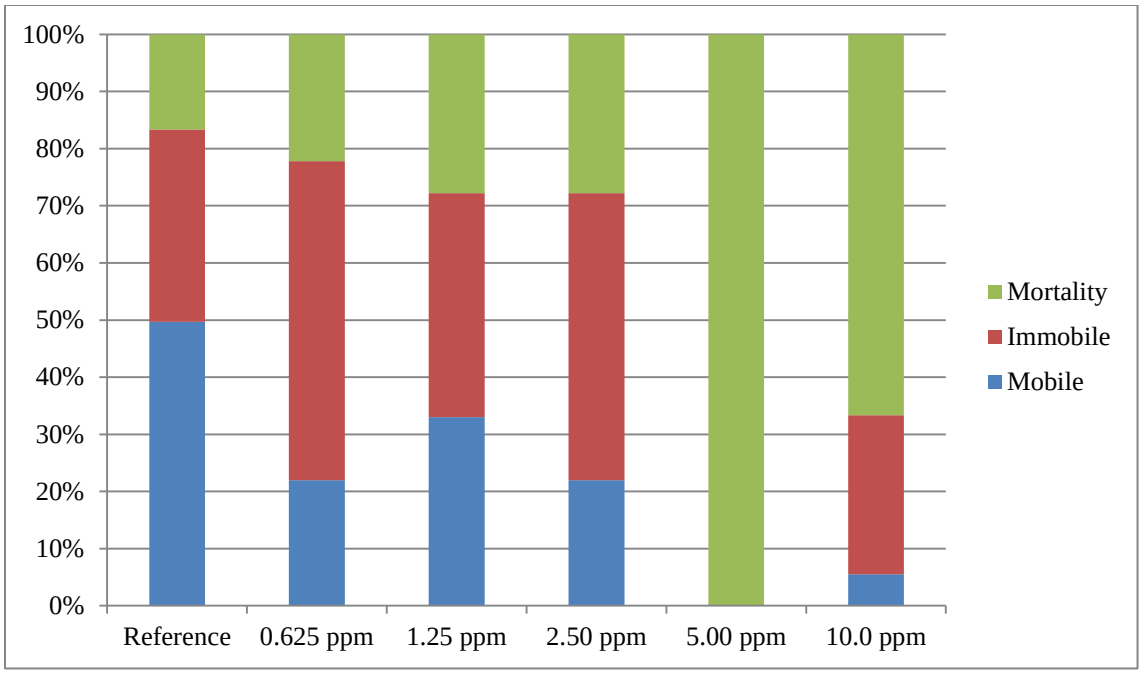

Figure 33 *Hyalella azteca* mortality at 24 hours in 4-chlorophenol

No mortality was observed after ten minutes and 1 hour of *H. azteca* exposure to 4-chlorophenol. After 24 hours however, some proportion of animals in each treatment exhibited mortality, as shown in Figure 33. This mortality partly contributed to the increased assigned locomotion and body score and the decrease mobilization of organisms.

Assessing *H. azteca* behaviour using a scoring systems was moderately successful. While organisms in the two highest 4-chlorophenol concentrations did show increasing locomotion and body scores over time, and these scores were statistically significantly higher than reference conditions, the high reference scores do call into question the test conditions and whether an external stress was forced upon the animals. The use of the positive toxicant 4-chlorophenol allowed the scoring system to be tested and created a partial library of behaviour data that can be referenced by future ecotoxicologists undertaking *Hyalella azteca* behaviour work.

Lumbriculus variegatus

All observed *Lumbriculus variegatus* behaviours under both reference and stress conditions were compiled into an ethogram, as seen in Figure 34.

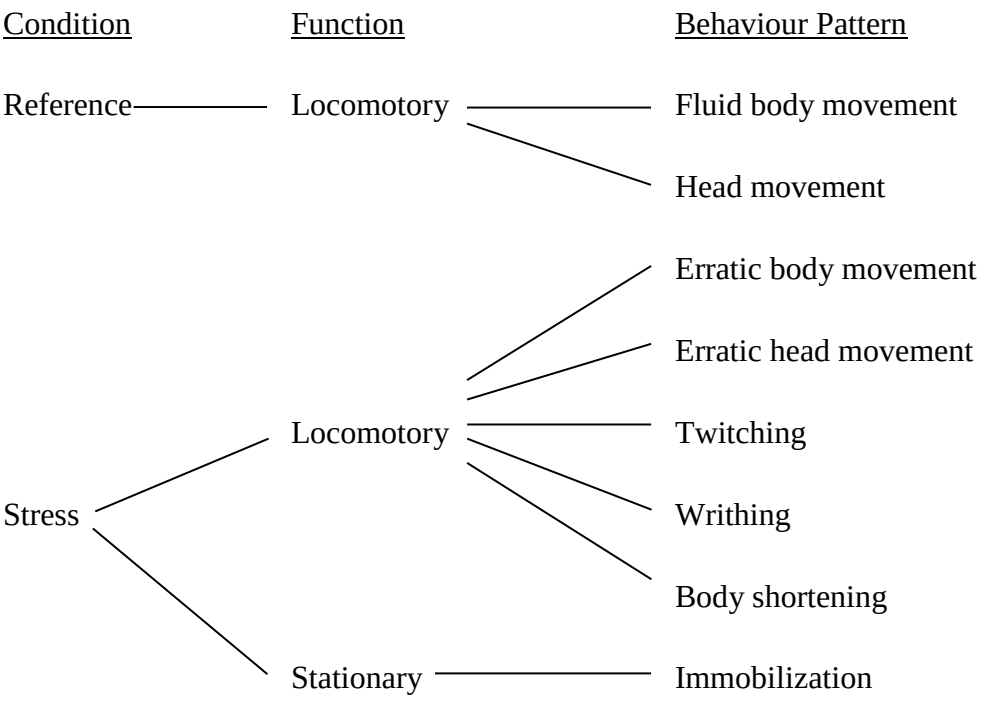

Figure 34 *Lumbriculus variegatus* ethogram

Building on the above ethogram, preliminary observations, work by Marshall (2009), and other *L. variegatus* behavioural studies in literature (see Introduction) three behaviour categories were chosen: locomotion style, body length, and mobilization. In the preliminary studies, these behaviours all showed a general dose-response relationship, with increasing concentrations of the positive toxicant leading to differing levels of each behaviour. Table 6 summarizes *L. variegatus* reference and stress behaviours.

Table 6 *Lumbriculus variegatus* reference versus stress behaviour for three behaviour categories

Behaviour	Reference Conditions	Stress Conditions
1. Locomotion style	• Fluid body movement; constant slow head movement • No twitching and/or writhing	• Erratic body and/or head movement • Sudden twitching movements and/or writhing of body
2. Body length	• Elongated body	• Shortening of body length • Curling, coiling, in C-shape
3. Mobilization	• No immobilization • Constant slow head movement	• Immobilization; no movement

In the same manner as the *D. magna* and *H. azteca* bioassays, *L. variegatus* behaviour was evaluated in a 5 concentration geometric dilution series of the positive toxicant 4-chlorophenol with six replicates; however, only one animal was used per replicate. Behaviour was evaluated for a 30 second interval at 10 minutes, 1 hour, and 24 hours after exposure. Locomotion style, body length and mobilization were evaluated simultaneously. The first behaviour evaluated, locomotion style, was scored according to the following 3-rank regime:

Score 0 - majority of the animals' locomotion style was that of the reference conditions; movement was fluid along the bottom of the vessel, with no twitching or writhing

Score 1 - majority of animals' locomotion style was erratic compared to reference conditions; number of twitches per minute ≤ 5 and/or intermittent writhing with breaks of rest

Score 2 - majority of animals' locomotion style was erratic compared to reference conditions; number of twitches per minute ≥ 5 and/or constant writhing and agitation with no breaks of rest; score of 2 was also assigned if the majority of animals were immobilized, either at the surface or on the bottom of the vessel.

Figure 35 illustrates mean locomotion score in the positive toxicant 4-chlorophenol over time.

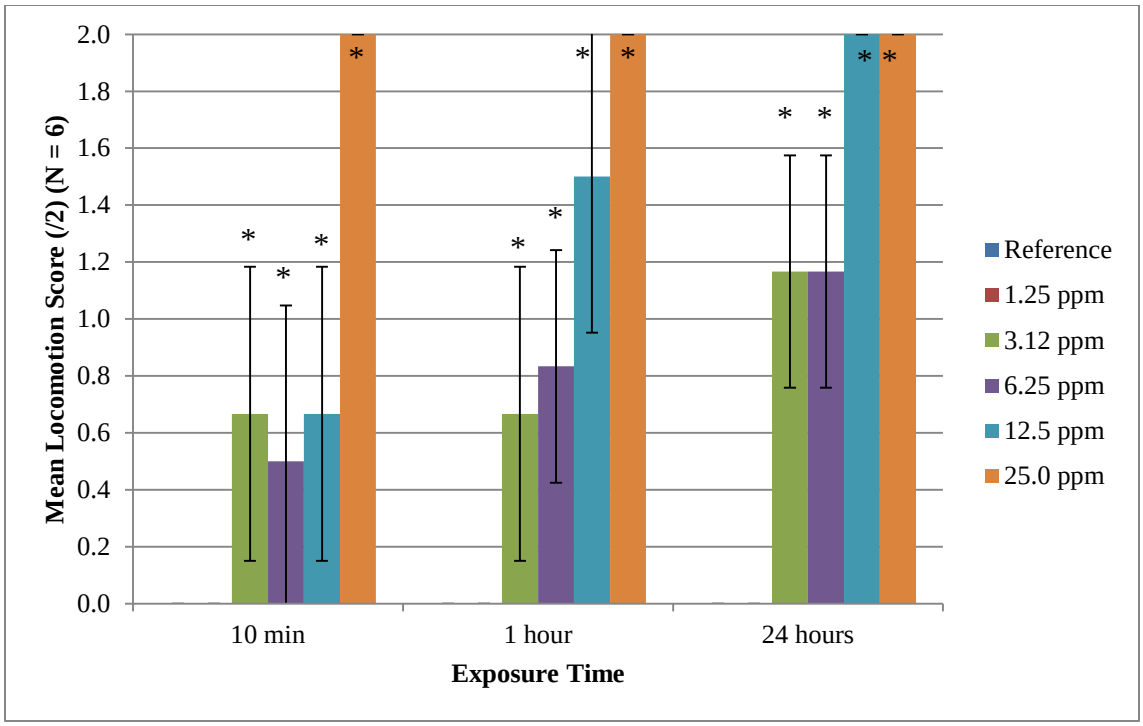

Figure 35 *Lumbriculus variegatus* mean locomotion score (/2) in 4-chlorophenol over time

Ten minutes after *L. variegatus* exposure to 4-chlorophenol, organisms in 3.12 ppm (χ^2 = 5.50, p = 0.019), 12.5 ppm (χ^2 = 5.50, p = 0.019) and 25.0 ppm (χ^2 = 11.0, p = 0.001) showed significantly higher locomotion scores compared to the reference. The reference and lowest 4-chlorophenol concentration, 1.25 ppm, showed locomotion scores of 0, indicating reference behaviour. After one hour of exposure, organisms in all 4-chlorophenol concentrations except the lowest showed significantly higher locomotion scores compared to the reference [3.12 ppm ((χ^2 = 5.5, p = 0.019), 6.25 ppm (χ^2 = 7.9, p = 0.005), 12.5 ppm (χ^2 = 9.8, p = 0.002) and 25.0 ppm (χ^2 = 11.0, p = 0.001)]. After 24 hours the same trend was shown; the locomotion scores in the four highest 4-chlorophenol concentrations were higher than at 1 hour [3.12 ppm ((χ^2 = 10.3, p = 0.001), 6.25 ppm (χ^2 = 10.3, p = 0.001), 12.5 ppm (χ^2 = 11.0, p = 0.001) and 25.0 ppm (χ^2 = 11.0, p = 0.001)]. In general, locomotion score, and therefore stress, increased as 4-chlorophenol concentration increased, as well as over time.

The second behaviour category evaluated was body length. Body length was evaluated qualitatively and was scored according to the following 3-rank regime:

Score 0 –animal shows no change in body length compared to that of the reference conditions; body is elongated and smooth with no bends, kinks or coiling

Score 1 – animal exhibits moderate kinking and/or coiling of the body

Score 2 – animal exhibits extreme kinking and/or coiling of the body and/or extreme body shortening (>50% original body length)

Figure 36 illustrates mean body scores in the positive toxicant 4-chlorophenol over time.

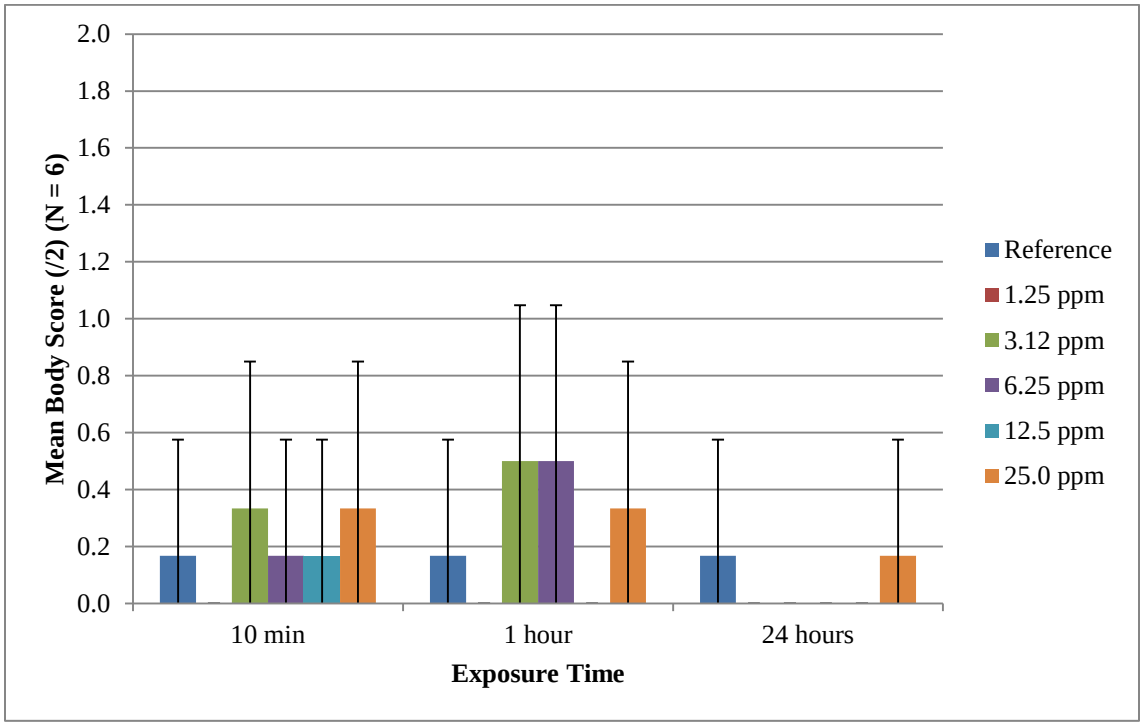

Figure 36 *Lumbriculus variegatus* mean body score (/2) in 4-chlorophenol over time

Ten minutes, 1 hour and 24 hours after exposure to 4-chlorophenol, no organisms showed significantly different body scores compared to the reference. No general biological trend between increasing 4-chlorophenol concentration and body score was observed.

The last behaviour evaluated was mobilization. *Lumbriculus variegatus* that were not immobilized for ≥ 5 seconds were tallied. Immobilized animals were usually in a coiled, body shortened position. Each vessel only contained one worm, so the mobility for each replicate was either 100% or 0%. Figure 37 illustrates mean % mobilization in 4-chlorophenol over time.

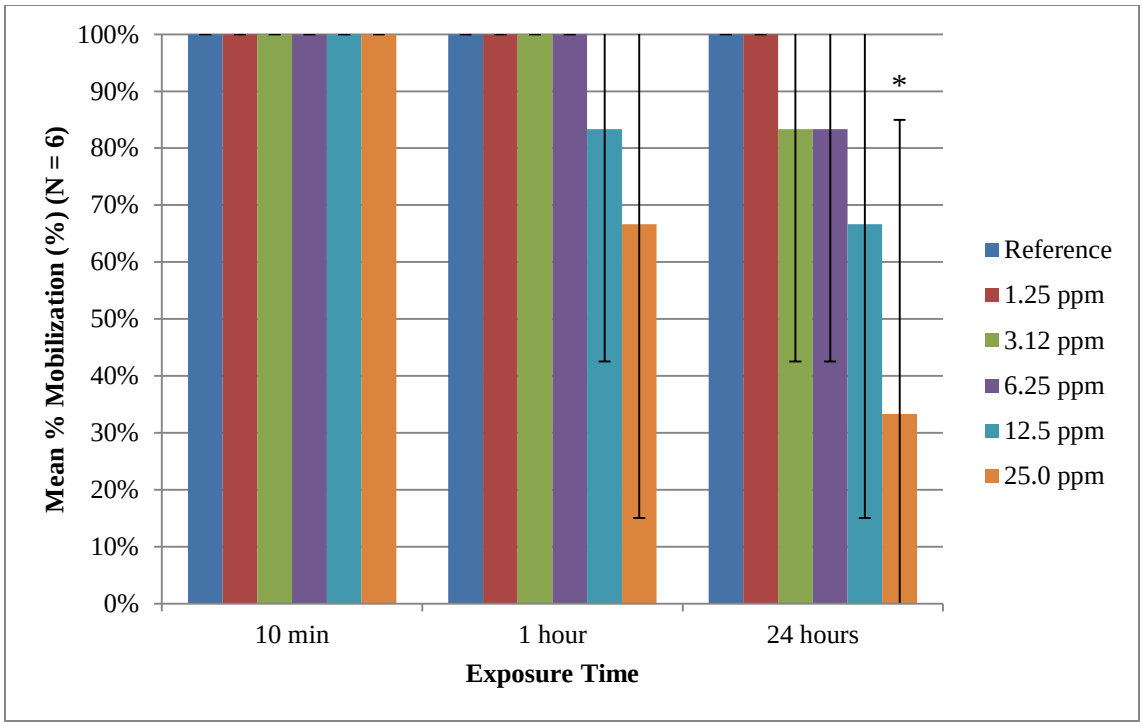

Figure 37 *Lumbriculus variegatus* mean percent mobilization in 4-chlorophenol over time

After ten minutes and one hour of exposure to 4-chlorophenol, all *Lumbriculus variegatus* showed mobility. After 24 hours of exposure, organisms in the highest concentration of 4-chlorophenol, 25.0 ppm, showed a significant decrease in mobility ($\chi^2 = 5.50$, $p = 0.019$). In general, *L. variegatus* mobility decreased as 4-chlorophenol concentration increased, as well as over time in the 4-chlorophenol treatments.

In addition to behaviour, any mortality was noted and confirmed under a dissecting microscope. Mortality was confirmed when the worm no longer showed blood movement through the dorsal vessel. No *Lumbriculus variegatus* mortality was seen in the 4-chlorophenol behaviour bioassay.

Assessing *L. variegatus* behaviour was moderately successful. Organisms in the highest 4-chlorophenol concentrations were easily distinguished from those in reference under blind conditions when assessing locomotion style and mobility, but not body length. Only a slight trend could be seen. Very high concentrations of the positive toxicant were needed to see body shortening or kinking.

Lemna minor

An ethogram was not created for *Lemna minor* because, as with most plants, it does not exhibit behaviours. Instead of behaviours, the appearance of the plant was evaluated under stress conditions. Building on preliminary observations, and *L. minor* studies in literature (see Introduction) three appearance categories were chosen: chlorosis, necrosis and colony break-up. In the preliminary studies, these appearances all showed a general dose-response relationship, with increasing concentrations of the positive toxicant leading to differing levels of each appearance. Table 7 summarizes *L. minor* reference and stress appearances.

Table 7 *Lemna minor* reference versus stress appearances for three appearance categories

Appearance Category	Reference Conditions	Stress Conditions
1. Chlorosis	• Bright green fronds	• Yellow-green or yellow fronds
2. Necrosis	• Bright green fronds	• White or brown areas on fronds
3. Colony break-up	• Colonies comprised of 3 to 5 fronds	• No colonies; fronds are separated and occur as individuals

In the same manner as the *D. magna* and *H. azteca* bioassays, *L. minor* appearance was evaluated in a 5 concentration geometric dilution series of the positive toxicant 4-chlorophenol. Photographs were taken from above of each vessel on days 0, 2 and 7. Each vessel was scored based on visual and photographic evidence in the three appearance categories on days 0, 2 and 7.

The first appearance evaluated, chlorosis, was scored according to the following 3-rank regime:

Score 0 - majority of fronds are bright green with no areas of yellowing

Score 1 - majority of fronds show areas of yellowing; chlorosis is minor

Score 2 - majority of fronds are yellow; major chlorosis

Figure 38 illustrates mean chlorosis score in the positive toxicant 4-chlorophenol over time.

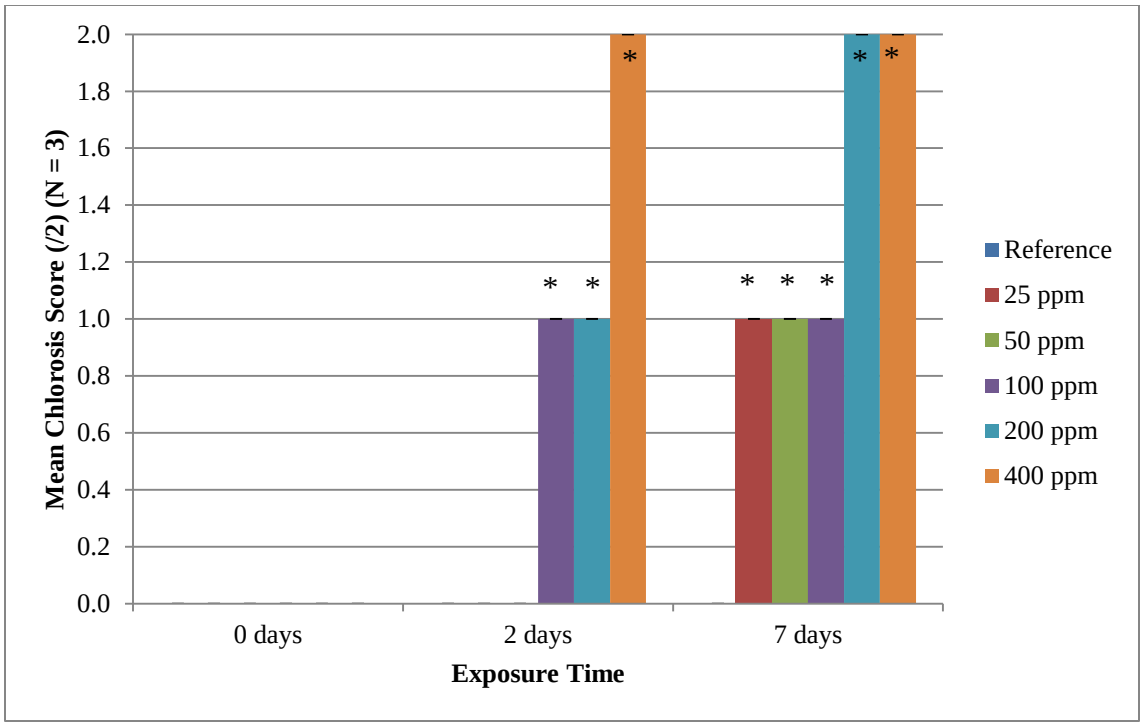

Figure 38 *Lemna minor* mean chlorosis score (/2) in 4-chlorophenol over time

All replicates started with two healthy, 3-frond colonies and thus all started with a chlorosis score of 0. After two days of exposure, fronds in the three highest concentrations of 100 ppm ($\chi^2 = 5.00$, $p = 0.025$), 200 ppm ($\chi^2 = 5.00$, $p = 0.025$) and 400 ppm ($\chi^2 = 5.00$, $p = 0.025$) showed significantly higher chlorosis scores compared to the reference. After 7 days of exposure, organisms in all concentrations of 4-chlorophenol showed elevated chlorosis scores compared to the reference (25 ppm ($\chi^2 = 5.00$, $p = 0.025$), 50 ppm ($\chi^2 = 5.00$, $p = 0.025$), 100 ppm ($\chi^2 = 4.00$, $p = 0.046$), 200 ppm ($\chi^2 = 5.00$, $p = 0.025$), 400 ppm ($\chi^2 = 5.00$, $p = 0.025$)). In general, chlorosis score increased as 4-chlorophenol concentration increased, as well as over time.

The second appearance category evaluated was necrosis and was scored according to the following 3-rank regime:

Score 0 – majority of fronds are bright green with no areas of white or brown

Score 1 – majority of fronds show small areas of white or brown tissue; necrosis is minor

Score 2 – majority of fronds show large areas of white or brown tissue; necrosis is major

Figure 39 illustrates mean necrosis score in the positive toxicant 4-chlorophenol over time.

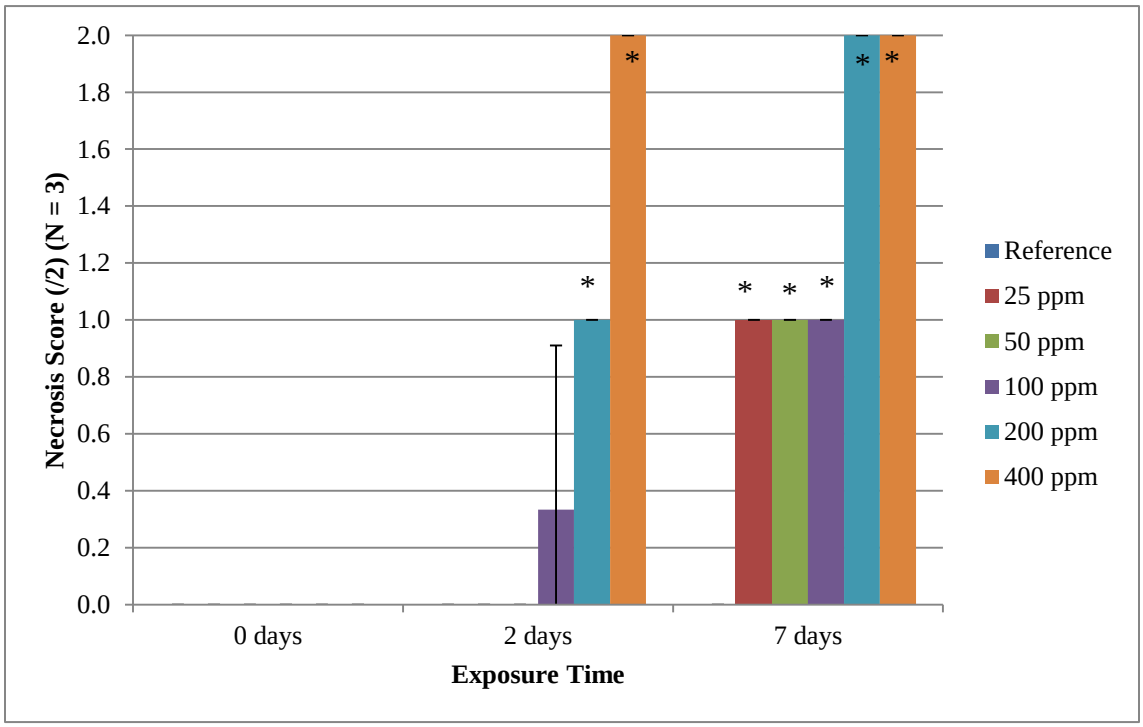

Figure 39 *Lemna minor* mean necrosis score (/2) in 4-chlorophenol over time

All replicates started with two healthy, 3-frond colonies and thus all started with a necrosis score of 0. After two days of exposure, fronds in the two highest concentrations of 200 ppm ($\chi^2 = 5.00$, $p = 0.025$) and 400 ppm ($\chi^2 = 5.00$, $p = 0.025$) showed significantly higher necrosis scores compared to the reference. After 7 days of exposure, organisms in all concentrations of 4-chlorophenol showed elevated necrosis scores compared to the reference (25 ppm ($\chi^2 = 5.00$, $p = 0.025$), 50 ppm ($\chi^2 = 5.00$, $p = 0.025$), 100 ppm ($\chi^2 = 4.00$, $p = 0.046$), 200 ppm ($\chi^2 = 5.00$, $p = 0.025$), 400 ppm ($\chi^2 = 5.00$, $p = 0.025$)). In general, necrosis scores increased as 4-chlorophenol concentrations increased, as well as over time.

The last appearance evaluated was colony break-up and was scored according to the following 3-rank regime:

Score 0 – all fronds are in colonies of 3+ fronds

Score 1 – some individual fronds, but most are in colonies of 3+ fronds

Score 2 – all fronds are individual; no colonies

Figure 40 illustrates mean colony break-up score in the positive toxicant 4-chlorophenol over time.

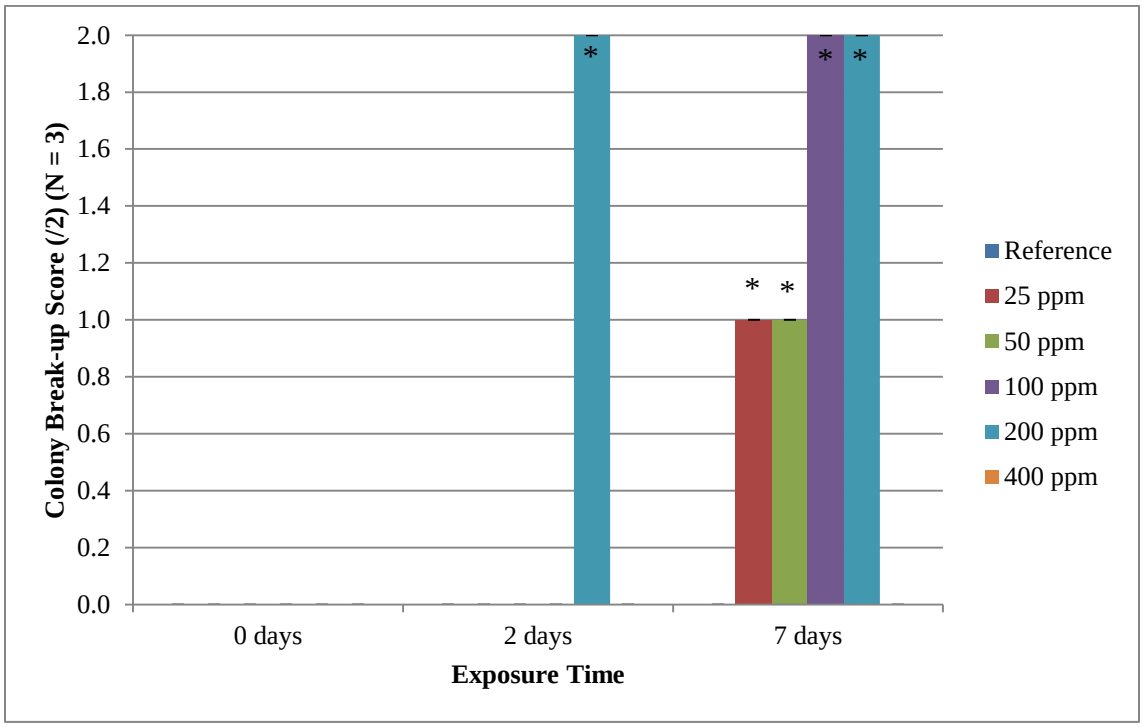

Figure 40 *Lemna minor* mean colony break-up score (/2) in 4-chlorophenol over time

All replicates started with two healthy, 3-frond colonies and thus all started with a colony break-up score of 0. After two days of exposure, fronds in 200 ppm ($\chi^2 = 5.00$, $p = 0.025$) showed a significantly higher colony break-up score compared to the reference. The highest 4-chlorophenol concentration, 400 ppm, caused such severe chlorosis and necrosis after 2 days of exposure that the fronds were dead and therefore the colonies did not separate. After 7 days of exposure, organisms in all concentrations of 4-chlorophenol except 400 ppm showed elevated colony break-up scores compared to the reference (25 ppm ($\chi^2 = 5.00$, $p = 0.025$), 50 ppm ($\chi^2 = 5.00$, $p = 0.025$), 100 ppm ($\chi^2 = 4.00$, $p = 0.046$), 200 ppm ($\chi^2 = 5.00$, $p = 0.025$)). In general, colony break-up scores increased as 4-chlorophenol concentrations increased, as well as over time.

Assessing *L. minor* appearance using a scoring system was successful. The highest concentrations of the positive toxicant could be easily distinguished from the reference.

3.2.2 Life Cycle

Positive toxicant life cycle bioassays were conducted with *Daphnia magna* and *Lemna minor*. *D. magna* were exposed to 4-chlorophenol from neonate to adult stage and ending after the first brood was produced. This was a partial life cycle test that involved only several sensitive life stages rather than the entire reproductive life cycle (i.e., from egg-to-egg) (Rand, 1995).

Hyalella azteca life cycle bioassays could not be conducted due to the lengthy nature of the species' reproductive cycle. Under our laboratory conditions the first brood was produced when *H. azteca* were 7.2 ± 0.45 weeks old (N = 5). This long time frame, combined with the need to gender-type the animals to ensure a proper male:female ratio in each vessel for mating to occur meant that *H. azteca* life cycle bioassays were too time-consuming for this study. *Lumbriculus variegatus* life cycle bioassays could not be conducted after attempts to induce reproduction under water-only conditions were unsuccessful. It is likely that *L. variegatus* require some substrate to induce reproduction. This study chose not to employ bioassays using substrate due to potential changes in contaminant bioavailability caused by partitioning to substrate. The exclusion of the two benthic species could suggest the elimination of the benthic route of chronic 4-chlorophenol exposure; however, as all the bioassays were conducted as water-only, sediment-ingestion and other routes of benthic exposure were at a minimum.

Daphnia magna

The *D. magna* partial life cycle bioassays was conducted with three concentrations of the positive toxicant 4-chlorophenol. A preliminary *D. magna* life cycle test (results not shown) showed no statistically significant impact to reproduction in 4-chlorophenol concentrations less than 0.625 ppm. A partial life cycle bioassay was set-up with 4-chlorophenol concentrations of 0.625, 1.25 and 2.50 ppm. After 14 days, all *D. magna* in the reference and two lowest concentrations of 4-chlorophenol had survived. Survival in the highest concentration of 4-chlorophenol, 2.50 ppm, was 87 ± 18% (N=5).

Three *D. magna* were in each vessel and together counted as a single replicate (N=1). Although all *D. magna* were from the same brood and were therefore genetically identical, the three animals did not release their brood at the same time. Therefore, to calculate the time-to-first brood, a weighted average was taken based on the number of neonates produced each day. This calculation is shown below:

Time-to-first brood = $\frac{(A\ x\ X)+(B\ x\ Y)+(C\ x\ Z)}{X+Y+Z}$

Where: A, B and C are the days on which the neonates were produced

X, Y, Z are the number of neonates produced on each day by the organisms in the vessel (three total), respectively

The mean time-to-first brood is shown in Figure 41.

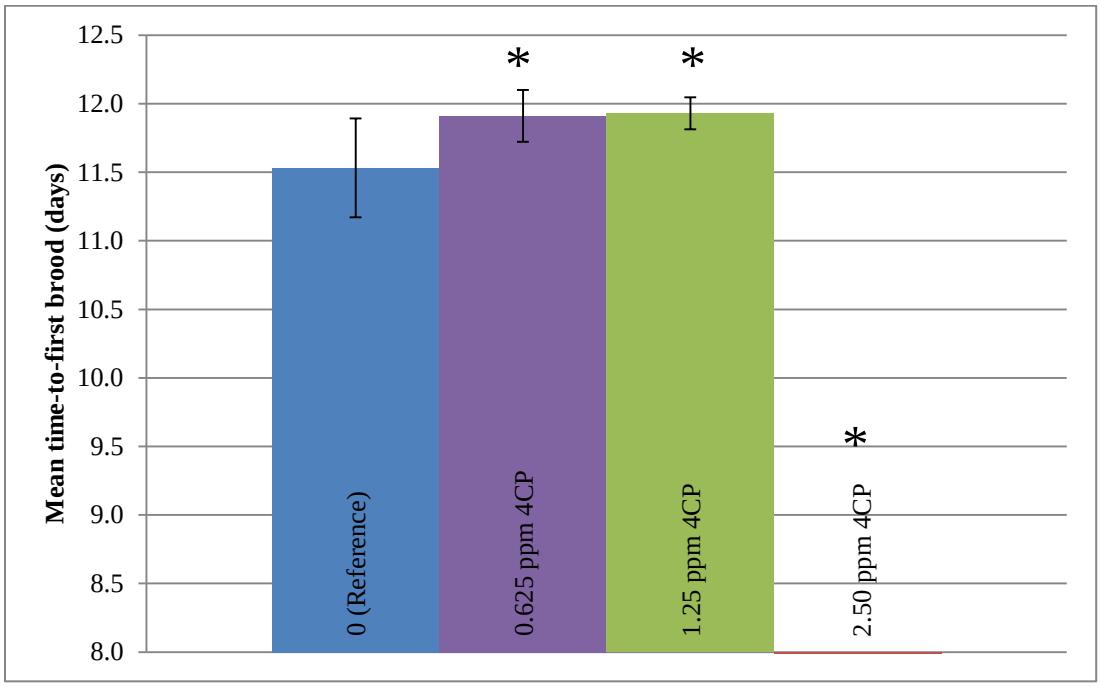

Figure 41 *Daphnia magna* mean time-to-first brood (days) for reference and 4-chlorophenol

The number of neonates present in each replicate were counted daily and divided by the total number of surviving adults in that replicate to achieve the mean brood size per surviving adult, shown in Figure 42.

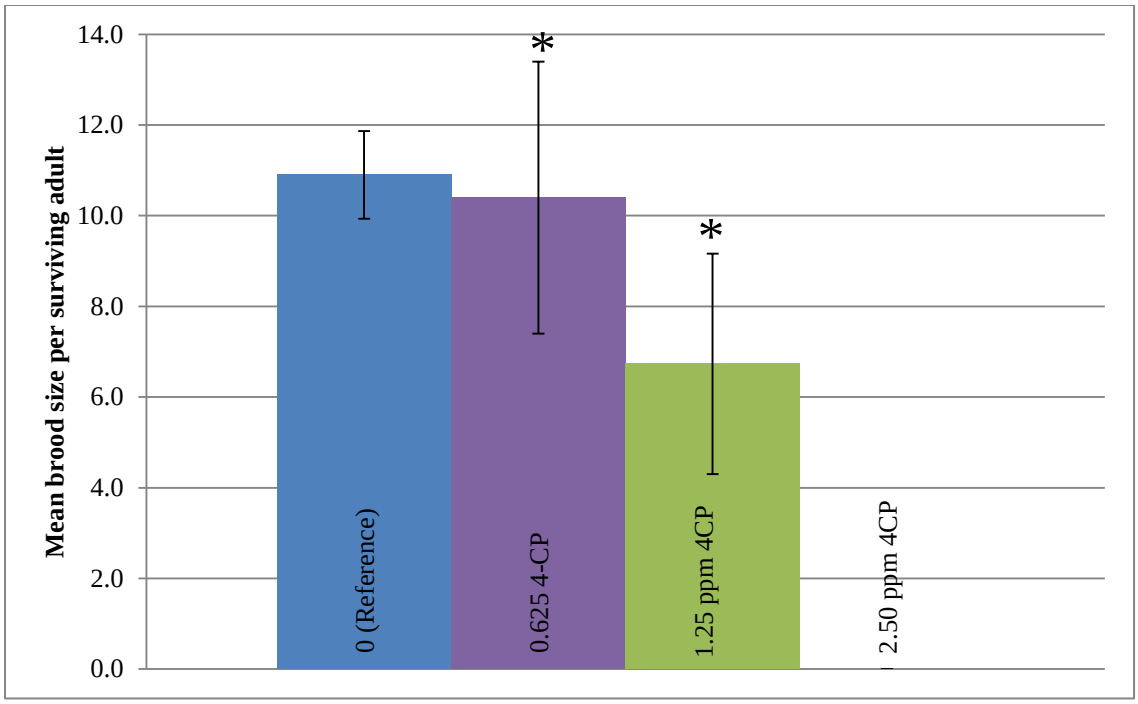

Figure 42 *Daphnia magna* mean first brood size per surviving adult for reference and 4-chlorophenol

Daphnia magna in the lowest 4-chlorophenol concentration of 0.625 ppm took longer to produce their first brood compared to the reference ($\chi^2 = 3.96$, $p = 0.047$) but the numbers of neonates produced were not significantly different ($\chi^2 = 0.49$, $p = 0.485$). *D. magna* in 1.25 ppm 4-chlorophenol took significantly longer to produce the first brood ($\chi^2 = 9.56$, $p = 0.002$) and produced significantly fewer neonates ($\chi^2 = 4.21$, $p = 0.040$). The highest concentration of 4-chlorophenol, 2.50 ppm, saw no neonates produced over the 14-day bioassay period. Animals in the highest concentration were observed to be smaller than those in reference conditions or lower concentrations of 4-chlorophenol; however, the *D. magna* appeared to develop normally, with eggs seen in the brood sacks. On day 13 of the test, a carapace was noticed in one of the 2.50 ppm vessels that had undeveloped eggs still in the brood sack. Closer inspection under a stereomicroscope confirmed the eggs had not developed. The highest concentration of 4-chlorophenol appeared to induce non-viable egg production, a phenomenon also described by other researchers (Vietoris, 2000 in Schmidt *et al.*, 2005).

Lemna minor

Lemna minor positive toxicant life cycle tests were conducted in SIS growth medium. Five concentrations of the positive toxicant 4-chlorophenol plus a control were prepared with SIS media. Growth was tracked by counting the number of individual fronds. Fronds were counted regardless of whether they were in a colony or not. As shown in Figure 43 and in Table 8, the lowest tested concentration of 4-chlorophenol, 25 ppm, resulted in 43% growth inhibition relative to the reference. Also shown in Figure 43 are the flattened growth curves of *L. minor* grown in 4-chlorophenol relative to the reference.

Table 8 *Lemna minor* growth rate and % growth inhibition after 7 days in 4-chlorophenol

4-chlorophenol Concentration (ppm)	Growth Rate	% Inhibition
0	0.47 ± 0.02	0 ± 3%
25.0	0.27 ± 0.03	44 ± 7%
50.0	0.15 ± 0.01	69 ± 2%
100	0.0 ± 0.00	100 ± 0%
200	0.01 ± 0.02	97 ± 5%
400	0.01 ± 0.02	97 ± 5%

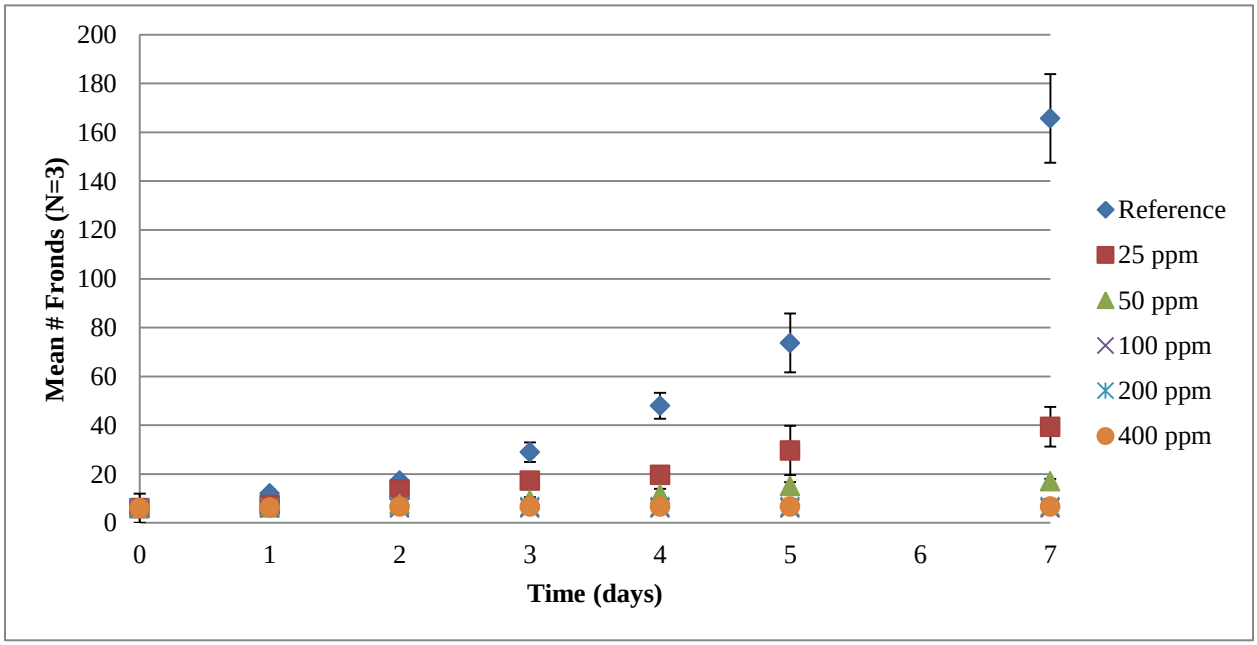

Figure 43 Growth of *Lemna minor* in 4-chlorophenol over time

After 7 days, nearly all 4-chlorophenol treatments showed significantly decreased growth, as measured in number of fronds, compared to reference conditions. 4-chlorophenol treatments of 25, 50, 200 and 400 ppm were conducted with N = 3 and showed statistically significant differences [(χ^2 = 3.86, p = 0.049), (χ^2 = 3.86, p = 0.049), (χ^2 = 3.97, p = 0.046), (χ^2 = 3.97, p = 0.046), respectively]. The 100 ppm 4-chlorophenol treatment was only conducted with N = 2 as a result of one vessel being knocked over. This decrease in N may have contributed to the non-significant difference in growth compared to the reference (χ^2 = 3.16, p = 0.076); however, the 100 ppm treatment follows the similar biological trend of decreased growth with increasing 4-chlorophenol concentration.

3.2.3 Summary: Behaviour and Reproduction

Behaviour and reproduction was observed in varying concentrations of the positive toxicant 4-chlorophenol with the goal of further refining behaviour toxicity methods and observing sub-lethal effects with reproductive endpoints. The purpose of using a positive toxicant in refining the bioassay methods was as an experimental control. 4-chlorophenol is a known toxicant to biota and is recommended by Environment Canada (1990) for use as a positive toxicant. *Daphnia magna*, *Hyalella azteca*, *Lumbriculus variegatus* behaviour and *Lemna minor* appearance was observed in increasing concentrations of 4-chlorophenol and scored according to the appropriate regime created after thorough observation of organism stress behaviour in a variety of positive toxicants. This created a library of behaviour/appearance data that can then be used by other scientists to compare their interpretation of the scoring system. For example, a researcher wanting to use the aforementioned methods to assess the toxicity of a compound would first run bioassays using 4-chlorophenol to learn what type of erratic behaviour constitutes a “1” or a “2” on the scoring system as well as to compare the data obtained with the library of data presented here. It is important that the described culturing methods also be implemented to ensure the use of organisms that pass the health criteria, as changes to biotic and abiotic factors may lead to differing results.

The use of 4-chlorophenol as a positive toxicant was moderately successful, with positive relationships seen between increasing 4-chlorophenol concentration and increasing stress behaviour observed. Some behaviours, for example *Hyalella azteca* body shortening, were not successful and further work should be conducted to replace this behaviour with another, more

easily observed one. The highest concentrations of 4-chlorophenol that would likely lead to death shortly after the 24 hour time frame were elucidated under blind conditions with relative ease, further demonstrating the use of this method as an early-warning system to assess sub-lethal and lethal toxicity. It was felt that the best behaviour endpoints were swimming/locomotion style and mobility of *Daphnia magna* and *Lumbriculus variegatus*. These endpoints were the easiest to observe and discern and provided the most significant results.

Daphnia magna and *Lemna minor* life cycle bioassays were conducted to further explore sub-lethal toxicity of the positive toxicant 4-chlorophenol. All three concentrations of 4-chlorophenol tested showed significantly delayed reproduction, and the two highest concentrations showed significantly fewer neonates produced in the first brood. *D. magna* in the lowest concentration of 4-chlorophenol showed no significant stress behaviour after 24 hours in 0.625 ppm 4-chlorophenol but did show delayed reproduction, indicating possible chronic effects of 4-chlorophenol to the development and reproductive systems. In both 1.25 and 2.50 ppm 4-chlorophenol, stress behaviours were seen after 24 hours and there were significant impacts to reproduction, with the first brood delayed and with fewer neonates compared to the reference. In 2.50 ppm 4-chlorophenol, aborted eggs were seen in moulted carapaces, a phenomenon described by other researchers (Vietoris, 2000 in Schmidt *et al.*, 2005). Delayed or decreased reproduction could be caused indirectly through physiological effects of 4-chlorophenol to the mother *D. magna* or directly by changes to egg development in the ovaries, causing abnormalities or miscarriages (Schmidt et al., 2005).

It should be noted that the *Daphnia magna* life cycle tests were conducted with approximately 5×10^4 algae cells/mL, which changed the bioavailability of 4-chlorophenol to the *D. magna* by an unknown factor. This change in bioavailability is demonstrated by the change in concentration that induced immobilization between the behaviour and life cycle bioassays. In the behaviour bioassay, 84% (N = 5) of neonate *D. magna* in 2.50 ppm 4-chlorophenol were immobilized after 24 hours; however, in the life cycle bioassay, all *D. magna* in the same concentration of 4-chlorophenol showed no immobility (during the period of observation) and 87% (N = 5) lived for the full 14 day test period. Nonetheless, if the 4-chlorophenol was adsorbed to the algae cells, it was still available to the *D. magna* through consumption of the algae.

3.3 Triclocarban

3.3.1 Behaviour

The behaviour of each species was observed in the antimicrobial triclocarban and scored according to the appropriate scoring regime, described in section 3.2.1. The methods were the same used to assess behaviour in the positive toxicant 4-chlorophenol. Figures illustrating mean behaviour/appearance criteria are presented for each organism with error bars representing standard deviations. Asterisks show treatments that were statistically significantly different ($\alpha = 0.05$) from reference conditions.

Daphnia magna

Daphnia magna behaviour was observed in four concentrations of TCC, a reference of aerated, dechlorinated MDW and a reference of the solvent carrier (0.001% DMSO) at three time points: 10 minutes, 1 hour and 24 hours after exposure. Three behaviours were assessed simultaneously: swimming style, movement through the water column and mobilization.

Swimming style was evaluated using a 3-rank regime with a score given to each replicate of between 0 and 2. Figure 44 illustrates mean swimming score in TCC over time.

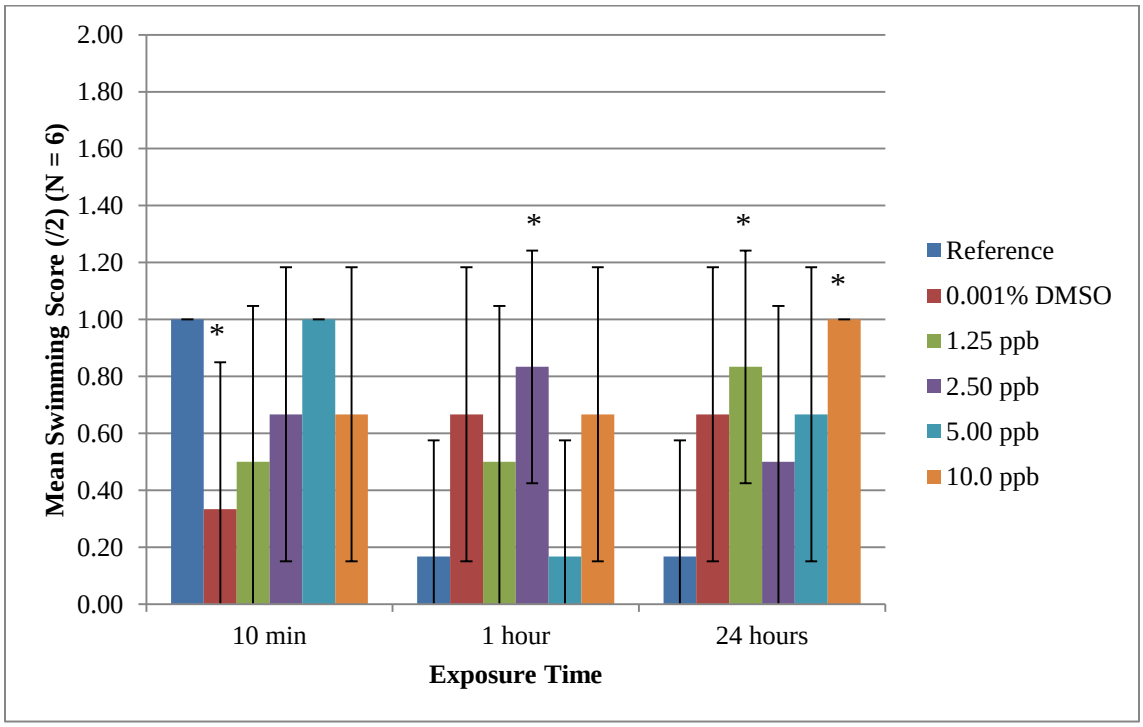

Figure 44 *Daphnia magna* mean swimming score in TCC over time

After 10 minutes of exposure to TCC, only *Daphnia magna* in the DMSO control had significantly different swimming score compared to the reference ($\chi^2 = 5.50$, $p = 0.019$); however, it should be noted that the reference score was 1.00 ± 0.00, indicating stress, likely leftover from transfer into the vessel. Due to this high level of stress seen in the reference, behaviour scores of the other treatments should not be treated with much certainty. After 1 hour of exposure, *D. magna* in 2.50 ppb TCC showed a significantly higher swimming score compared to the reference ($\chi^2 = 4.89$, $p = 0.027$). After 24 hours, *D. magna* in 1.25 ppb ($\chi^2 = 4.89$, $p = 0.027$) and 10.0 ppb TCC ($\chi^2 = 7.86$, $p = 0.005$) showed significantly higher swimming scores compared to the reference, indicating stress behaviour. The highest observed swimming score at 24 hours, 1.00 ± 0.00 in 10.0 ppb TCC, is within the range of the published 48-hour LC_{50} value of 10-20 ppb (TCC Consortium, 2002) and therefore may be an early-warning of lethality.

While only some TCC treatments showed swimming scores that were significantly higher than reference scores, the general positive trend of increasing score or stress behaviour with increasing TCC concentration, especially after 24 hours of exposure, may indicate sub-lethal toxicity of TCC to *D. magna*.

Movement through the water column was evaluated by counting the total number of times the three animals in the vessel crossed either of the two thresholds marked on the test vessel. Figure 45 illustrates mean number of boundary crossings in TCC over time.

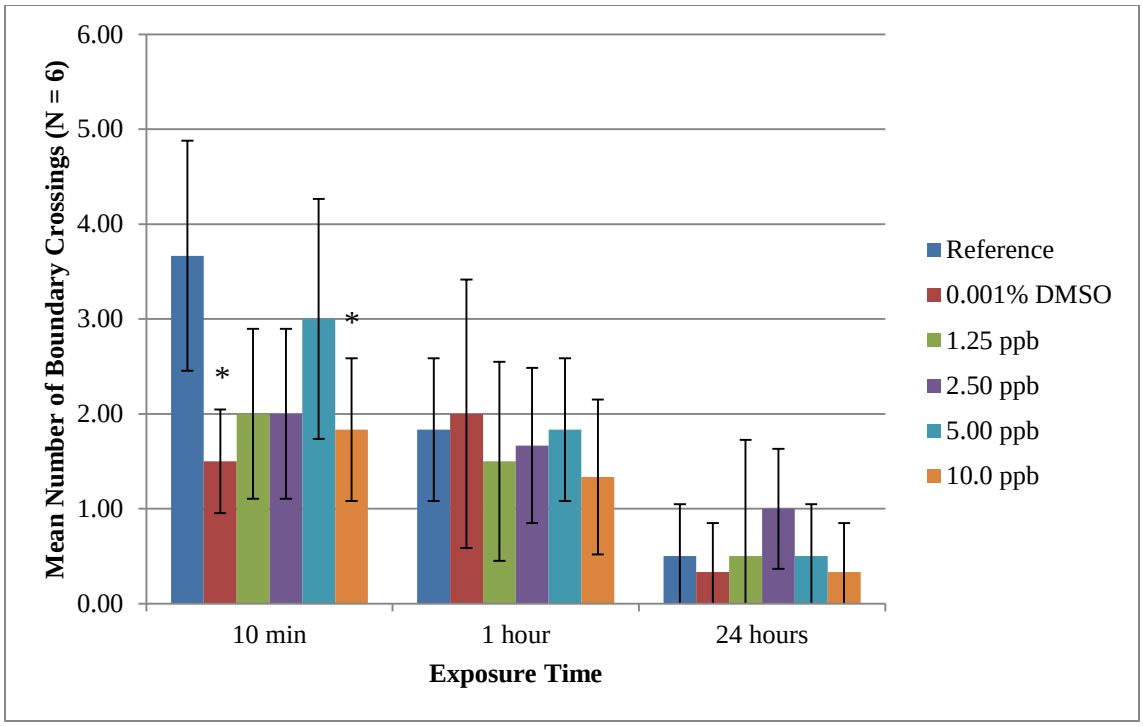

Figure 45 *Daphnia magna* mean number of threshold crossings in TCC over time

After 10 minutes of exposure to TCC, *Daphnia magna* in the DMSO control ($\chi^2 = 7.39$, $p = 0.007$) and 1.25 ppb, ($\chi^2 = 4.59$, $p = 0.032$), 2.50 ppb ($\chi^2 = 4.59$, $p = 0.032$) and 10.0 ppb TCC ($\chi^2 = 5.71$, $p = 0.017$) all showed significantly fewer boundary crossings compared to the reference; however, as with swimming style, there is likely stress leftover from transfer. After one and 24 hours, no treatments showed significantly different numbers of boundary crossings compared to the reference. In addition, there was no general trend within any of the three time points.

Mobility was evaluated by counting the number of *D. magna* in each vessel (out of 3) that were not immobile for > 2 seconds. All animals were mobile in all treatments at all time points. Accordingly, no mortality was observed in any treatment at any time point.

In summary, *Daphnia magna* appeared to exhibit slight sub-lethal toxicity at TCC concentrations approaching the 48-hour LC_{50} as shown by the elevated swimming style score, but not by movement through the water column or by mobility. Normal *D. magna* swimming style is a steady saltatory movement in straight lines. This is so *D. magna* can travel to food patches quickly and escape rapidly from predators. Changes to swimming style, such as those

induced by 10.0 ppb TCC after 24 hours, include erratic swimming characterized by short bursts of swimming with multiple changes in direction and even spinning behaviour. These behaviours could affect efficient foraging of food sources as well as increase susceptibility to predation. Fast, erratic swimming is likely an avoidance or escape behaviour that is probably seen in response to chemicals as an attempt to move to a less contaminated area (Dodson *et al*., 1995; Ren *et al*., 2007). Spinning is believed to be a type of escape behaviour; however, it likely increases predation as it attracts visual predators (Dodson *et al*., 1995). Although not seen in response to TCC, immobilization is an important behaviour that could be either caused by lack of energy (Untersteiner *et al*., 2003) or a loss of muscle co-ordination due to chemical action (Untersteiner *et al*., 2003; Schmidt *et al*., 2005). Finally, although no significant difference to *D. magna* water column movement was seen in response to TCC, this behaviour has been shown in literature to be indicative of chemical stress (Kieu et al., 2001; Michels et al., 2001; Martins et al., 2007). If *D. magna* are unable to move throughout the water column, they are more vulnerable to predation by visual hunters and their ability to effectively find food patches could be affected (Ryan and Dodson, 1998).

Hyalella azteca

Hyalella azteca behaviour was observed in four concentrations of TCC, a reference of aerated, dechlorinated MDW and a reference of the solvent carrier (0.001% DMSO) at three time points: 10 minutes, 1 hour and 24 hours after exposure. Three behaviours were assessed simultaneously: locomotion style, body length and mobilization.

Locomotion style was evaluated using a 3-rank regime with a score given to each replicate of between 0 and 2. Figure 46 illustrates mean locomotion score in TCC over time.

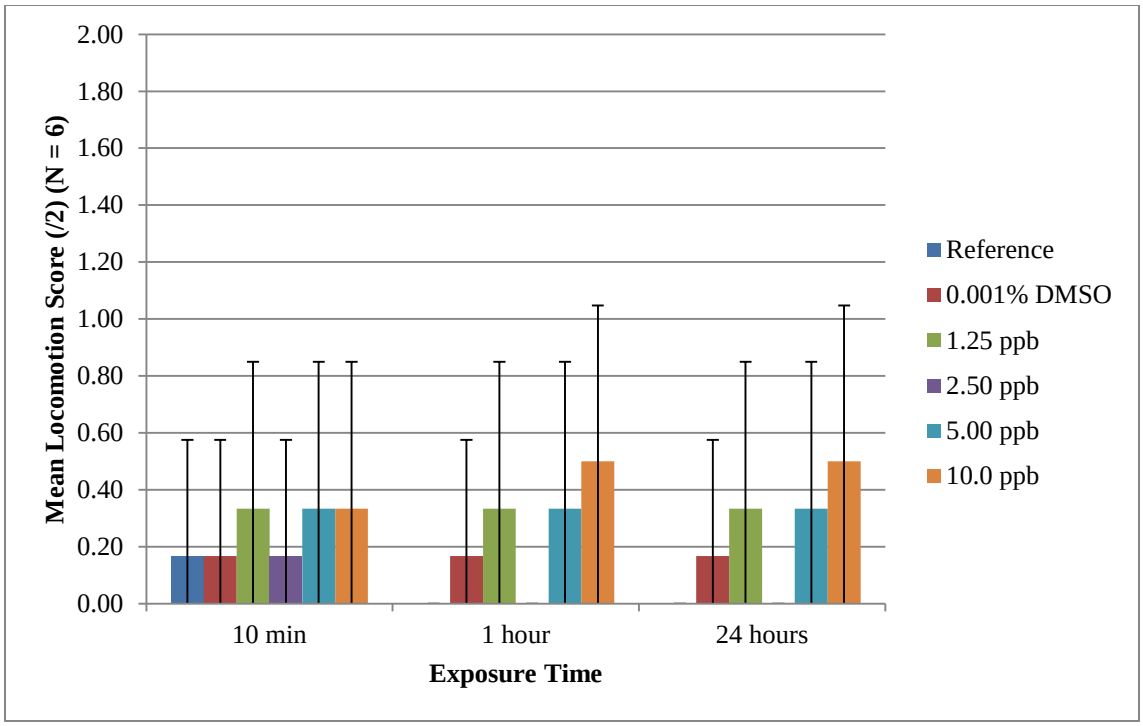

Figure 46 *Hyalella azteca* mean locomotion score (/2) in TCC over time

No treatments showed significantly different locomotion scores from the reference after any time point. With the exception of 2.50 ppb TCC there may be a slight positive biological trend of increasing locomotion score with increasing TCC concentration; however, it is not statistically significant.

Body shortening was evaluated using a 3-rank regime with a score given to each replicate of between 0 and 2.

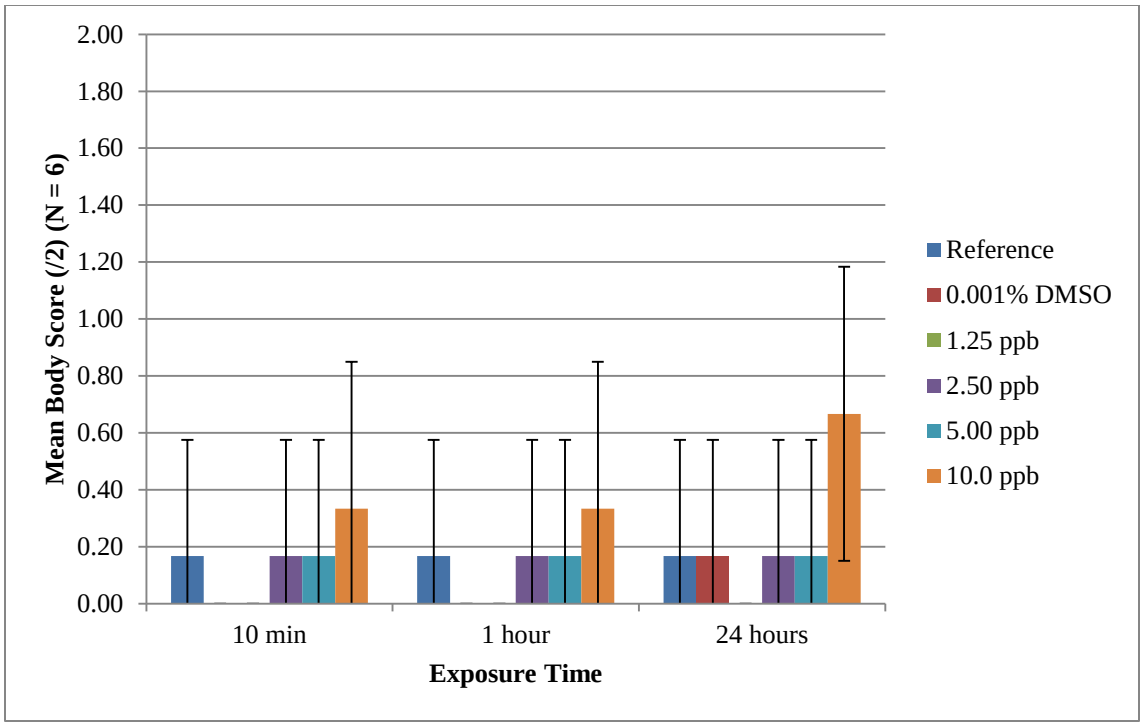

Figure 47 *Hyalella azteca* mean body score (/2) in TCC over time

Similar to *H. azteca* locomotion score, no treatments showed significantly different body scores compared to the reference at any time point. With the exception of 1.25 ppb TCC there may be a slight positive biological trend of increasing body score with increasing TCC concentration; however, it is not significant.

Mobility was evaluated by counting the number of *Hyalella azteca* in each vessel (out of 3) that were not immobile for > 10 seconds. Figure 48 illustrates mean percent mobilization in 4-chlorophenol over time.

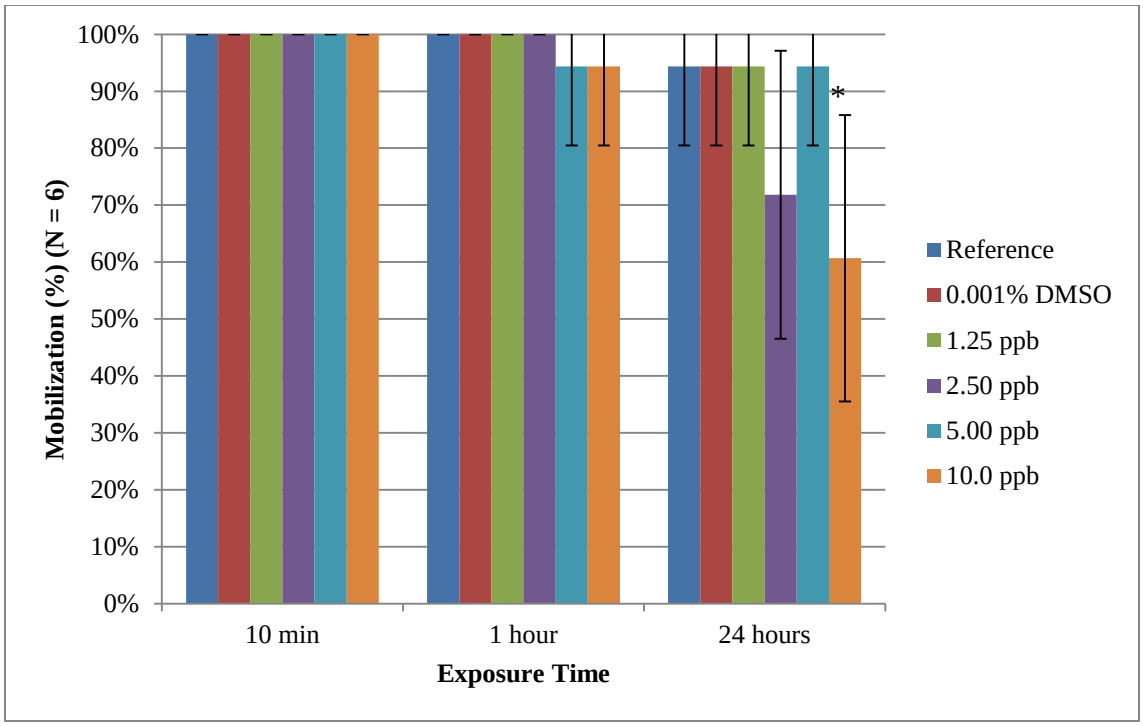

Figure 48 *Hyalella azteca* mean percent mobilization in TCC over time

After 10 minutes and 1 hour of exposure, no treatments showed significantly different mobility compared to the reference. After 24 hours, *Hyalella azteca* in 10.0 ppb TCC showed significantly decreased mobility compared to the reference ($\chi^2 = 5.16$, $p = 0.023$). This decrease in mobility was partially due to one instance of lethality. Although no lethality was seen in other treatments it is a common occurrence even under reference conditions and therefore the significant decrease in mobility seen at 10.0 ppb TCC cannot be interpreted as a sub-lethal toxicity.

In summary, no toxicity of TCC at concentrations up to 10.0 ppb was seen in *Hyalella azteca*. Although no significant changes in behaviour were seen in TCC treatments, the behaviours that were scored are ecologically-relevant and should be discussed. Swimming allows *H. azteca* to cross distances more rapidly than crawling and is important to move between food sources and to forage within the water column (Wang *et al*., 2004; Marshall, 2009). Inhibited swimming behaviours and immobility could increase vulnerability to predation, and decrease foraging and mating abilities (Marshall, 2009).

Lumbriculus variegatus

Lumbriculus variegatus behaviour was observed in four concentrations of TCC, a reference of aerated, dechlorinated MDW and a reference of the solvent carrier (0.001% DMSO) at three time points: 10 minutes, 1 hour and 24 hours after exposure. Three behaviours were assessed simultaneously: locomotion style, body length and mobilization.

Locomotion style was evaluated using a 3-rank regime with a score given to each replicate of between 0 and 2. Figure 49 illustrates mean locomotion score in TCC over time.

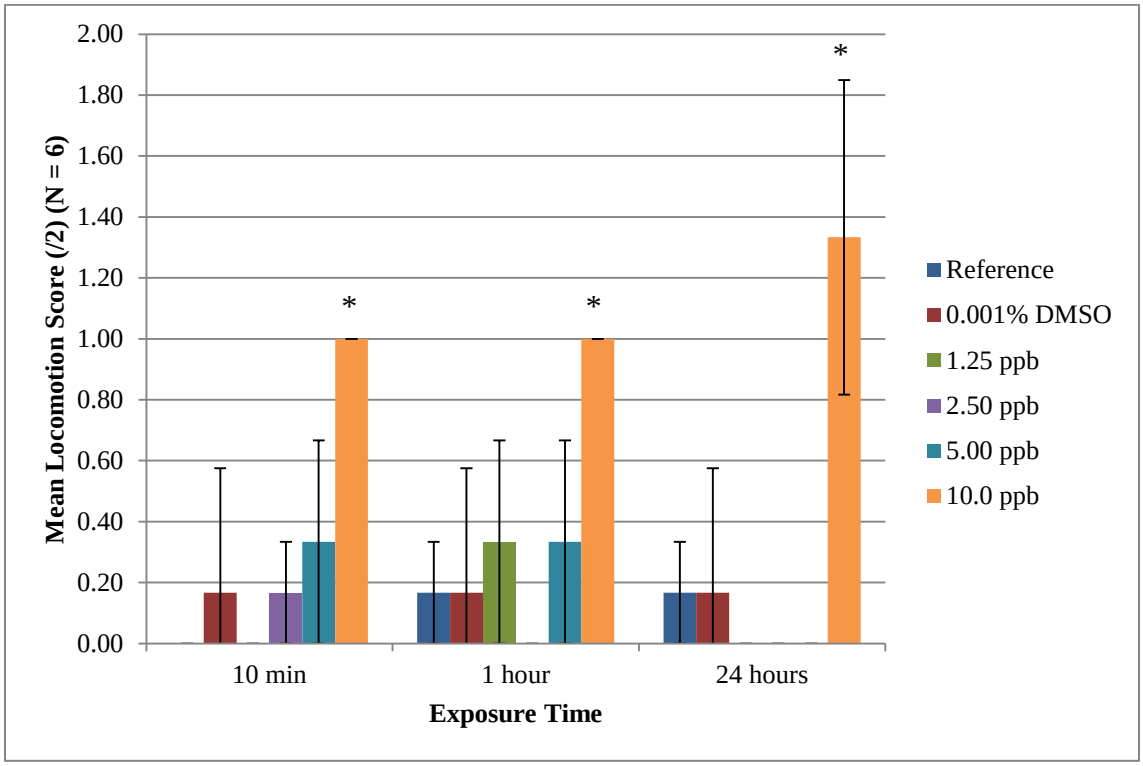

Figure 49 *Lumbriculus variegatus* mean locomotion score (/2) in TCC over time

At 10 minutes, 1 hour and 24 hours after exposure, *Lumbriculus variegatus* in 10.0 ppb TCC showed significantly higher locomotion scores compared to the reference [(χ^2 = 11.00, p = 0.001), (χ^2 = 7.86, p = 0.005) and (χ^2 = 7.66, p = 0.006), respectively].

Body length was evaluated using a 3-rank regime with a score given to each replicate of between 0 and 2. Figure 50 illustrates mean body score in TCC over time.

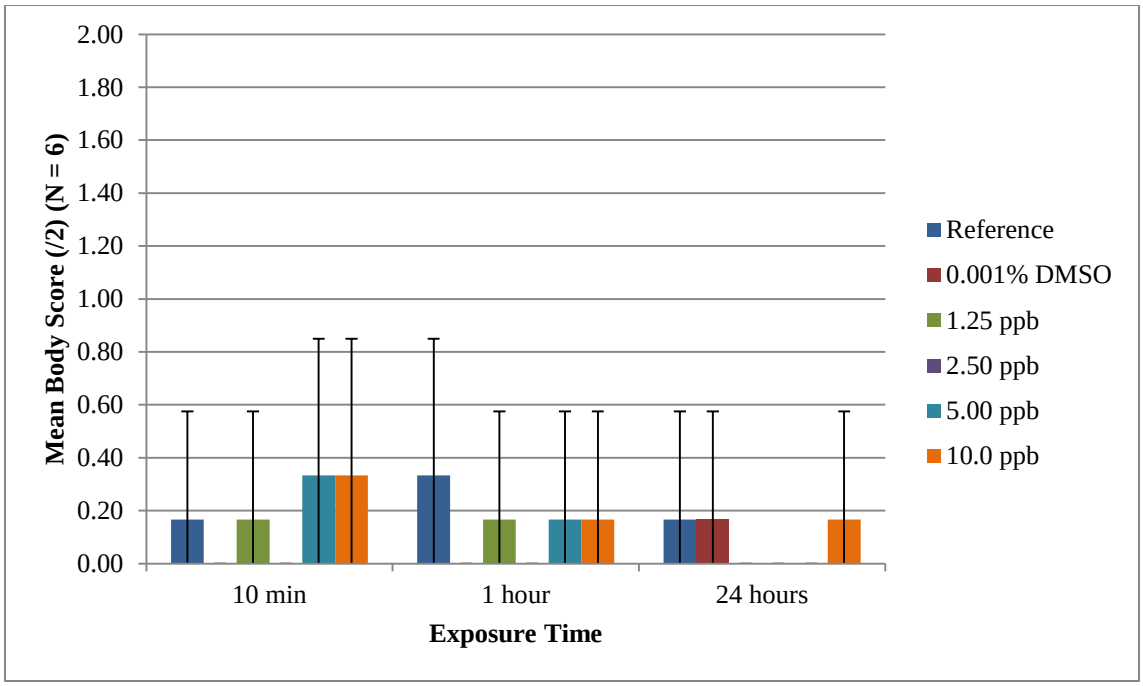

Figure 50 *Lumbriculus variegatus* mean body score (/2) in TCC over time

No treatments showed significantly different locomotion scores from the reference after any time point and no general trend between body score and TCC concentration was apparent.

Mobility was evaluated by counting the *Lumbriculus variegatus* that were not immobile for > 10 seconds. Each vessel only contained one worm, so the mobility for each replicate was either 100% or 0%. All worms were mobile in all treatments at all time points. Accordingly, no mortality was observed.

In summary, *Lumbriculus variegatus* appeared to exhibit slight sub-lethal toxicity at 10.0 ppb TCC as shown by the elevated locomotion score, but not by body score or mobility. This concentration was not close to the lethal concentration for *L. variegatus*. Previous work in the laboratory (unpublished) observed no lethality after 72 hours in concentrations of TCC up to 500 ppb. Normal *L. variegatus* locomotion in a water-only setting with a flat, hard plastic or glass bottom and no substrate is characterized by fluid body movements and peristaltic crawling (Drewes and Cain, 1999) with no twitching or writhing. Writhing was seen in 10.0 ppb TCC, indicating stress. Writhing and twitching may affect ability to escape predation, as the constant contraction of circular and longitudinal muscles likely uses large amounts of energy that are then

unavailable for escape. Also, changes to locomotion behaviour may affect *Lumbriculus variegatus* foraging and mating behaviours.

A major limitation to the study of *Lumbriculus variegatus* behaviour was the inability to observe *L. variegatus* in a substrate. Experiments were run under water-only conditions to ensure no change occurred in the bioavailability of the tested contaminant. Consequently, it is not known how the reference behaviour under substrate conditions (see section 1.6.4, *Lumbriculus variegatus* feeding and behaviour) changes in response to a contaminant such as TCC. Marshall (2009) found decreased burrowing behaviours in response to chemical contamination; this behaviour should be studied in more detail in future *L. variegatus* behaviour projects.

Lemna minor

Lemna minor appearance was observed in five concentrations of TCC, a reference of SIS media and a reference of the solvent carrier (0.001% DMSO) at three time points: 0, 2 and 7 days after exposure. Three appearances were assessed: the presence of chlorosis, necrosis and colony break-up. No presence of chlorosis, necrosis or colony break-up was observed in any treatment after 7 days. Accordingly, no figures are shown. Concentrations of TCC up to 40.0 ppb showed no impact in terms of appearance to *L. minor*.

Although not observed under tested concentrations of TCC, chlorosis, necrosis and colony break-up are important, ecologically-relevant endpoints that have been studied extensively (Wang, 1986; Wang and Williams, 1988; Wang, 1990; Clement and Bouvet, 1993; Radic *et al*., 2010). Chlorosis presents as yellow tissue and occurs when cells lack chlorophyll (Wang and Williams, 1988). A decrease in chlorophyll means a decrease in photosynthesis and subsequently a decrease in energy production for the plant. Necrosis, or the death of localized patches of tissue, also causes decreased photosynthesis and break-up of tissue. *Lemna minor* fronds exist as colonies, perhaps to increase buoyancy or to increase water surface coverage; break-up of these colonies may lead to decreased buoyancy and decreased potential light gathering.

3.3.2 Life Cycle

Life cycle bioassays were conducted following the same methods used to assess the positive toxicant, 4-chlorophenol. *Lumbriculus variegatus* and *Hyalella azteca* were not used for life cycle bioassays (see section 3.2.2). *Daphnia magna* and *Lemna minor* were exposed to four

concentrations of TCC: 5.00, 10.0, 20.0, and 40.0 ppb, along with a reference of aerated, dechlorinated MDW and a solvent reference (0.01% DMSO).

Daphnia magna

Three neonates were placed in each treatment replicate (N = 5). The day(s) on which their first brood was released as well as the number of neonates comprising that first brood were recorded as endpoints. The time-to-first brood was calculated as a weighted average (see section 3.2.3). The mean first brood size and mean time-to-first brood for treatments with surviving adults are shown in Figure 51 and Figure 52, respectively.

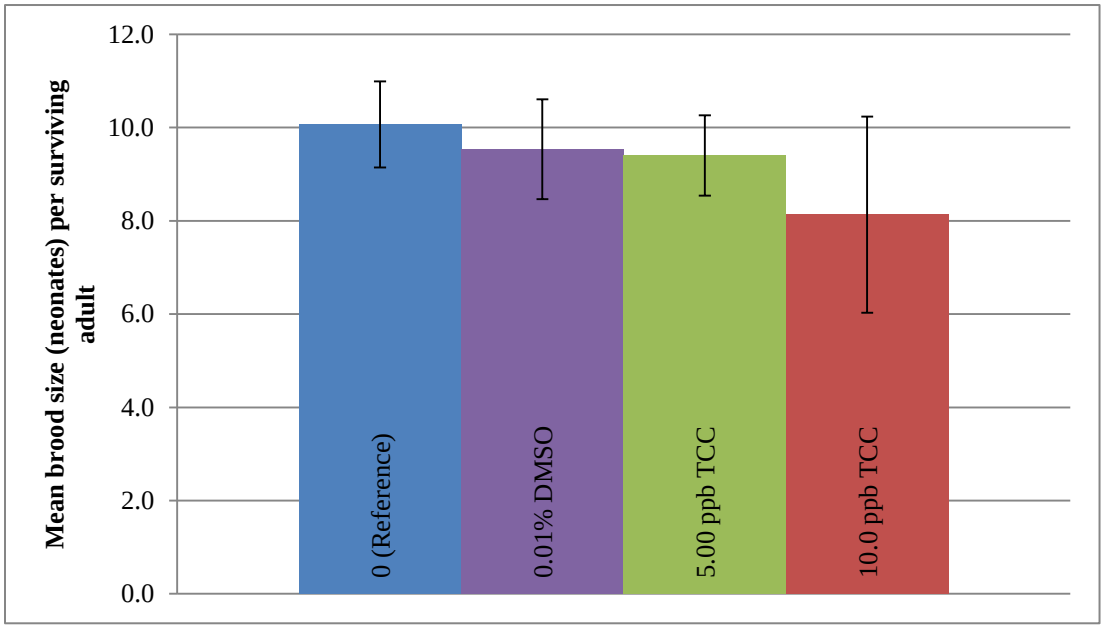

Figure 51 *Daphnia magna* mean brood size (neonates) for treatments with surviving adults

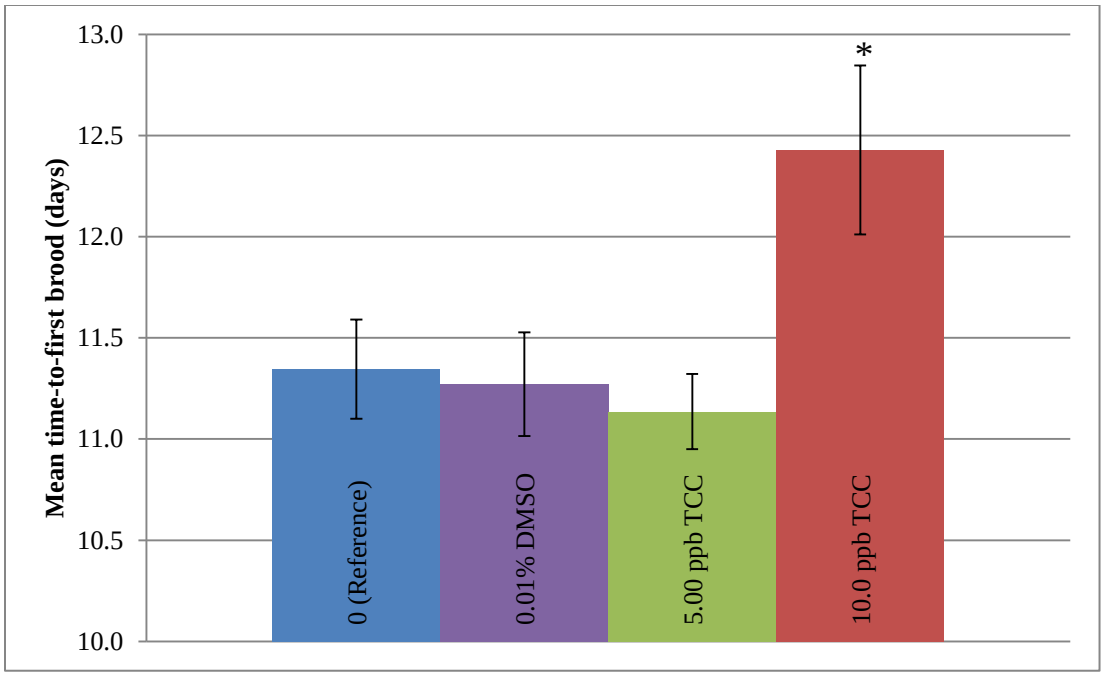

Figure 52 *Daphnia magna* mean time-to-first brood for treatments with surviving adults

After 14 days, all *Daphnia magna* in the reference, 0.01% DMSO, 5.00 and 10.0 ppb TCC concentrations had survived. *D. magna* in 20.0 ppb TCC did not survive past 5 days, and *D. magna* in 40.0 and 80.0 ppb did not survive past 48 hours. *D. magna* in the 10.0 ppb TCC, the highest concentration where the animals survived the full 14 day test period, took longer to produce their first brood compared to the reference ($\chi^2 = 6.94$, $p = 0.008$) but the number of neonates produced were not significantly different ($\chi^2 = 2.52$, $p = 0.113$). Other concentrations of TCC and the DMSO reference were not significantly different from the reference for either endpoint.

Lemna minor

Lemna minor TCC life cycle tests were conducted in SIS growth medium. Four concentrations of the TCC plus a control of SIS media and of the solvent (0.01% DMSO) were tested. Growth was tracked by counting the number of individual fronds. As shown in Figure 53 and Table 9, the highest test concentration of TCC, 40.0 ppm, resulted in 8% growth inhibition relative to the reference; however, the number of fronds was not significantly different from the reference ($\chi^2 = 2.33$, $p = 0.127$).

Table 9 *Lemna minor* growth rate and % growth inhibition after 7 days in TCC

TCC Concentration (ppb)	Growth Rate	% Inhibition
0	0.38 ± 0.03	0 ± 7%
0.01% DMSO	0.38 ± 0.05	0 ± 12%
5.00	0.38 ± 0.01	0 ± 2%
10.0	0.39 ± 0.01	-2 ± 2%
20.0	0.35 ± 0.03	7 ± 8%
40.0	0.35 ± 0.01	8 ± 3%

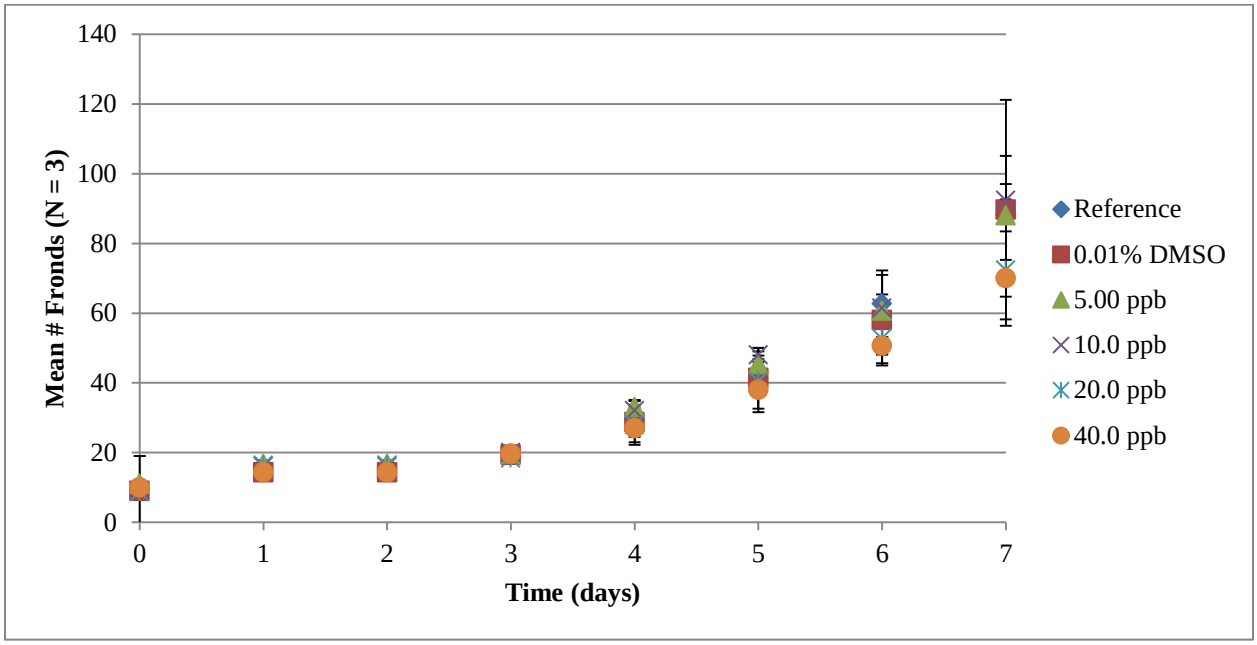

Figure 53 Growth of *Lemna minor* in TCC over time

Growth of *Lemna minor* in reference satisfied the health criteria (see section 3.1.5); however, growth in the TCC life cycle bioassay compared to the positive toxicant life cycle bioassay was considerably less. The growth rate in the TCC life cycle bioassay was 0.38 ± 0.03 compared to 0.47 ± 0.02 in the positive toxicant life cycle bioassay. The likely reason for this decrease in growth is a decrease in lab temperature. The optimal temperature for growing *L. minor* is 25 ± 2°C. During the positive toxicant life cycle bioassay, the lab temperature was approaching 25°C, compared to a few weeks later when the TCC bioassay was run and the lab temperature was 19 ± 2°C.

3.3.3 Summary: TCC

Behaviour and reproduction was observed in environmentally-relevant concentrations of TCC up to 10.0 ppb. Slight sub-lethal toxicity to *Daphnia magna* was seen after 24 hours of exposure; however, increased stress behaviour was only seen in concentrations approaching the 48 hour LC_{50} and therefore more likely represents an early-warning to lethality rather than sub-lethal toxicity. In terms of reproduction, however, an increased time-to-first brood was seen in 10.0 ppb TCC. As discussed in section 3.2.3, *D. magna* life cycle tests were conducted with approximately 5×10^4 algae cells/mL, which changed the bioavailability of TCC to the *D. magna* by an unknown factor. Still, the TCC was still available to the *D. magna* through consumption of the algae. The delay in release of the first brood caused by 10.0 ppb TCC could have ecological consequences for *D. magna* populations and freshwater communities as a whole.

Overall, TCC was found to exert no sub-lethal or lethal toxicity to *Hyalella azteca*, *Lumbriculus variegatus* or *Lemna minor* at environmentally-relevant concentrations up to 10.0 ppb in water-only, static conditions. At 10.0 ppb, TCC significantly delayed reproduction in *Daphnia magna*, indicating chronic toxicity. The full mechanism of action of TCC is not known. TCC is an anilide, a class of compounds shown to induce cell death by adsorbing and destroying the cytoplasmic membrane (McDonnell, 2007 in Snyder and O'Connor, 2013). TCC has also shown a potential for endocrine disruption . TCC was found to stimulate embryo production in the freshwater mudsnail (Giudice and Young, 2010) and enhance induced tubercle formation in fathead minnows (Ankley *et al*., 2010). The cause of the delayed reproduction in *D. magna* in beyond the scope of this work; however, possible explanations such as direct changes to egg development or indirect changes through physiological effects of TCC to the mother *D. magna* (Schmidt et al., 2005) that exist for chemical contaminants in general could be applied.

4.0 OVERALL SUMMARY AND FUTURE DIRECTIONS

4.1 Overall Summary

This study utilized *Daphnia magna*, *Hyalella azteca*, *Lumbriculus variegatus* and *Lemna minor* to: (1) implement culturing procedures that produce healthy, age-synchronized organisms for toxicity testing; (2) further refine sub-lethal behaviour and reproduction toxicity methods; and (3) assess the impact of the antimicrobial triclocarban. These objectives were complementary and built upon each other. The behaviour and reproduction toxicity bioassays could not have been accomplished without healthy, age-synchronized organisms from the culturing methods. In turn, the toxicity assessment of TCC could not have been accomplished without first implementing the culturing methods and refining the behaviour and reproduction methods.

Many endpoints were utilized to assess toxicity: a total of twelve behaviours and three reproductive endpoints for the four organisms. While some endpoints showed sub-lethal toxicity of TCC, others did not. To this end, it is important to note that single endpoints may give false positives or negatives. The behaviour endpoints showed only early-warning lethality of TCC to *Daphnia magna*; however, reproduction showed chronic toxicity. Three important statements can be drawn regarding the use of bioassays in this study:

(1) We must use bioassays to assess impact, not chemical analysis. TCC has been found in surface and wastewaters in concentrations of 0.012–6.75 ppb (see section 1.7.1); however, these concentrations are meaningless in terms of biological impact. Does 6.75 ppb TCC affect *Daphnia magna* swimming behaviour or reproduction? Only bioassays can assess *true* impact.

(2) We must use multiple species to assess impact. Different species have different sensitivities to toxicants (Phipps *et al.*, 1995) and inhabit different areas of the environment (e.g., benthos, water column, water-air interface) and are therefore exposed to different levels of the contaminant based on partitioning. Using multiple species attempts to minimize over- or under-estimation of toxicity. This difference is species sensitivity as was seen in this study. For example, TCC was found to affect

the swimming behaviour of *Daphnia magna* at 10.0 ppb, but not *Lumbriculus variegatus*, likely due to *L. variegatus'* tough integument built for living in the benthos.

(3) We must choose the toxicity endpoints carefully. Sub-lethal endpoints such as behaviour and reproduction are more sensitive than lethality. If only lethality was observed in this study, no impact of TCC to any of the organisms would have been seen. By assessing the sub-lethal endpoint of reproduction, it was found that 10.0 ppb TCC significantly delayed reproduction.

In conclusion, this study utilized bioassays to assess the toxicity of the widely found surface water contaminant, triclocarban. Behaviour and reproduction were demonstrated as useful endpoints while refining these methods using 4-chlorophenol and were successfully implemented in the toxicity assessment of TCC. Within the environmentally-relevant concentrations (one slightly above), the endpoints used were discerning enough to see impact. TCC at 10.0 ppb was found to delay reproduction in *Daphnia magna*. This delay could result in population, community and ecosystem-level responses.

4.2 Future Directions

This thesis is a part of ongoing behaviour research in the McCarthy ecotoxicology research group. This work built upon existing methods put forth by Marshall (2009) and will no doubt be built upon by future researchers. It is recommended that future work disregard the 10 minutes time point as there is too much stress left over from the transfer of the organisms into the test vessels. In addition, behaviour work with *Hyalella azteca* was found to be quite difficult due to the very small size of the juveniles. Future work should look at different *H. azteca* behaviours or perhaps utilize older and larger animals, at the potential sacrifice of age sensitivity, to be able to better observe the behaviours.

Very few non-industry sponsored TCC toxicity assessments have been reported in the literature. Since this work showed delayed reproduction in *Daphnia magna*, it is highly recommended that future work investigate chronic toxicity further and include a full life cycle test (i.e. <24 hour old neonate through to death) for *Daphnia magna* as well as *Hyalella azteca* and *Lumbriculus variegatus*. This research only used water-only exposures for toxicity

assessment. Considering TCC is a hydrophobic compound, future work should utilize spiked sediment and sediment bioassays to more accurately assess toxicity.

The ultimate goal of this line of research is to develop a holistic, multi-species, early-warning biomonitoring technology that can be implemented within drinking water facilities to help detect potentially dangerous contaminants. The behaviours and methods refined in this research will contribute to a library of response patterns to various types of contaminants that could then be used in developing this technology.

Appendix I. Wastewater Treatment and Whole Effluent Toxicity Testing

WWTP effluent is the treated liquid waste from households, industries, commercial establishments, institutions, and sometimes urban runoff (Environment Canada, 2001; Servos et al., 2001; CCME, 2006). The composition of WWTP effluent is greatly affected by the community that produces the wastewater and the level of treatment employed (Environment Canada, 2001). The demographics, community attitude towards pollution and presence of industry greatly affects the input of chemicals into the wastewater stream (Environment Canada, 2001). While most metals and chemicals found in municipal wastewater come from industrial and business sources, domestic wastes contain also contain important contaminants such as cleaning agents, personal care products, pharmaceuticals, brominated flame retardants, paint, grease and oils (Chambers et al., 1997; Environment Canada, 2001; CCME, 2006). In addition to industrial, business and domestic sources, municipal wastewater can also contain stormwater if the municipality employs a combined sewer system (Environment Canada, 2001). A large proportion of stormwater is made up of urban runoff, and therefore contains substantial amounts of grease, oil, road salt, metals and organic contaminants such as polycyclic aromatic hydrocarbons (PAHs) (Chambers et al., 1997; Environment Canada, 2001). Stormwater can also contain nutrients such as nitrogen and phosphorus from fertilizers, insecticides and herbicides from lawn and garden runoff, and debris, grit and deposited air pollutants (Chambers et al., 1997; Environment Canada, 2001).

WWTP effluents are the primary source of many important contaminants of our aquatic environment, including biological oxygen demand (BOD), total suspended solids (TSS), nutrients such as nitrogen and phosphorus, organic chemicals, and metals (Environment Canada, 2001). Compared to industrial effluents, WWTP effluents discharge nearly three times more phosphorus, eight times more nitrogen, three times more PCBs and seven times more mercury into Canada's lakes (Environment Canada, 2001). The significance of pollution by WWTP effluent is so important that it was identified as a major problem in 10 out of the 17 International Joint Commission Areas of Concern in 1985 (Environment Canada, 2001).

Impacts of WWTP Effluent on the Environment

The severity of the impact that WWTP effluent has on the receiving aquatic environment depends on, among many other factors, the level of wastewater treatment employed, the quality of the incoming raw (untreated) water, the number and type of industries and commercial businesses connected to a municipal wastewater system and household habits (Environment Canada, 2001). The level of wastewater treatment is the major factor in effluent quality, with treatment plants that employ the more complex secondary and tertiary treatment discharging better quality effluent than those with the primitive primary treatment (Environment Canada, 2001).

The impact of WWTP effluent on the environment may have acute, or short-term effects that are characterized by specific events and show effects over an identifiable period of time (Harremoes, 1988). Alternatively, they may be chronic, showing accumulated or cumulative effects over long periods of time (Harremoes, 1988). WWTP effluent discharge into aquatic environments has the potential to cause overall environmental degradation as well as direct toxicity and bioaccumulation to aquatic organisms (Chambers et al., 1997). Environmental degradation, such as eutrophication caused by phosphorus and nitrogen addition, or by physical changes such as temperature change or addition of total suspended solids (TSS) can occur (Chambers et al., 1997). The aquatic habitat could also be degraded by BOD addition causing dissolved oxygen stress (Chambers et al., 1997). Direct, acute toxicity to aquatic organisms could occur due to addition of ammonia, nitrite, chlorine, metals, chlorinated solvents, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), chlorinated phenols, and many more chemicals in the WWTP effluent (Chambers et al., 1997). Common receiving aquatic environments include surface waters, such as lakes, rivers and streams or wetlands, but WWTP effluent can also be used to recharge groundwater aquifers by percolation, deep-well injection or land application (WEF, 2008). In some water scarce areas, WWTP effluent can be re-used for irrigation, drinking water, as a source of water for constructed wetlands, or as cooling water for industrial purposes (WEF, 2008).

Municipal Wastewater Treatment in Canada

The treatment and disposal of municipal wastewater has always been a local or municipal issue, with the level of treatment and disposal method varying across Canada (Environment Canada, 2010). Historically, sewage and other wastewater was disposed of in nearby waterways,

where it dispersed or was whisked downstream, away from the population. Cities across Canada began building wastewater treatment facilities in the early 1900s, with the first being Toronto's Main Sewage Treatment Plant in 1910 (City of Toronto, 2012), followed by Calgary in 1918 (City of Calgary, 2012), Winnipeg in 1937 (City of Winnipeg, 2011), Vancouver in 1961 (Metro Vancouver, 2011), Montreal in 1987 (Ville de Montreal, 2012) and Halifax in 2008 (Halifax Regional Municipality, 2011).

Currently, approximately 97% of the Canadian population on municipal sewers is served by some level of wastewater treatment; however, this level varies from coast to coast (Environment Canada, 2001). Ontario and Manitoba employ almost exclusively secondary treatment while Saskatchewan and Alberta employing the even better tertiary, or additional treatment (Environment Canada, 2011). However, Environment Canada estimates that 3.2% of the Canadian population, or approximately one million people, the majority in Newfoundland and Labrador and the territories and other coastal communities, are served by a sewer system that provides no treatment besides preliminary screening and grit removal only (CCME, 2006; Environment Canada, 2011). Environment Canada also estimates that over 150 billion litres of untreated municipal wastewater is being discharged to Canadian surface waters each year (Environment Canada, 2010). Furthermore, an additional 18.1% of Canadians are served by a system that only employs primary treatment (Environment Canada, 2011). In total, nearly 20% of all Canadians, or 6.8 million people are served by sewer systems that produce untreated or inadequately treated effluent compared to Canada's national effluent standards of secondary treatment.

Municipal Wastewater Treatment in Ontario

Before the mid-1900s, municipalities in Ontario simply deposited their wastewater directly into surrounding bodies of water, including the Great Lakes (MOE, 1990). For example, Toronto's first sewer, built in 1836, carried stormwater and human sewage underground and deposited it into Toronto Bay (Brace, 1993). While the idea was that the sewage would disperse naturally, in reality it ended up on the shores of Toronto Bay and ultimately contaminated Toronto's drinking water source (Brace, 1993). By the 1860s, Toronto Bay was polluted well out from the shore by sewage (Brace, 1993). It was estimated that in 1891 Toronto was discharging 12 tons of untreated solid matter in Toronto Bay per day and that accumulated

sludge measured three to four feet on the bottom of the harbour (Brace, 1993). After the link between sewage and disease was established in the late 1880s, water treatment facilities, for both drinking water and wastewater, were established (Brace, 1993). Ontario's first wastewater treatment plant was built in 1910 and located in Toronto, on the shores of Lake Ontario (City of Toronto, 2012). The facility, then the Main Treatment Plant and now Ashbridges Bay Treatment Plant, had the capacity to treat 150 000 cubic meters of wastewater per day (City of Toronto, 2012). Early treatment processes at the Main Treatment Plant included sludge beds, settling tanks and screening chambers (Metropolitan Toronto Works, 1920).

By 1930, 70 Ontario municipalities had wastewater treatment plants; however, the onset of the Great Depression, combined with population growth resulted in outdated and overloaded facilities (OMEE, 1990). In addition, World War II introduced a new source of pollution, industrial waste, into the municipal wastewater (OMEE, 1990). A report by the International Joint Commission found that between 1912 and 1949 the Great Lakes showed a 3- to 4-fold increase in bacteria levels, and that oxygen demand from industrial waste was greater than from the sewage produced by the surrounding population of 3.5 million (OMEE, 1990).

In 1953 there were 18 wastewater treatment plants in the Toronto area alone, with many operating over their capacity (City of Toronto, 2012). Beginning in 1954 the 18 plants were been consolidated into four large facilities, with the smaller plants converted into wastewater pumping stations (City of Toronto, 2012). Today Toronto employs four wastewater treatment plants: Ashbridges Bay (previously called the Main Treatment Plant), North Toronto, Highland Creek and Humber (City of Toronto, 2012).

Municipal Wastewater Treatment Processes

Municipal wastewater treatment is a multi-stage process that is designed to clean wastewater of debris and organic solids so it may be replaced to natural bodies of water (Sonune and Ghate, 2004; WEF, 2008). Incoming (raw) municipal wastewater is typically 99.94% liquid and 0.06% solid (WEF, 2008). Historically, the main goal of wastewater treatment was to remove solids and floating debris, reduce biological oxygen demand (BOD) and disinfect to remove pathogens (Sonune and Ghate, 2004). More recently, additional municipal wastewater

treatment processes have been designed to remove other sources of pollution, such as ammonia, phosphorus, and trace resistant, organic substances (US EPA, 2004).

The municipal wastewater treatment process is linear; incoming wastewater undergoes a series of treatment steps, beginning with preliminary treatment, followed by primary, secondary and tertiary, or additional treatments. The study facility employs preliminary, primary and secondary treatment, followed by disinfection.

Preliminary Treatment

Incoming wastewater first undergoes preliminary treatment. The goal of preliminary treatment is to remove large particulates and odour, and to measure and control the wastewater flow (WEF, 2008). First, screening removes large debris, including large solids and trash, using a series of coarse to fine bars and/or screens (US EPA, 2004; WEF, 2008). Next, a series of grit chambers is used to settle fine, non-biodegradable particles such as sand, rocks, coffee grounds, and cinders (WEF, 2008). Grit removal reduces potential blockages in the equipment and reduces accumulation of solids in downstream processes (US EPA, 2004; WEF, 2008). Odour control is employed where necessary to reduce the risk of build-up of explosive or toxic gases such as hydrogen sulfide and other anaerobic by-products of degradation (WEF, 2008). Methods such as pre-chlorination, iron salts and pre-aeration are used (WEF, 2008). Finally, preliminary treatment can also involve measuring and controlling the incoming flow of wastewater through flow equalization to ensure optimum performance of the wastewater treatment facility (WEF, 2008).

Primary Treatment

Following preliminary treatment, wastewater is further separated from solids using sedimentation tanks (WEF, 2008). The goal of primary treatment is to remove dissolved organic and inorganic substances as well as suspended solids and floatables (US EPA, 2004; WEF, 2008). In the sedimentation tanks, the flow of wastewater is slowed to allow suspended particles to flocculate and settle, often with the aid of chemicals (WEF, 2008). Floatables such as grease and scum float to the top and are skimmed from the surface (WEF, 2008).

Secondary Treatment

The goal of secondary treatment is to remove organic matter from the wastewater (US EPA, 2004). Several biological treatment processes are employed, including attached growth

processes such as trickling filters and rotating biological contactors, and suspended growth processes such as activated sludge (US EPA, 2004). The Ashbridges Bay facility utilizes the activated sludge method, the most widely used biological treatment process, to remove organic matter that causes biological oxygen demand (BOD) (WEF, 2008; Toronto Water, 2011). In short, the activated sludge process is a primarily aerobic open culture system that utilizes a mixture of microorganisms to digest organic matter (WEF, 2008).

Tertiary (Additional) Treatment

Some wastewater treatment facilities utilize additional treatment to disinfect the wastewater, or to control nitrogen or resistant organic substances (US EPA, 2004). Methods for disinfection include addition of chlorine, ozone or ultraviolet radiation (US EPA, 2004; WEF, 2008).

WWTP Effluent Toxicity Research

A plethora of studies have assessed acute and chronic toxicity of a single or group of contaminants commonly found in WWTP effluent; however, comparatively few studies have attempted to assess the acute and/or chronic toxicity of whole WWTP effluent. All laboratory studies assessing whole effluent toxicity of WWTP effluent found some level of toxicity, at either the acute (Esvelt et al., 1973; Logue et al., 1989; Pessala et al., 2004; Hernando et al., 2005; Ra et al., 2008; Pignanta et al., 2012), or acute and chronic levels (Neiheisel et al., 1988; Schroder et al., 1991; Rutherford et al., 1994; Bailey et al., 2000; Manusadzianas et al., 2003; Ra et al., 2007). In general, it was found that increasing wastewater treatment levels such as secondary and tertiary treatment decreased toxicity.

In addition, some researchers focused on assessing WWTP effluent toxicity mixed with receiving water to better estimate actual toxicity in the environment (Di Marzio et al., 2005; Pignata et al., 2012), with toxicity being found in some waters and not in others. Apart from performing toxicity tests in the laboratory, some studies performed *in situ* tests to constantly expose test organisms to effluent-contaminated waters in environmental conditions (Birge et al., 1989; Maltby et al., 2000). While most studies utilized variants of the whole effluent toxicity (WET) test, some researchers pre-concentrated effluent samples to mimic bioaccumulation of organic contaminants in aquatic organisms (Wang et al., 2003). Clearly, toxicity of WWTP

effluent greatly depends on the input of contaminants into wastewater, and the level of treatment employed, as well as the characteristics of the receiving waters.

Whole Effluent Toxicity Testing

Whole effluent toxicity (WET) tests were first developed in the 1950s as a way to assess the acute toxicity of an effluent to a receiving water with the goal of ensuring that "wastewaters discharged into receiving water systems do not adversely affect aquatic life" (Grothe et al., 1995). Today, WET testing is an integral part of the US EPA's water quality initiatives (Grothe et al., 1995). WET tests involve exposing an aquatic test organism to a series of concentrations of an effluent sample where during and/or after a given duration, observations of the organism's response such as mortality, growth, and reproduction are made (Denton and Norberg-King, 1995; SETAC, 2004). These observations are then used to estimate the toxicity of the sample through statistical analysis to quantify the effects observed during the test (Denton and Norberg-King, 1995; SETAC, 2004). The main advantage of WET testing is that it allows for measurement of the potential toxicity of all chemicals in an effluent, including additive and synergistic effects as well as daughter metabolites (Chapman, 2000; SETAC, 2004). Although it is hoped that WET testing is predictive of receiving water ecological effects, in reality the gap between toxicity in the laboratory and toxicity in the receiving water may be unrealistic and therefore the toxicity risk may be over or under estimated (Dorn, 1995). WET tests may be overprotective due to the conservative nature of laboratory tests as well as differences in model species sensitivity (Chapman, 2000). Conversely, WET tests may also be underprotective because the multiple stresses that exist on an organism in the environment are not replicated in the laboratory, and laboratory experiments cannot account for food chain effects or all possible endpoints (Chapman, 2000).

Appendix II. Discussion of Behaviour in Government Toxicity Testing Protocols

Daphnia sp.

Regulatory Body	Title	Reference #	Date	Test Conditions	Endpoints	Discussion of Behaviour
ASTM	Standard guide for conducting *Daphnia magna* life-cycle toxicity tests	E1193-97	2004	21-day life cycle test Chemicals Static-renewal, flow-through	Final number alive Final weight Number of progeny per daphnid or total number young Time-to-first brood Young per adult reproduction day	Recommends observing first and second-generation daphnids for aberrant behavior, such as inability to maintain position in water column, uncoordinated swimming and cessation of feeding. Suggests that behavioural endpoints are often difficult to quantify and might not be suitable endpoints; however, they might be useful for interpreting effects on survival and growth and for deciding wehther the test should be extended beyond the minimum duration.
Environment Canada	BTM: Acute Lethality Test Using *Daphnia* spp.	EPS1/RM/11	July 1990, Amend-ments May 1996	48-h acute lethality Chemicals Effluents, leachates, elutriates Static	Mortality	States that the test is invalid if > 10% of the daphnids in either the control or solvent control exhibits atypical/stressed behaviour (e.g., immobility). States that if daphnid survival and behaviour (e.g., mobility, circling, floating) in laboratory control water should be compared to that shown in a sample of receiving water to distinguish any overt toxic responses that may be attributable to contaminants within the upstream water. In test results, atypical/stressed daphnid behaviour in each test solution are to be observed at the beginning and end of test, as a minimum.
	BTM: Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*	EPS1/RM/14	2nd Ed. Dec 2000	48-h acute lethality Effluents Static	Mortality	Recommends that observations of daphnid behaviour be noted during the test. States that the test is invalid if > 10% of the daphnids in either the control or solvent control exhibits atypical/stressed behaviour (e.g., immobility).
ISO	Water quality – determination of the inhibition of mobility of *Daphnia magna* Straus (Cladocera, Crustacea) – acute toxicity test	ISO 6341	3rd Ed. 1996			*Cannot review ($).*
	Water quality – determination	ISO 10706	1st Ed. 2000			*Cannot review ($).*

	of long term toxicity of substances to *Daphnia magna*					
OECD	OECD guidelines for the testing of chemicals: *Daphnia* sp., acute immboilisation test	202	2004	48-h acute test Chemicals Static	Immobilization	States that in addition to immobility, abnormal behaviour should be noted.
	OECD guidelines for the testing of chemicals: *Daphnia magna* reproduction test	211	2008	21-day reproduction test Chemicals Semi-static, flow-through	Living offspring produced per parent animal alive Survival of parent animals Time to production of first brood	Not mentioned.
Ontario MOE	*Daphnia magna* acute lethality testing of effluents and leachates, v.9.0	E3439	2010	48-h acute test Effluent and leachates Static	Immobilization Mortality	Test is not acceptable if > 10% mortality or abnormal behaviour in control.
US EPA	Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 5th Ed.	EPA-821-R-02-012	2002	24,48, 96-h acute test Effluents and receiving waters Static, static-renewal, flow-through	Mortality	Not mentioned as an endpoint.
	Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms, 4th Ed.	EPA-821-R-02-013	2002	Maximum 8-day reproduction test Effluents and receiving waters Static-renewal	Survival Reproduction	Not mentioned as an endpoint.

Lemna sp.

Regulatory Body	Title	Reference #	Date	Test Conditions	Endpoints	Discussion of Behaviour
ASTM	Standard guide for conducting static toxicity tests with *Lemna gibba* G3	E1415-91(2004)e1	2004	7-day test Chemicals Static	Inhibition of growth	No mention of appearance as end-point, but do not have access to entire document ($).
Environment Canada	Biological test method: Test for measuring the inhibition of growth using the freshwater macrophyte, *Lemna minor*	EPS 1/RM/37	2007	7-day test Chemicals Static, static-renewal	Increase in number of fronds Dry weight	Recommends that observations of chlorosis, necrosis, gibbosity, colony destruction, root destruction and loss of buoyancy should be made and recorded.
ISO	Water quality – determination of the toxic effect of water constituents and waste water on duckweed (*Lemna minor*) – duckweed growth inhibition test	ISO 20079	2005			*Cannot review ($).*
OECD	OECD guidelines for the testing of chemicals: *Lemna* sp. growth inhibition test	202	2004	7-day test Chemicals Static, semi-static, flow-through	Inhibition of growth Total frond area Dry or fresh weight	Recommends that changes in plant development, e.g. in frond size, appearnace, indication of necrosis, chlorosis or gibbosity, colony break-up or loss of buoyancy, and in root length and appearance be noted.
US EPA	Ecological effects test guidelines: OPPTS 850.4400 aquatic plant toxicity test used *Lemna* spp., tiers I and II	EPA-712-C-96-156	1996	7, 14-day test Chemicals Static-renewal		Recommends that any change in frond development or appearance including increase in number, decrease in size, necrosis, chlorsis should be recorded.

Hyalella azteca

Regulatory Body	Title	Reference #	Date	Test Conditions	Endpoints	Discussion of Behaviour
ASTM	Standard test method for measuring the toxicity of sediment-associated contaminants with freshwater invertebrates	E1706-05	2010	10-day test Sediments Static renewal	Survival Growth	Mentions that test organisms should not show apparent stress (unusual behavior) in culturing. States that all chambers should be checked daily and observations made to assess test organism behavior such as sediment avoidance.
Environment Canada	BTM: Test for survival and growth in sediment using the freshwater amphipod *Hyalella azteca*	EPS 1/RM/33	1997	14-day test Sediments Static, static-renewal	Survival Growth	Recommends observations of amphipods in chamber seen emerged from sediment, and their behaviour.
Ontario MOE	*Hyalella azteca* KCl acute lethality testing of chemicals	SOP HA3.v5	2012	96-hour test Chemicals Static	Survival	Acceptability criteria includes no abnormal behaviour in control.
US EPA	Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates	EPA-600-R-99-064	2nd Ed., 2000	10-day test Sediments Static, water-renewal	Survival Growth	Recommends checking chambers daily to make organism behaviour observations such as sediment avoidance or burrowing activity.
	Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates	EPA-600-R-99-064	2nd Ed., 2000	42-day test Sediments Static-renewal, flow-through	Survival Growth Reproduction	Recommends checking chambers daily to make organism behaviour observations such as sediment avoidance.

Lumbriculus variegatus

Regulatory Body	Title	Reference #	Date	Test Conditions	Endpoints	Discussion of Behaviour
OECD	OECD guidelines for the testing of chemicals: sediment-water *Lumbriculus* toxicity test using spiked sediment	225	2007	28-day Spiked sediments Static	Survival Reproduction	Recommends observing worms during exposure to assess visually any behavioural differences, e.g. sediment avoidance, fecal pellets visible on the sediment surface, compared to controls.
US EPA	Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates	EPA-600-R-99-064	2nd Ed. 2000	28-day bioaccumulation test Sediments Static-renewal, flow-through	Bioaccumulation	Recommends checking chambers daily to make organism behaviour observations such as sediment avoidance or burrowing activity; however, notes that burrowing may be difficult to see.
Ontario MOE	Bioaccumulation of sediment-associated contaminants in freshwater organisms	SOP BIOACC.v1.2	2011	28-day bioaccumulation test Sediments Static	Survival Growth	Acceptability criteria includes to altered behaviour or signs of stress (e.g., lack of burrowing, etc.)

Appendix III. Modified Bristol's Media Preparation (MOE, 2012b)

The following outlines the preparation of Modified Bristol's Media as per MOE (2012b) for non-axenic culturing of green alga *P. subcapitata* and *C. fusca*, with the following four modifications:

1. Replaced 2.5 g calcium chloride dihydrate ($CaCl_2\ 2H_2O$, mw = 147.014 g/mol) with 1.89 g anydrous calcium chloride ($CaCl_2$, mw = 110.98 g/mol).

$$\frac{2.5\text{ g CaCl2 2H2O}}{147.014\text{ g/mol}} = \frac{x}{110.98\text{ g/mol}}$$

$$(2.5\text{ g } CaCl_2\ 2H_2O)(110.98\text{ g/mol}) = (x\text{ g } CaCl_2)(147.014\text{ g/mol})$$

$$x = 1.89\text{ g } CaCl_2$$

2. Replaced 31 g potassium hydroxide (KOH, mw = 56.11 g/mol), or 5 mL of a 0.552 M KOH solution with 0.46 mL of a 6 M KOH solution.

$$\frac{31\text{ g}}{1\text{ L}} \text{x } \frac{1\text{ mol}}{56.11\text{ g}} = 0.552\frac{\text{mol}}{\text{L}}\text{ KOH}$$

$$C_1V_1 = C_2V_2$$

$$(6\text{ mol/L})V_1 = (0.552\text{ mol/L})(0.005\text{ L})$$

$$V_1 = 0.46\text{ mL}$$

3. Replaced 0.18 g molybdenum trioxide (MoO_3, mw = 143.94 g/mol) with 0.30 g sodium molybdate dihydrate ($Na_2MoO_4\ 2H_2O$, mw = 241.964 g/mol).

$$\frac{0.18\text{g MoO3}}{143.94\text{ g/mol}} = \frac{x}{241.964\text{ g/mol}}$$

$$(0.18\text{ g } MoO_3)(241.964\text{ g/mol}) = (x\text{ g } Na_2MoO_4\ 2H_2O)(143.94\text{ g/mol})$$

$$x = 0.30\text{ g } Na_2MoO_4\ 2H_2O$$

4. Replaced 0.125 g cobalt nitrate hexahydrate ($Co(NO_3)\ 6H_2O$, mw = 291.03 g/mol) with 0.079 g anhydrous cobalt dinitrate ($Co(NO_3)_2$, mw = 182.943 g/mol).

$$\frac{0.125\text{ Co(NO3)6H2O}}{291.03\text{ g/mol}} = \frac{x}{182.943\text{ g/mol}}$$

$$(0.125\text{ } Co(NO_3)\ 6H_2O)(182.943\text{ g/mol}) = (x\text{ g } CaCl_2)(291.03\text{ g/mol})$$

$$x = 0.079\text{ g } Co(NO_3)_2$$

All stocks solutions were prepared in deionized water using volumetric flasks and an analytical balance. All stocks were kept in glass media bottles in the dark at 2-6°C. Macro nutrient stocks were prepared in 500 mL volumes. Minor nutrient stocks were prepared in 250 mL volumes. The micronutrient stock was prepared in a 1 L volume.

Table 1. Modified Bristol's Medium: Macro Nutrients Stocks

Stock Solution	Concentration (g/L)
1. Sodium Nitrate, $NaNO_3$	25
2. Calcium chloride, $CaCl_2$	1.89
3. Magnesium sulfate heptahydrate, $MgSO_4$ $7H_2O$	7.5
4. Dipotassium hydrogen phosphate, K_2HPO_4	7.5
5. Monopotassium phosphate KH_2PO_4	17.5
6. Sodium chlorine, NaCl	2.5

Table 2. Modified Bristol's Medium: Minor Nutrients Stocks

Stock Solution	Concentration (g/L)
7. Disodium ethylene diamine tetracetic acid, Na_2EDTA	50
8. Potassium hydroxide, KOH	6 mol/L
9. Ferric chloride hexahydrate, $FeCl_3$ $6H_2O$	4.84
10. Boric acid, H_3BO_3	11.4

Table 3. Modified Bristol's Medium: Micro Nutrients Stocks

Stock Solution	Concentration (g/L)
Manganese chloride, $MnCl_2$ $4H_2O$	0.385
Zinc sulfate heptahydrate, $ZnSO_4$ $7H_2O$	2.205
Sodium molybdate dihydrate, Na_2MoO_4 $2H_2O$	0.30
Copper sulfate pentahydrate, $CuSO_4$ $5H_2O$	0.395
Cobalt dinitrate, $Co(NO_3)_2$	0.079

The medium was prepared in 3.5 L batches in 4 L glass jars. To prepare one 3.5 L batch, 3 L of deionized water was first added to the 4 L glass jar. Stock solutions were measured using a graduated cylinder or pipette and added to the jar as outlined in Table 4.

Table 4. Preparation of 3.5 L batch of Modified Bristol's Medium

Stock Solution	mL
1. Sodium Nitrate, $NaNO_3$	17.5
2. Calcium chloride, $CaCl_2$	17.5
3. Magnesium sulfate heptahydrate, $MgSO_4$ $7H_2O$	17.5
4. Dipotassium hydrogen phosphate, K_2HPO_4	17.5
5. Monopotassium phosphate KH_2PO_4	17.5
6. Sodium chlorine, NaCl	17.5
7. Disodium ethylene diamine tetracetic acid, Na_2EDTA	1.75
8. Potassium hydroxide, KOH	0.16
9. Ferric chloride hexahydrate, $FeCl_3$ $6H_2O$	1.75
10. Boric acid, H_3BO_3	1.75
11. Micronutrients	7.0

The medium was stirred with a magnetic stir bar and a stir plate, and the pH was adjusted to 7.0 ± 0.1 with 6 M KOH. The medium was allowed to stir for several more minutes and topped up to 3.5 L with deionized water. The stir bar was removed, and the medium was inoculated with algae as per section 2.1.5 *Daphnia* culture medium.

Appendix IV. Preparation of Se and Vitamin B12 Stocks for Daphnia Culture Medium Supplementation

Selenium and vitamin B12 stocks were prepared for *Daphnia* culture medium supplementation as per MOE (2012a).

A 3 mg/L Se (mw = 78.96 g/mol) stock was prepared from sodium selenate decahydrate. 1.4 mg of sodium selenate decahydrate (Na_2SeO_4 $10H_2O$, mw = 369.09 g/mol) was weighed out using an analytical balance and dissolved in 100 mL deionized water in a volumetric flask. The selenium stock solution was poured into a media bottle and kept in the dark at 2-6°C. The stock can be kept up to 12 months. The *Daphnia* culture medium is supplemented once weekly at a rate of 1 mL Se stock per 1 L of medium for a final concentration of 3 μg per 1 L of culture medium.

$$\frac{3 \text{ mg Se}}{78.96 \text{ g/mol}} = \frac{x}{369.09 \text{ g/mol}}$$

(3 mg Se)(369.09 g/mol) = (x g $Na_2SeO_4 10H_2O$)(78.96 g/mol)

x = 14.02 mg $Na_2SeO_4 10H_2O$

A 10 mg/L vitamin B12 ($C_{63}H_{88}CoN_{14}O_{14}P$, mw = 1355.37 g/mol) stock was prepared. 0.001 g of vitamin B12 was weight out using an anaytical balance and dissolved in 100 mL of deionized water in a volumetric flask to give a final concentration of 1 mg Se/100 mL, or 10 mg/L. The vitamin B12 stock was poured into an amber bottle and kept in the dark at 2-6°C. The stock can be kept up to 4 weeks. The *Daphnia* culture medium is supplemented once weekly at a rate of 0.1 mL vitamin B12 stock per 1 L of medium for a final concentration of 1 ug vitamin B12 per 1 L of culture medium.

Appendix V. Swedish Standard Media Preparation (OECD, 2006)

The following outlines the preparation of Swedish Standard Media as per OECD (2006) for culturing of *L. minor*, with the following modification: stocks I, II, III, IV and VI were prepared as 10x stocks. Stock V was prepared as per OECD (2006) as a 1x stock.

All stocks solutions were prepared in deionized water using volumetric flasks and an analytical balance. All stocks were kept in glass media bottles in the dark at 2-6°C. Macro nutrient stocks were prepared in 500 mL volumes. Stocks I, II, III, IV and VI were prepared in 100 mL volumes. Stock V was prepared in a 1 L volume.

Table 1. SIS Media Stock Preparation

Stock Solution		Concentration (g/L)
I	$NaNO_3$	85.0
	KH_2PO_4	13.4
II	$MgSO_4\ 7H_2O$	150
III	$CaCl_2\ 2H_2O$	72
IV	Na_2CO_3	40
V	H_3BO_3	1.0
	$MnCl_2\ 4H_2O$	0.20
	$Na_2MoO_4\ 2H_2O$	0.010
	$ZnSO_4\ 7H_2O$	0.050
	$CuSO_4\ 5H_2O$	0.0050
	$Co(NO_3)_2\ 6H_2O$	0.010
VI	$FeCl_3\ 6H_2O$	1.7
	Na_2 EDTA $2H_2O$	2.8

The medium was prepared in 1 L batches in 1 L glass beakers. To prepare one 1 L batch, 900 mL of deionized water was first added to the beaker. Stock solutions were measured using a micropipette and added to the beaker as outlined in Table 2.

Table 2. Preparation of 1 L batch of SIS Media

Stock Solution (10x)	mL
I	1.50
II	0.75
III	0.75
IV	0.75
V (1x stock)	1.50
VI	0.75

The medium was stirred with a magnetic stir bar and a stir plate, and the pH was adjusted to 6.5 ± 0.2 with 1 M HCl. The medium was allowed to stir for several more minutes and topped up to 1 L with deionized water. The stir bar was removed, and the medium was poured into the appropriate vessel for *L. minor* culturing.

Appendix VI. Raw Data

Culturing Data

Daphnia magna

		Brood Number									
Brood Stock	**Time to first brood (days)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
11-Mar-A/B	10	11	25	31	54	40	38	41	48	15	41
12-Mar	11	10	32	38	57	30	13	47	13		
14-Mar	11	12	31	34	13	18	32	16			
24-Mar	10	8	15	10	13	22	35	46	42	44	33
16-Apr	10	12	12	21	28	32	45	52	37	32	
22-Apr	10	4	13	24	39	39	41	43	45	24	
23-Apr	9	9	21	33	42	35	39	44	42	30	
09-May	11	21	38	32	36	33	39				
16-May	10	18	32	17	39	42					
26-May	10	11	22								
28-May	11	15	24								
29-May	10	13	18								

Note: All data is number of neonates produced by adult in health jar, except for time-to-first brood (days)

Hyalella azteca

	Brood Stock A			**Brood Stock B**			**Brood Stock C**			**Brood Stock D**		
Date	# Adults	# Pairs	# Juveniles	# A	# P	# J	# A	# P	# J	# A	# P	# J
18-Sep-12	28	4	12									
26-Sep-12	22	5	51	22	7							
03-Oct-12	27	6	80	21	1	20	29	4	32			
11-Oct-12	27	7	38	19	3	19	28	11	7			
17-Oct-12	26	2	85	18+7	1+3	7	28	5	72			
25-Oct-12	26	4	61	25	6	43	27	6	11			
31-Oct-12	26	3	24	25	8	12	27	5	45			
07-Nov-12	26	7	25	24	5	34	24	6	31			
14-Nov-12	25+5	6+2	28	22+8	4+4	17	25+5	5+2	63			
21-Nov-12	29+1	5	96	27+3	8+1	56	28+2	9+1	43			
28-Nov-12	22+8	4+4	124	26+4	5+2	46	30	5	49			
08-Mar	13	2	8	16	4	18	18	4	38	10+10	1+1	44
15-Mar	13+17	1+4	12	15+15	3+5	45	17+13	5+1	25	18	3	25
22-Mar	29	2	6	25	3	29	26	6	48	17	4	12
05-Apr	24	4	68	22	7	92	25	8	112	13	3	92
13-Apr	22	2	48	21	3	53	22	3	55	14+17	4+2	16
20-Apr	22	4	24	18	6	46	22	10	49	27	5	59
27-Apr	20+10	1+5	33	18+12	2+6	70	20+10	3+5	51	24+6	2+3	25

04-May	30	8	58	30	4	43	30	9	54	26	1	28
11-May	28	4	73	25	4	86	25	3	48	23	2	3
18-May	27	5	29	24	4	5	25	6	38	19+10	1+4	8
25-May	26	4	47	21	3	43	25	6	72	26	4	28
01-Jun	26	9	67	20+10	5+2	21	24	6	43	24	4	34
08-Jun	22	3	38	28	4	57	24	6	54	24	2	29
16-Jun	22	6	40	28	8	29	24	7	63	21	1	20

Bioassay Data

Daphnia magna
Behaviour bioassay in 4-chlorophenol

Time (hours)	[4-CP] (ppm)	Replicate	Swimming Score	# Boundary crossings	Mobilization %
0.17	0	1	1	5	100%
0.17	0	2	1	5	100%
0.17	0	3	0	3	100%
0.17	0	4	2	0	100%
0.17	0	5	1	4	100%
0.17	0	6	1	4	100%
0.17	0.625	1	0	2	100%
0.17	0.625	2	1	4	100%
0.17	0.625	3	0	5	100%
0.17	0.625	4	0	5	100%
0.17	0.625	5	0	3	100%
0.17	0.625	6	0	4	100%
0.17	1.25	1	1	7	100%
0.17	1.25	2	1	5	100%
0.17	1.25	3	2	5	100%
0.17	1.25	4	1	5	100%
0.17	1.25	5	1	3	100%
0.17	1.25	6	2	6	100%
0.17	2.5	1	1	1	100%
0.17	2.5	2	1	3	100%
0.17	2.5	3	1	2	100%
0.17	2.5	4	1	1	100%
0.17	2.5	5	0	1	100%
0.17	2.5	6	1	3	100%
0.17	5	1	0	2	100%
0.17	5	2	2	0	100%
0.17	5	3	1	1	100%
0.17	5	4	2	1	100%
0.17	5	5	1	2	100%

0.17	5	6	2	2	100%
0.17	10	1	2	0	0%
0.17	10	2	2	0	0%
0.17	10	3	2	0	0%
0.17	10	4	2	0	0%
0.17	10	5	2	0	0%
0.17	10	6	2	0	0%
1	0	1	1	5	100%
1	0	2	1	5	100%
1	0	3	0	3	100%
1	0	4	2	0	100%
1	0	5	1	4	100%
1	0	6	1	4	100%
1	0.625	1	0	2	100%
1	0.625	2	1	4	100%
1	0.625	3	0	5	100%
1	0.625	4	0	5	100%
1	0.625	5	0	3	100%
1	0.625	6	0	4	100%
1	1.25	1	1	7	100%
1	1.25	2	1	5	100%
1	1.25	3	2	5	100%
1	1.25	4	1	5	100%
1	1.25	5	1	3	100%
1	1.25	6	2	6	100%
1	2.5	1	1	1	100%
1	2.5	2	1	3	100%
1	2.5	3	1	2	100%
1	2.5	4	1	1	100%
1	2.5	5	0	1	100%
1	2.5	6	1	3	100%
1	5	1	0	2	100%
1	5	2	2	0	100%
1	5	3	1	1	100%
1	5	4	2	1	100%
1	5	5	1	2	100%
1	5	6	2	2	100%
1	10	1	2	0	0%
1	10	2	2	0	0%
1	10	3	2	0	0%
1	10	4	2	0	0%
1	10	5	2	0	0%
1	10	6	2	0	0%

24	0	1	0	1	100%
24	0	2	0	1	100%
24	0	3	0	0	100%
24	0	4	0	0	100%
24	0	5	0	0	66%
24	0	6	0	1	100%
24	0.625	1	0	2	100%
24	0.625	2	0	1	100%
24	0.625	3	0	1	100%
24	0.625	4	0	2	100%
24	0.625	5	1	1	100%
24	0.625	6	1	0	100%
24	1.25	1	2	0	0%
24	1.25	2	2	1	66%
24	1.25	3	1	1	100%
24	1.25	4	1	1	66%
24	1.25	5	2	0	33%
24	1.25	6	1	1	100%
24	2.5	1	2	0	33%
24	2.5	2	2	0	0%
24	2.5	3	2	0	33%
24	2.5	4	2	0	0%
24	2.5	5	2	0	33%
24	2.5	6	2	0	0%
24	5	1	2	0	0%
24	5	2	2	0	33%
24	5	3	2	0	33%
24	5	4	2	0	0%
24	5	5	2	0	0%
24	5	6	2	0	33%
24	10	1	2	0	0%
24	10	2	2	0	0%
24	10	3	2	0	0%
24	10	4	2	0	0%
24	10	5	2	0	0%
24	10	6	2	0	0%

Life cycle bioassay in 4-chlorophenol

Age of Adults (Days)	Control-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
11	10	0	10	18	33	10	0	20	19	30
12	21	38	21	16	0	21	34	11	13	0

13	0	2	0	0	0	0	0	0	0	0

Age of Adults (Days)	0.625 ppm 4CP-1	0.625-2	0.625-3	0.625-4	0.625-5
11	0	8	9	11	0
12	31	24	9	23	28
13	0	0	13	0	0

Age of Adults (Days)	1.25 ppm 4CP-1	1.25-2	1.25-3	1.25-4	1.25-5
11	0	3	5	0	0
12	24	14	18	27	9
13	1	0	0	0	0

Age of Adults (Days)	2.50 ppm 4CP-1	2.50-2	2.50-3	2.50-4	2.50-5
11	0	0	0	0	0
12	0	0	0	0	0
13	0	0	0	0	0

Behaviour bioassay in TCC

Time (hours)	[TCC] (ppb)	Replicate	Swimming Score	# Boundary Crossings	Mobilization %
0.17	0	1	1	5	100%
0.17	0	2	1	3	100%
0.17	0	3	1	2	100%
0.17	0	4	1	3	100%
0.17	0	5	1	5	100%
0.17	0	6	1	4	100%
0.17	DMSO	1	0	1	100%
0.17	DMSO	2	0	1	100%
0.17	DMSO	3	1	2	100%
0.17	DMSO	4	1	2	100%
0.17	DMSO	5	0	2	100%
0.17	DMSO	6	0	1	100%
0.17	1.25	1	0	1	100%
0.17	1.25	2	0	1	100%
0.17	1.25	3	1	3	100%
0.17	1.25	4	1	2	100%
0.17	1.25	5	1	3	100%
0.17	1.25	6	0	2	100%
0.17	2.5	1	1	1	100%
0.17	2.5	2	0	1	100%
0.17	2.5	3	0	2	100%
0.17	2.5	4	1	3	100%

0.17	2.5	5	1	2	100%
0.17	2.5	6	1	3	100%
0.17	5	1	1	2	100%
0.17	5	2	1	4	100%
0.17	5	3	1	2	100%
0.17	5	4	1	3	100%
0.17	5	5	1	5	100%
0.17	5	6	1	2	100%
0.17	10	1	1	1	100%
0.17	10	2	1	2	100%
0.17	10	3	1	3	100%
0.17	10	4	1	2	100%
0.17	10	5	0	1	100%
0.17	10	6	0	2	100%
1	0	1	0	2	100%
1	0	2	0	1	100%
1	0	3	1	2	100%
1	0	4	0	2	100%
1	0	5	0	3	100%
1	0	6	0	1	100%
1	DMSO	1	1	0	100%
1	DMSO	2	0	2	100%
1	DMSO	3	1	1	100%
1	DMSO	4	1	2	100%
1	DMSO	5	1	4	100%
1	DMSO	6	0	3	100%
1	1.25	1	1	0	100%
1	1.25	2	0	1	100%
1	1.25	3	0	2	100%
1	1.25	4	1	2	100%
1	1.25	5	1	3	100%
1	1.25	6	0	1	100%
1	2.5	1	1	0	100%
1	2.5	2	1	2	100%
1	2.5	3	0	2	100%
1	2.5	4	1	2	100%
1	2.5	5	1	2	100%
1	2.5	6	1	2	100%
1	5	1	0	3	100%
1	5	2	0	2	100%
1	5	3	0	1	100%
1	5	4	0	2	100%
1	5	5	0	1	100%

1	5	6	1	2	100%
1	10	1	1	1	100%
1	10	2	1	2	100%
1	10	3	1	0	100%
1	10	4	0	1	100%
1	10	5	1	2	100%
1	10	6	0	2	100%
24	0	1	0	1	100%
24	0	2	0	0	100%
24	0	3	0	1	100%
24	0	4	1	0	100%
24	0	5	0	1	100%
24	0	6	0	0	100%
24	DMSO	1	1	0	100%
24	DMSO	2	1	0	100%
24	DMSO	3	0	1	100%
24	DMSO	4	1	1	100%
24	DMSO	5	0	0	100%
24	DMSO	6	1	0	100%
24	1.25	1	1	0	100%
24	1.25	2	1	0	100%
24	1.25	3	1	0	100%
24	1.25	4	0	3	100%
24	1.25	5	1	0	100%
24	1.25	6	1	0	100%
24	2.5	1	0	0	100%
24	2.5	2	1	1	100%
24	2.5	3	0	2	100%
24	2.5	4	1	1	100%
24	2.5	5	0	1	100%
24	2.5	6	1	1	100%
24	5	1	1	1	100%
24	5	2	1	0	100%
24	5	3	1	0	100%
24	5	4	0	1	100%
24	5	5	0	1	100%
24	5	6	1	0	100%
24	10	1	1	0	100%
24	10	2	1	1	100%
24	10	3	1	1	100%
24	10	4	1	0	100%
24	10	5	1	0	100%
24	10	6	1	0	100%

Life cycle bioassay in TCC

Survival in TCC

Age (Days)	5-1	5-2	5-3	5-4	5-5	10-1	10-2	10-3	10-4	10-5	20-1	20-2	20-3	20-4	20-5	40-1	40-2	40-3	40-4	40-5
1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	2	3	3	3
3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	0	0	1	0	0
4	3	3	3	3	3	3	3	3	3	3	1	2	1	0	2	0	0	0	0	0
5	3	3	3	3	3	3	3	3	3	3	1	2	1	0	1	0	0	0	0	0
6	3	3	3	3	3	3	3	3	3	3	1	2	0	0	1	0	0	0	0	0
7	3	3	3	3	3	3	3	3	3	3	1	1	0	0	0	0	0	0	0	0
8	3	3	3	3	3	3	3	3	3	3	1	1	0	0	0	0	0	0	0	0
9	3	3	3	3	3	3	3	3	3	3	1	1	0	0	0	0	0	0	0	0
10	3	3	3	3	3	3	3	3	3	3	0	0	0	0	0	0	0	0	0	0
11	3	3	3	3	3	3	3	3	3	3	0	0	0	0	0	0	0	0	0	0
12	3	3	3	3	3	3	3	3	3	3	0	0	0	0	0	0	0	0	0	0
13	3	3	3	3	3	3	3	3	3	3	0	0	0	0	0	0	0	0	0	0
14	3	3	3	3	3	3	3	3	3	3	0	0	0	0	0	0	0	0	0	0

Note: all alive (3/3) in all control and DMSO treatment replicates

Neonates produced in TCC

Age (Days)	Control-1	C-2	C-3	C-4	C-5
11	20	21	21	27	9
12	9	13	11	0	20
13	0	0	0	0	0

Age (Days)	DMSO-1	DMSO-2	DMSO-3	DMSO-4	DMSO-5
11	17	11	22	27	26
12	9	18	12	0	1
13	0	0	0	0	0

Age (Days)	5 ppm-1	5-2	5-3	5-4	5-5
11	26	19	20	25	31
12	0	10	10	0	0
13	0	0	0	0	0
14	0	0	0	0	0

Age (Days)	10ppm - 1	10-2	10-3	10-4	10-5
11	0	0	0	0	0
12	15	22	14	5	6
13	0	0	10	24	24
14	0	0	1	0	1

Note: No adults survived to produce first brood in 20 or 40 ppb TCC

Hyalella azteca

Behaviour bioassay in 4-chlorophenol

Time	[4-CP] (ppm)	Replicate	Locomotion Score	Body Score	Mobilization %
0.17	0	1	0	0	100%
0.17	0	2	0	0	100%
0.17	0	3	0	0	100%
0.17	0	4	0	0	100%
0.17	0	5	0	0	100%
0.17	0	6	0	0	100%
0.17	0.625	1	1	0	100%
0.17	0.625	2	1	0	100%
0.17	0.625	3	1	0	100%
0.17	0.625	4	0	0	100%
0.17	0.625	5	1	0	100%
0.17	0.625	6	1	0	100%
0.17	1.25	1	0	0	100%
0.17	1.25	2	0	0	100%
0.17	1.25	3	0	0	100%
0.17	1.25	4	0	0	100%
0.17	1.25	5	0	0	100%
0.17	1.25	6	0	0	100%
0.17	2.5	1	0	0	100%
0.17	2.5	2	0	0	100%
0.17	2.5	3	0	0	100%
0.17	2.5	4	0	0	100%
0.17	2.5	5	0	0	100%
0.17	2.5	6	0	0	100%
0.17	5	1	0	0	100%
0.17	5	2	1	0	100%
0.17	5	3	0	0	100%
0.17	5	4	0	0	100%

0.17	5	5	1	0	100%
0.17	5	6	0	0	100%
0.17	10	1	1	1	100%
0.17	10	2	1	0	100%
0.17	10	3	1	0	100%
0.17	10	4	1	1	100%
0.17	10	5	2	1	100%
0.17	10	6	1	1	100%
1	0	1	1	0	100%
1	0	2	1	0	100%
1	0	3	1	0	100%
1	0	4	1	0	100%
1	0	5	1	1	100%
1	0	6	1	1	100%
1	0.625	1	0	0	100%
1	0.625	2	0	0	100%
1	0.625	3	0	0	100%
1	0.625	4	1	0	100%
1	0.625	5	0	0	100%
1	0.625	6	0	0	100%
1	1.25	1	0	0	100%
1	1.25	2	0	0	100%
1	1.25	3	1	0	100%
1	1.25	4	0	0	100%
1	1.25	5	0	0	100%
1	1.25	6	0	0	100%
1	2.5	1	0	0	100%
1	2.5	2	0	0	100%
1	2.5	3	1	0	100%
1	2.5	4	0	0	100%
1	2.5	5	0	0	100%
1	2.5	6	0	0	100%
1	5	1	1	1	100%
1	5	2	1	1	66%
1	5	3	0	1	100%
1	5	4	1	1	100%
1	5	5	1	1	100%
1	5	6	1	1	66%
1	10	1	1	2	100%
1	10	2	2	2	100%
1	10	3	1	1	100%
1	10	4	2	2	66%
1	10	5	2	2	66%

1	10	6	1	1	100%
24	0	1	1	1	33%
24	0	2	1	1	100%
24	0	3	1	0	66%
24	0	4	1	1	66%
24	0	5	2	2	0%
24	0	6	2	2	33%
24	0.625	1	2	2	33%
24	0.625	2	2	2	33%
24	0.625	3	2	2	33%
24	0.625	4	1	2	33%
24	0.625	5	2	2	0%
24	0.625	6	2	2	0%
24	1.25	1	1	2	33%
24	1.25	2	2	2	33%
24	1.25	3	2	2	33%
24	1.25	4	1	1	66%
24	1.25	5	2	2	0%
24	1.25	6	2	2	33%
24	2.5	1	2	2	0%
24	2.5	2	2	2	33%
24	2.5	3	2	2	33%
24	2.5	4	1	1	66%
24	2.5	5	2	2	0%
24	2.5	6	2	2	0%
24	5	1	2	2	0%
24	5	2	2	2	0%
24	5	3	2	2	0%
24	5	4	2	2	0%
24	5	5	2	2	0%
24	5	6	2	2	0%
24	10	1	2	2	33%
24	10	2	2	2	0%
24	10	3	2	2	0%
24	10	4	2	2	0%
24	10	5	2	2	0%
24	10	6	2	2	0%

Behaviour bioassay in TCC

Time	[TCC] (ppb)	Replicate	Locomotion Score	Body Score	Mobilization %
0.17	0	1	0	0	100%
0.17	0	2	0	0	100%
0.17	0	3	1	0	100%
0.17	0	4	0	1	100%
0.17	0	5	0	0	100%
0.17	0	6	0	0	100%
0.17	DMSO	1	0	0	100%
0.17	DMSO	2	0	0	100%
0.17	DMSO	3	1	0	100%
0.17	DMSO	4	0	0	100%
0.17	DMSO	5	0	0	100%
0.17	DMSO	6	0	0	100%
0.17	1.25	1	0	0	100%
0.17	1.25	2	1	0	100%
0.17	1.25	3	0	0	100%
0.17	1.25	4	0	0	100%
0.17	1.25	5	1	0	100%
0.17	1.25	6	0	0	100%
0.17	2.5	1	0	0	100%
0.17	2.5	2	1	0	100%
0.17	2.5	3	0	0	100%
0.17	2.5	4	0	0	100%
0.17	2.5	5	0	1	100%
0.17	2.5	6	0	0	100%
0.17	5	1	0	0	100%
0.17	5	2	0	0	100%
0.17	5	3	1	0	100%
0.17	5	4	0	0	100%
0.17	5	5	1	1	100%
0.17	5	6	0	0	100%
0.17	10	1	1	1	100%
0.17	10	2	0	0	100%
0.17	10	3	1	0	100%
0.17	10	4	0	0	100%
0.17	10	5	0	1	100%
0.17	10	6	0	0	100%
1	0	1	0	0	100%
1	0	2	0	0	100%

1	0	3	0	0	100%
1	0	4	0	1	100%
1	0	5	0	0	100%
1	0	6	0	0	100%
1	DMSO	1	0	0	100%
1	DMSO	2	0	0	100%
1	DMSO	3	1	0	100%
1	DMSO	4	0	0	100%
1	DMSO	5	0	0	100%
1	DMSO	6	0	0	100%
1	1.25	1	0	0	100%
1	1.25	2	1	0	100%
1	1.25	3	0	0	100%
1	1.25	4	0	0	100%
1	1.25	5	1	0	100%
1	1.25	6	0	0	100%
1	2.5	1	0	0	100%
1	2.5	2	0	0	100%
1	2.5	3	0	0	100%
1	2.5	4	0	0	100%
1	2.5	5	0	1	100%
1	2.5	6	0	0	100%
1	5	1	0	0	100%
1	5	2	0	0	100%
1	5	3	1	0	66%
1	5	4	0	0	100%
1	5	5	1	1	100%
1	5	6	0	0	100%
1	10	1	1	1	100%
1	10	2	0	0	100%
1	10	3	1	1	100%
1	10	4	1	0	66%
1	10	5	0	1	100%
1	10	6	0	0	100%
24	0	1	0	0	100%
24	0	2	0	0	100%
24	0	3	0	0	100%
24	0	4	0	1	66%
24	0	5	0	0	100%
24	0	6	0	0	100%
24	DMSO	1	0	0	100%
24	DMSO	2	0	0	100%
24	DMSO	3	1	0	66%

24	DMSO	4	0	0	100%
24	DMSO	5	0	1	100%
24	DMSO	6	0	0	100%
24	1.25	1	0	0	100%
24	1.25	2	1	0	100%
24	1.25	3	0	0	66%
24	1.25	4	0	0	100%
24	1.25	5	1	0	100%
24	1.25	6	0	0	100%
24	2.5	1	0	0	100%
24	2.5	2	0	0	66%
24	2.5	3	0	0	66%
24	2.5	4	0	0	66%
24	2.5	5	0	1	33%
24	2.5	6	0	0	100%
24	5	1	0	0	100%
24	5	2	0	0	100%
24	5	3	1	0	100%
24	5	4	0	0	100%
24	5	5	1	1	66%
24	5	6	0	0	100%
24	10	1	1	1	33%
24	10	2	0	0	33%
24	10	3	1	1	66%
24	10	4	1	1	66%
24	10	5	0	1	100%
24	10	6	0	0	66%

Lumbriculus variegatus

Behaviour bioassay in 4-chlorophenol

Time (hours)	[4-CP] (ppm)	Replicate	Locomotion Score	Body Score	Mobilization %
0.17	0	1	0	1	100%
0.17	0	2	0	0	100%
0.17	0	3	0	0	100%
0.17	0	4	0	0	100%
0.17	0	5	0	0	100%
0.17	0	6	0	0	100%
0.17	1.25	1	0	0	100%
0.17	1.25	2	0	0	100%
0.17	1.25	3	0	0	100%
0.17	1.25	4	0	0	100%

0.17	1.25	5	0	0	100%
0.17	1.25	6	0	0	100%
0.17	3.12	1	1	1	100%
0.17	3.12	2	1	0	100%
0.17	3.12	3	0	0	100%
0.17	3.12	4	1	0	100%
0.17	3.12	5	0	1	100%
0.17	3.12	6	1	0	100%
0.17	6.25	1	0	0	100%
0.17	6.25	2	1	0	100%
0.17	6.25	3	0	0	100%
0.17	6.25	4	1	0	100%
0.17	6.25	5	1	0	100%
0.17	6.25	6	0	1	100%
0.17	12.5	1	1	0	100%
0.17	12.5	2	1	0	100%
0.17	12.5	3	0	0	100%
0.17	12.5	4	1	0	100%
0.17	12.5	5	0	0	100%
0.17	12.5	6	1	1	100%
0.17	25	1	2	1	100%
0.17	25	2	2	0	100%
0.17	25	3	2	0	100%
0.17	25	4	2	0	100%
0.17	25	5	2	0	100%
0.17	25	6	2	1	100%
1	0	1	0	0	100%
1	0	2	0	1	100%
1	0	3	0	0	100%
1	0	4	0	0	100%
1	0	5	0	0	100%
1	0	6	0	0	100%
1	1.25	1	0	0	100%
1	1.25	2	0	0	100%
1	1.25	3	0	0	100%
1	1.25	4	0	0	100%
1	1.25	5	0	0	100%
1	1.25	6	0	0	100%
1	3.12	1	1	1	100%
1	3.12	2	1	1	100%
1	3.12	3	0	0	100%
1	3.12	4	1	1	100%
1	3.12	5	0	0	100%

1	3.12	6	1	0	100%
1	6.25	1	1	1	100%
1	6.25	2	1	0	100%
1	6.25	3	1	0	100%
1	6.25	4	1	1	100%
1	6.25	5	1	0	100%
1	6.25	6	0	1	100%
1	12.5	1	1	0	100%
1	12.5	2	1	0	100%
1	12.5	3	2	0	100%
1	12.5	4	1	0	100%
1	12.5	5	2	0	100%
1	12.5	6	2	0	0%
1	25	1	2	0	100%
1	25	2	2	1	100%
1	25	3	2	0	100%
1	25	4	2	1	100%
1	25	5	2	0	0%
1	25	6	2	0	0%
24	0	1	0	0	100%
24	0	2	0	1	100%
24	0	3	0	0	100%
24	0	4	0	0	100%
24	0	5	0	0	100%
24	0	6	0	0	100%
24	1.25	1	0	0	100%
24	1.25	2	0	0	100%
24	1.25	3	0	0	100%
24	1.25	4	0	0	100%
24	1.25	5	0	0	100%
24	1.25	6	0	0	100%
24	3.12	1	1	0	100%
24	3.12	2	1	0	100%
24	3.12	3	1	0	100%
24	3.12	4	2	0	0%
24	3.12	5	1	0	100%
24	3.12	6	1	0	100%
24	6.25	1	1	0	100%
24	6.25	2	1	0	100%
24	6.25	3	1	0	100%
24	6.25	4	1	0	100%
24	6.25	5	1	0	100%
24	6.25	6	2	0	0%

24	12.5	1	2	0	100%
24	12.5	2	2	0	100%
24	12.5	3	2	0	0%
24	12.5	4	2	0	100%
24	12.5	5	2	0	100%
24	12.5	6	2	0	0%
24	25	1	2	0	0%
24	25	2	2	0	0%
24	25	3	2	1	0%
24	25	4	2	0	100%
24	25	5	2	0	100%
24	25	6	2	0	0%

Behaviour bioassay in TCC

Time (hours)	[TCC] (ppb)	Replicate	Locomotion Score	Body Score	Mobilization %
0.17	0	1	0	0	100%
0.17	0	2	0	0	100%
0.17	0	3	0	0	100%
0.17	0	4	0	1	100%
0.17	0	5	0	0	100%
0.17	0	6	0	0	100%
0.17	DMSO	1	0	0	100%
0.17	DMSO	2	1	0	100%
0.17	DMSO	3	0	0	100%
0.17	DMSO	4	0	0	100%
0.17	DMSO	5	0	0	100%
0.17	DMSO	6	0	0	100%
0.17	1.25	1	0	0	100%
0.17	1.25	2	0	0	100%
0.17	1.25	3	0	0	100%
0.17	1.25	4	0	1	100%
0.17	1.25	5	0	0	100%
0.17	1.25	6	0	0	100%
0.17	2.5	1	0	0	100%
0.17	2.5	2	1	0	100%
0.17	2.5	3	0	0	100%
0.17	2.5	4	0	0	100%
0.17	2.5	5	0	0	100%
0.17	2.5	6	0	0	100%
0.17	5	1	0	0	100%
0.17	5	2	0	0	100%

0.17	5	3	0	0	100%
0.17	5	4	1	1	100%
0.17	5	5	1	1	100%
0.17	5	6	0	0	100%
0.17	10	1	1	1	100%
0.17	10	2	1	0	100%
0.17	10	3	1	0	100%
0.17	10	4	1	1	100%
0.17	10	5	1	0	100%
0.17	10	6	1	0	100%
1	0	1	0	0	100%
1	0	2	0	1	100%
1	0	3	1	0	100%
1	0	4	0	0	100%
1	0	5	0	1	100%
1	0	6	0	0	100%
1	DMSO	1	0	0	100%
1	DMSO	2	1	0	100%
1	DMSO	3	0	0	100%
1	DMSO	4	0	0	100%
1	DMSO	5	0	0	100%
1	DMSO	6	0	0	100%
1	1.25	1	1	0	100%
1	1.25	2	0	0	100%
1	1.25	3	0	0	100%
1	1.25	4	1	1	100%
1	1.25	5	0	0	100%
1	1.25	6	0	0	100%
1	2.5	1	0	0	100%
1	2.5	2	0	0	100%
1	2.5	3	0	0	100%
1	2.5	4	0	0	100%
1	2.5	5	0	0	100%
1	2.5	6	0	0	100%
1	5	1	0	0	100%
1	5	2	0	0	100%
1	5	3	0	0	100%
1	5	4	1	0	100%
1	5	5	1	1	100%
1	5	6	0	0	100%
1	10	1	1	0	100%
1	10	2	1	0	100%
1	10	3	1	1	100%

1	10	4	1	0	100%
1	10	5	1	0	100%
1	10	6	1	0	100%
24	0	1	1	0	100%
24	0	2	0	0	100%
24	0	3	0	1	100%
24	0	4	0	0	100%
24	0	5	0	0	100%
24	0	6	0	0	100%
24	DMSO	1	0	0	100%
24	DMSO	2	1	1	100%
24	DMSO	3	0	0	100%
24	DMSO	4	0	0	100%
24	DMSO	5	0	0	100%
24	DMSO	6	0	0	100%
24	1.25	1	0	0	100%
24	1.25	2	0	0	100%
24	1.25	3	0	0	100%
24	1.25	4	0	0	100%
24	1.25	5	0	0	100%
24	1.25	6	0	0	100%
24	2.5	1	0	0	100%
24	2.5	2	0	0	100%
24	2.5	3	0	0	100%
24	2.5	4	0	0	100%
24	2.5	5	0	0	100%
24	2.5	6	0	0	100%
24	5	1	0	0	100%
24	5	2	0	0	100%
24	5	3	0	0	100%
24	5	4	0	0	100%
24	5	5	0	0	100%
24	5	6	0	0	100%
24	10	1	2	1	100%
24	10	2	1	0	100%
24	10	3	1	0	100%
24	10	4	2	0	100%
24	10	5	1	0	100%
24	10	6	1	0	100%

Lemna minor

Appearance bioassay in 4-chlorophenol

Time (days)	[4-CP] (ppm)	Rep	Chlorosis Score	Necrosis Score	Colony Break-up Score
0	0	1	0	0	0
0	0	2	0	0	0
0	0	3	0	0	0
0	25	1	0	0	0
0	25	2	0	0	0
0	25	3	0	0	0
0	50	1	0	0	0
0	50	2	0	0	0
0	50	3	0	0	0
0	100	1	0	0	0
0	100	2	0	0	0
0	100	3	0	0	0
0	200	1	0	0	0
0	200	2	0	0	0
0	200	3	0	0	0
0	400	1	0	0	0
0	400	2	0	0	0
0	400	3	0	0	0
2	0	1	0	0	0
2	0	2	0	0	0
2	0	3	0	0	0
2	25	1	0	0	0
2	25	2	0	0	0
2	25	3	0	0	0
2	50	1	0	0	0
2	50	2	0	0	0
2	50	3	0	0	0
2	100	1	1	1	0
2	100	2	1	0	0
2	100	3	1	0	0
2	200	1	1	1	2
2	200	2	1	1	2
2	200	3	1	1	2
2	400	1	2	2	0
2	400	2	2	2	0
2	400	3	2	2	0
7	0	1	0	0	0
7	0	2	0	0	0
7	0	3	0	0	0

7	25	1	1	1	1
7	25	2	1	1	1
7	25	3	1	1	1
7	50	1	1	1	1
7	50	2	1	1	1
7	50	3	1	1	1
7	100	1			
7	100	2	1	1	2
7	100	3	1	1	2
7	200	1	2	2	2
7	200	2	2	2	2
7	200	3	2	2	2
7	400	1	2	2	0
7	400	2	2	2	0
7	400	3	2	2	0

Life cycle bioassay in 4-chlorophenol

Time (Days)	Concentration (ppm)	Replicate	# Fronds
0	0	1	6
0	0	2	6
0	0	3	6
0	25	1	6
0	25	2	6
0	25	3	6
0	50	1	6
0	50	2	6
0	50	3	6
0	100	1	6
0	100	2	6
0	100	3	6
0	200	1	6
0	200	2	6
0	200	3	6
0	400	1	6
0	400	2	6
0	400	3	6
1	0	1	13
1	0	2	13
1	0	3	10
1	25	1	6
1	25	2	9

1	25	3	7
1	50	1	6
1	50	2	7
1	50	3	6
1	100	1	6
1	100	2	6
1	100	3	6
1	200	1	6
1	200	2	6
1	200	3	7
1	400	1	6
1	400	2	7
1	400	3	6
2	0	1	18
2	0	2	17
2	0	3	17
2	25	1	10
2	25	2	15
2	25	3	16
2	50	1	8
2	50	2	8
2	50	3	7
2	100	1	6
2	100	2	6
2	100	3	6
2	200	1	6
2	200	2	6
2	200	3	8
2	400	1	6
2	400	2	8
2	400	3	6
3	0	1	29
3	0	2	33
3	0	3	25
3	25	1	15
3	25	2	19
3	25	3	18
3	50	1	10
3	50	2	10
3	50	3	8
3	100	1	6
3	100	2	6
3	100	3	6

3	200	1	6
3	200	2	6
3	200	3	8
3	400	1	6
3	400	2	8
3	400	3	6
4	0	1	50
4	0	2	52
4	0	3	42
4	25	1	18
4	25	2	22
4	25	3	19
4	50	1	13
4	50	2	13
4	50	3	9
4	100	1	6
4	100	2	6
4	100	3	6
4	200	1	6
4	200	2	6
4	200	3	8
4	400	1	6
4	400	2	8
4	400	3	6
5	0	1	75
5	0	2	85
5	0	3	61
5	25	1	18
5	25	2	35
5	25	3	36
5	50	1	16
5	50	2	16
5	50	3	13
5	100	1	
5	100	2	6
5	100	3	6
5	200	1	6
5	200	2	6
5	200	3	8
5	400	1	6
5	400	2	8
5	400	3	6
7	0	1	163

7	0	2	185
7	0	3	149
7	25	1	30
7	25	2	43
7	25	3	45
7	50	1	17
7	50	2	18
7	50	3	16
7	100	1	
7	100	2	6
7	100	3	6
7	200	1	6
7	200	2	8
7	200	3	6
7	400	1	6
7	400	2	8
7	400	3	6

Appearance bioassay in TCC

All scores at all time points 0.

Life cycle bioassay in TCC

Time (Days)	[TCC] (ppb)	Replicate	# Fronds
0	Reference	1	6
0	Reference	2	6
0	Reference	3	6
0	0.01% DMSO	1	6
0	0.01% DMSO	2	6
0	0.01% DMSO	3	6
0	5	1	6
0	5	2	6
0	5	3	6
0	10	1	6
0	10	2	6
0	10	3	6
0	20	1	6
0	20	2	6
0	20	3	6
0	40	1	6
0	40	2	6
0	40	3	6
1	Reference	1	7

1	Reference	2	12
1	Reference	3	10
1	0.01% DMSO	1	10
1	0.01% DMSO	2	8
1	0.01% DMSO	3	9
1	5	1	10
1	5	2	11
1	5	3	12
1	10	1	9
1	10	2	10
1	10	3	11
1	20	1	10
1	20	2	8
1	20	3	8
1	40	1	11
1	40	2	9
1	40	3	10
2	Reference	1	14
2	Reference	2	17
2	Reference	3	15
2	0.01% DMSO	1	15
2	0.01% DMSO	2	14
2	0.01% DMSO	3	14
2	5	1	16
2	5	2	17
2	5	3	17
2	10	1	16
2	10	2	16
2	10	3	16
2	20	1	18
2	20	2	16
2	20	3	15
2	40	1	14
2	40	2	14
2	40	3	15
3	Reference	1	18
3	Reference	2	21
3	Reference	3	20
3	0.01% DMSO	1	20
3	0.01% DMSO	2	18
3	0.01% DMSO	3	20
3	5	1	18
3	5	2	19

3	5	3	22
3	10	1	18
3	10	2	22
3	10	3	20
3	20	1	20
3	20	2	17
3	20	3	18
3	40	1	20
3	40	2	18
3	40	3	21
4	Reference	1	24
4	Reference	2	34
4	Reference	3	31
4	0.01% DMSO	1	36
4	0.01% DMSO	2	24
4	0.01% DMSO	3	26
4	5	1	31
4	5	2	33
4	5	3	35
4	10	1	31
4	10	2	34
4	10	3	31
4	20	1	33
4	20	2	28
4	20	3	23
4	40	1	28
4	40	2	25
4	40	3	28
5	Reference	1	39
5	Reference	2	48
5	Reference	3	43
5	0.01% DMSO	1	51
5	0.01% DMSO	2	34
5	0.01% DMSO	3	39
5	5	1	44
5	5	2	47
5	5	3	45
5	10	1	50
5	10	2	46
5	10	3	48
5	20	1	50
5	20	2	38
5	20	3	33

5	40	1	37
5	40	2	38
5	40	3	39
6	Reference	1	54
6	Reference	2	73
6	Reference	3	61
6	0.01% DMSO	1	73
6	0.01% DMSO	2	50
6	0.01% DMSO	3	51
6	5	1	61
6	5	2	59
6	5	3	62
6	10	1	59
6	10	2	66
6	10	3	59
6	20	1	60
6	20	2	52
6	20	3	46
6	40	1	53
6	40	2	48
6	40	3	51
7	Reference	1	72
7	Reference	2	106
7	Reference	3	86
7	0.01% DMSO	1	126
7	0.01% DMSO	2	70
7	0.01% DMSO	3	73
7	5	1	93
7	5	2	87
7	5	3	84
7	10	1	96
7	10	2	94
7	10	3	87
7	20	1	90
7	20	2	68
7	20	3	59
7	40	1	76
7	40	2	66
7	40	3	68

End of raw data

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