Assessing behavioural and physiological responses of three aquatic invertebrates to atrazine and tributyltin in a multi-species, early-warning biomonitoring technology

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Abstract

Assessing behavioural and physiological responses of three aquatic invertebrates to atrazine and tributyl tin in a multi-species, early-warning biomonitoring technology

Recent global events and anthropogenic changes to the natural environment have raised concerns about the quality of drinking water consumed by the public throughout the world. Traditional chemical testing is slow and is not performed for all possible contaminants, necessitating the development of innovative new technology to detect and mitigate threats to human health. The development of a multi-species, early-warning biomonitoring technology, based on behavioural and physiological changes in aquatic organisms, greatly furthers this goal. In this study, changes in movement behaviour and respiration rates were examined in three aquatic species, *Daphnia magna, Hyalella azteca*, and *Lumbriculus variegatus,* exposed to varying concentrations of TBT and atrazine, using digital video analysis and direct oxygen measurement. Different parameters of movement were examined and evaluated for inclusion in a multi-species, early-warning biomonitoring technology and the utility of incorporating these parameters into a model to determine classes and concentrations of various contaminants is discussed. An evaluation of whether or not direct measurement of oxygen consumption rates is feasible and useful for inclusion in a multi-species, early-warning biomonitoring technology is also discussed.

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List of Abbreviations

AChE – Acetycholinesterase

BEWS – Biological Early Warning System

CTAB - Cetyltrimethylammonium bromide

DBT – Dibutyltin

DMSO – Dimethysulfoxide

DO – Dissolved Oxygen

EC – Effective Concentration

EU – European Union

FSH – Follicle Stimulating Hormone

IARC – International Agency for Research on Cancer

IC_{50} – the concentration of a contaminant which inhibits a given behaviour or other parameter in 50% of organisms in a bioassay over a given period of time

IMO – International Maritime Organization

LC_{50} – the concentration of a contaminant which is lethal to 50% of organisms in a bioassay over a given period of time

LH – Luteinizing Hormone

MBT – Monobutyltin

MFB – Multi-species Freshwater Biomonitor

NOEC – No Observable Effects Concentration

NSERC – Natural Sciences and Engineering Research Council

PCB – polychlorobiphenyl

PCP - pentachlorophenol

ppb – Parts Per Billion

ppm - Parts Per Million

ppt – Parts Per Trillion

ROS – Reactive Oxygen Species

TBT – Tributyltin

USEPA – United States Environmental Protection Agency

WHO – World Health Organization

1.0 Introduction

1.1 Project Background

Recent global events and anthropogenic changes to the natural environment have raised concerns about the quality of drinking water consumed by the public throughout the world. The monitoring of water quality is important in order to identify potentially toxic substances introduced to the environment, both purposefully and accidentally, which may cause adverse health effects in humans. The development of new ways in which to detect these compounds is important in order to protect human health and ensure that drinking water is highly palatable (Martins *et al.*, 2007a). Past advancements in the field of water treatment have included the development of organism-based systems which can help to detect contaminants in freshwater. Such systems that monitor behavioural responses of organisms and changes in behaviour let operators know a contaminant is present. These systems have become associated with the term BEWS, Biological Early Warning Systems, and have been developed for use in Europe with the hopes that they may someday be widespread at water treatment plants throughout the world. Although the potential exists for these systems to be quite useful, they have several limitations and their full utility has not been seen to date.

This thesis is part of a large-scale NSERC project aimed at developing a more holistic, real-time multi-organism early-warning biomonitoring technology which will be fully implemented at a water treatment facility in southern Ontario within the next five years. This technology will build upon currently developed BEWS and provide a new, much more effective, organism-based way for water treatment facility operators to detect specific contaminants which may be entering public water supplies.

The technology we will be developing is unique in several ways, and expands past the limitations of currently developed BEWS. This technology will be unique in that it will encompass the responses of several phyla of organisms at the same time, something which has not been seen in past BEWS, which have typically relied on the responses of only one organism at a time. As different organisms have different tolerances to chemical contaminants it is possible that the risks posed by these contaminants could be over- or underestimated based on responses by only one organism (McCarthy, 2007). Using a suite of organisms will allow for greater

sensitivity of the technology and will help to ensure that even very low concentrations of a contaminant are detected. In this thesis project, three test species are used which inhabit various compartments of the aquatic environment. These include *Daphnia magna*, a free-swimming crustacean which inhabits the water column, *Hyalella azteca*, an arthropod which lives at the sediment-water interface, and *Lumbriculus variegatus*, a sediment-dwelling aquatic worm. Simultaneously conducted thesis projects are also examining changes in plant species, protists and bivalves. The use of many different classes of organisms will help to detect sub-lethal concentrations of many types of contaminants due to the variation of sensitivity across a spectrum of species.

Our technology will also incorporate a modelling component, something which has been lacking in previous BEWS. In previously developed systems, the changes in behaviour of an organism have been used to alert researchers and plant operators that a chemical contaminant is present. These systems are not able to provide any information about the specific type of contaminant or the concentration that may be present, merely that a chemical is present. Extensive chemical testing is then required to determine the exact type of contaminant and whether it is present at a concentration which may be of concern for human health. By performing laboratory-based bioassays, such as those discussed in this thesis, specific responses by an organism to a given concentration of contaminant can be determined. This information can then be incorporated into a model which can be used by water treatment plant operators to determine the exact type and concentration of contaminant, greatly reducing the need for more testing and helping to determine if concentrations present are in fact of danger to human health. This project is also important in that it is being developed in conjunction with a water treatment facility and will eventually be implemented in a real-world setting. The use of past BEWS has mostly taken place in a laboratory setting, with limited field application. Our technology will use multiple species in the field and will help to improve operations efficiency at a functioning water treatment plant.

Finally, our project is unique in that it will incorporate changes in both organism behaviour and physiology. Past BEWS have focused mainly on changes in organism behaviour, while for the most part ignoring changes in physiological parameters which can also be easily and rapidly detected and are highly sensitive to contaminants in the environment. The technology

exists to rapidly detect changes in many physiological parameters, including organism respiration. Changes in physiology may be more sensitive to certain chemicals than behavioural changes, and therefore monitoring respiration is highly important to ensure that low levels of contaminants are consistently detected. Changes in the respiration rate of organisms can also be modelled to help discriminate the exact chemical and its concentration level. Simultaneous evaluation of behaviour and physiology will allow for a more complete picture of the exact impacts a contaminant may be having at an organismal level and will allow for better discrimination between various classes of contaminants.

1.2 Biological Early Warning Systems (BEWS)

Research in the 1990s lead to the development of systems commonly referred to as Biological Early Warning Systems (BEWS). The aim of this project is not to develop a BEWS, but to create a much more holistic multi-organism based early-warning biomonitoring technology capable of identifying specific classes and concentrations of contaminants. However, it is important to understand the principles, importance, limitations, and applications of currently existing systems in order to fully comprehend how the current project will move beyond these systems and contribute to this exciting area of research.

1.2.1 Background

An emerging method of water quality control is the use of Biological Early Warning Systems (BEWS), systems which employ living organisms to monitor the level of contaminants in drinking water sources. The development of BEWS for monitoring of water quality supports United Nations Agenda 21, which aims to protect and manage the world's freshwater (Gerhardt *et al.*, 2006b). These systems are commonly used in Europe (Lechelt *et al.*, 2000; Watson *et al.*, 2007) and the use of technologies based on their main principles would be greatly beneficial in Canadian water treatment plants in order to detect potentially dangerous aquatic contaminants before they enter the drinking water supply.

In BEWS, changes in behavioural and physiological parameters of living organisms are used to detect sub-lethal concentrations of pollutants and to monitor overall water quality (Michels *et al.*, 1999; Kieu *et al.*, 2001). By continuously monitoring certain behavioural and physiological measures under normal conditions, it is possible to detect changes in these parameters which may be induced by stressors such as low levels of chemical pollutants and

other changes in water quality (Michels *et al.,* 1999; Watson *et al.,* 2007). These changes can be detected rapidly and are made in response to contaminant concentrations which are much lower than those associated with mortality in the organisms (Kieu *et al.,* 2001). Changes can be induced in response to both organic and inorganic compounds and are therefore useful for monitoring a variety of contaminants at one time (Mikol *et al*., 2007).

BEWS generally consist of three components including test organisms, an automated detection system, and an alarm system (Gerhardt *et al.,* 2006b). BEWS must be fully-automated, easy to operate, provide reliable alarm generation, and be highly sensitive. Detection systems typically consist of video-based biomonitoring or non-contact bioelectric systems which are attached to computers which can rapidly interpret changes in organism behaviour (Gerhardt *et al.,* 2006b). These systems require organisms which have rapid, reproducible responses to contaminants which can be easily monitored and evaluated (Van Hoof *et al.,* 1994). Organisms currently employed in BEWS include fish, crustaceans, mussels, benthic invertebrates, algae, and bacteria (Lechelt *et al,* 2000; Bisthoven *et al.,* 2004; Mikol *et al.*, 2007). These systems typically only monitor the behaviour of one type of organism at a given time. The potential for development of multi-species, early-warning biomonitoring technologies using other representatives of the aquatic environment, as well as multiple species simultaneously, is possible, and is an exciting area study which this project aims to examine. A number of criteria should be taken into consideration when selecting organisms to use in a multi-species, early-warning biomonitoring technology. It is suggested that organisms should be economically important (e.g. commercial or recreational species), representative of other species in the ecosystem, or be a keystone species (Bunn, 1995). By using species such as these, it is possible to simultaneously protect ecologically important species in a given area by determining if changes in water quality are having an impact on native populations, as well as detecting changes in incoming drinking water quality (Underwood and Petersen, 1988; Bunn, 1995). Ideally more than one species will be used in a multi-species, early-warning biomonitoring technology, as different organisms are sensitive to different levels and types of contaminants (Bunn, 1995). Some BEWS are capable of monitoring the behaviour of multiple organisms (ie the Multispecies Freshwater Biomonitor discussed below) (Gerhardt, 2007b). However, limited application with multiple species has been performed in the past in field settings, an area which this project aims to expand upon.

The Multispecies Freshwater Biomonitor (MFB)

The MFB is based on the use of quadropole impedance technology, where the test organism is held in a flow-through chamber connected to two pairs of electrodes (Gerhardt *et al.,* 2006). One pair of electrodes creates a high frequency alternating current and the other pair senses impedance changes in the frequency of the currents, which are generated by the movement of the organism within the test chamber (Gerhardt *et al.,* 2006b). Different behaviours will generate different electrical patterns and from this a behavioural "fingerprint" for the organism can be developed based on the percentage of time spent doing each type of behaviour (Gerhardt *et al.,* 2003). Activity frequencies are determined under control conditions, and if a certain activity occurs at 10% higher or lower than expected frequency, an alarm will sound (Gerhardt *et al.,* 2003). An alarm will also sound if more than 50% of organisms are "inactive" and not generating any behavioural signals (Gerhardt *et al.,* 2003). The MFB is unique in that it has a high number of individual chambers allowing for monitoring of many organisms at the same time and can be used to monitor the behaviour of more than one species at a time, allowing for increased sensitivity (Gerhardt *et al.,* 2006b). Field applications of the MFB at water treatment facilities using multiple species of organisms has been limited to date, an area of study which this project aims to improve upon.

1.2.2 Advantages and Disadvantages

The use of BEWS has several advantages over traditional water quality monitoring methods. Behavioural and physiological changes are often the first changes seen in aquatic organisms and are much more rapidly induced endpoints than changes in reproduction and morphology (Gerhardt and Bisthoven, 1995; Michels *et al.,* 1999). The detection of changes in behaviour and physiology is also much more rapid than traditional chemical testing, which requires significant preparation time, skilled staff to perform tests, and is not possible to do in a continuous online manner (Van Hoof *et al.,* 1994; Green *et al.,* 2003). In addition, traditional chemical analyses are only performed for a limited number of chemicals which may be present in incoming water to be treated (Van Hoof *et al.,* 1994). The use of traditional BEWS allows for the monitoring of many chemicals at one time but not for discrimination amongst different types of contaminants. When some type of pollutant is detected using these systems, it is possible to use further chemical and physical testing to identify the contaminant, which may or may not normally be tested for (Van Hoof *et al.,* 1994; Mikol *et al.,* 2007). Because of their continuous

nature, BEWS can detect pollution "pulses", sporadic events that may be missed by static chemical tests (Bisthoven *et al.*, 2004; Gerhardt *et al.*, 2006b). Accidental spills from point and non-point sources can therefore be rapidly detected and water-inflow to treatment plants can be stopped before the contaminant enters the circulation system (Mikol *et al.*, 2007). BEWS can also be used together with traditional chemical testing to increase public confidence in drinking water providers and to encourage potential polluters to prevent accidental discharges (Gerhardt *et al.*, 2006b; Mikol *et al.*, 2007). A further advantage is that costs associated with BEWS are also lower than traditional chemical testing and other forms of continuous water monitoring (Green *et al.*, 2003). Most BEWS are fully-automated, allowing for low operating, monitoring, and maintenance costs (Bisthoven *et al.*, 2004). BEWS are also useful in terms of ecosystem monitoring in that they take into account the reactions of biota to environmental stress and give an idea of the bioavailability and effects of compounds on organisms which are present in natural environments outside of the water treatment facility (Martins *et al.*, 2007a). BEWS have the potential to be useful in field applications as they are small, portable, and cost less than a permanent water quality testing station. This may allow for a larger number of stations to be installed throughout a watershed to provide a more accurate picture of water quality in the ecosystem (Gerhardt *et al.*, 2006b).

As mentioned previously and re-emphasized here, despite the many benefits, traditional BEWS have several limitations which could potentially be improved over the course of this project. Most BEWS which have already been developed, with the exception of the Multispecies Freshwater Biomonitor (discussed above), are based on the responses of only one organism to contaminant stress (McCarthy, 2007). Differing species of organisms may be more or less sensitive to different classes of contaminants, and thus the use of only a single species in a traditional BEWS may over- or underestimate the risk posed by a given chemical in incoming water supplies (McCarthy, 2007). By using multiple species representing different trophic levels and phyla, this project aims to develop a more holistic, comprehensive early-warning detection system using a modelling-based approach. The responses of a variety of organisms to several classes of chemical and biological contaminants will be determined in order to determine more detail about the specific nature of a threat to Canadian water supplies. By evaluating multiple patterns of response and creating dose-response models, greater evidence will be available to treatment plant managers as to the exact stressor present in incoming water, thus facilitating

decision making with regards to further treatment and/or temporary shut-down of facilities (McCarthy, 2007). Traditional BEWS are often inadequate as single organisms are able to detect that a stressor is present, but give no clue as to the exact nature of that contaminant. By using a modelling-based approach with several organisms, behavioural response fingerprints may be developed in order to figure out the exact type of chemical present in the water.

1.2.3 Potential Applications

BEWS have numerous potential applications. In additional to their use in monitoring pollutant levels in drinking water supplies, they have been used in the past in anti-terrorism applications in order to test for the addition of chemical warfare agents into drinking water supplies (Butterworth *et al.,* 2002; Green *et al.,* 2003). The potential for early-warning systems to detect biological agents such as anthrax, *Clostridium perfringens,* botulinum, aflatoxin, and ricin has also been suggested; however, more research in this area is required (Foran and Brosnan, 2000). A multi-species, early warning biomonitoring technology could also be used to protect a number of freshwater environments, regardless of whether they are being used a source of water for human consumption or not. It is possible to use a multi-species, early warning biomonitoring technology for large-scale watershed protection, in testing of water quality in nature conservation areas, and in water testing of areas where fisheries are located to ensure that contaminants are not affecting fish which humans may consume (Mikol *et al.,* 2007; Bisthoven *et al.,* 2004). A multi-species, early warning biomonitoring technology could also have industrial applications for industries which discharge effluent into receiving bodies of water (Mikol *et al.,* 2007). Contaminant levels can be monitored using a multi-species, early warning biomonitoring technology to ensure that they remain below regulated values and do not affect the aquatic life in the bodies of water into which the effluent is being released. BEWS have also been used to monitor discharge from contaminated sites where remediation is taking place, to ensure that contaminants are no longer present in the aquatic environment (van der Schalie *et al.,* 2001). Several European nations have installed BEWS in rivers at national borders to help detect chemical pulses and to determine if international permit violations are taking place (Gerhardt *et al.,* 2006b). In 1986, a BEWS known as the Dynamic *Daphnia* test detected low levels of atrazine in the Rhine River after a chemical spill 500 km upstream just hours after the event had taken place (Butterworth *et al.,* 2002). The rapid detection of this event increased interest in

Europe in the development of these systems and subsequently many single-organism monitoring systems have been installed along major waterways on the continent.

1.3 Relevance of Behavioural and Movement Quantification

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, 1994). Behaviour is highly adaptable and the type, intensity and time of occurrence of various behaviours can be modified by organisms in response to external stimuli, such as chemical contaminants (Gerhardt, 2007b). The study of changes in behaviour in response to chemical contaminants in the environment is a rapidly expanding field known as behavioural ecotoxicology (Beitinger, 1990; Doving, 1991; Scherer, 1991; Clotfelter *et al.,* 2004; Scott and Sloman, 2004; Zala and Penn, 2004; Mills *et al.,* 2006; Cunha *et al.,* 2008). Behaviour is a response to contaminants that can be visually measured (Gerhardt *et al.,* 2008). Observations of changes in behaviour are non-lethal, so organisms can be used for biomonitoring for extended periods of time (Gerhardt *et al.,* 2006b). Behavioural responses are detected extremely rapidly and are estimated to take one-tenth of the time needed to evaluate changes in life history traits or mortality in response to low concentrations of contaminants (Dodson *et al.,* 1995). Responses are mediated by chemo- or visual reception of contaminants in the environment (Ren *et al.,* 2009).

Behaviours are often the first obvious thing to change in response to pollutants and are easy to observe due to their effects at the whole organism level (Gerhardt *et al.,* 2006b). Mobility is a type of behaviour which is highly important to aquatic organisms. Changes in movement can often provide important information about the overall physiological condition and fitness of organisms (Baillieul and Scheunders, 1998). Changes in movement behaviour in single organisms are closely related to changes at the cellular, population and ecosystem level (Wicklum *et al.,* 1997; Bunn, 1995). Movement is an integration of responses of physiological, nervous, sensorial and muscular systems and can therefore be indicative of changes at a sub-organismal level (Untersteiner *et al.,* 2003). Examining changes in movement behaviour can also help to predict and prevent detrimental impacts at higher organization levels (Bunn, 1995). Changes in movement can be linked to many ecologically-relevant functions such as reproduction, foraging and predation (Doving, 1991). If reproduction and foraging success decrease significantly due to changes in movement behaviour, populations could decline which

could seriously affect ecosystem functioning. Populations could also face a decline due to increased predation caused by an inability to avoid predators.

1.4 Relevance of Respiration Quantification

Respiration is a necessary physiological process for all organisms and changes in respiration brought on by exposure to contaminants could have an extremely detrimental impact at organismal, population and ecosystem level (Martins *et al.,* 2007a). Changes in respiration and metabolism should be examined at the same time as other fitness parameters in order to understand the full effect that a contaminant is having on an organism (Knops *et al.,* 2001). Changes in respiration can potentially be used as an early-warning biomonitoring characteristic (Geiger and Buikema, 1981). Oxygen-level monitoring offers several major advantages; it is very rapid, is non-invasive and does not induce stress in organisms, and can be used to detect sub-lethal levels of contaminants (Martins *et al.,* 2007a). Changes in respiration rates in response to contaminants has been examined but limited applications of this type of monitoring have been incorporated into past BEWS, especially when examined simultaneously with behavioural parameters. This project aims to utilize the two measures at the same time to provide a more holistic picture of organism-level changes induced in response to chemical contaminants.

Disturbances caused by contaminant exposure often result in energy-demanding processes in order to compensate and adapt to stress (Knops *et al.,* 2001). Under low levels of stress, the metabolic rate and aerobic respiration rate of organisms which use oxygen should increase (Knops *et al.,* 2001), while increased and prolonged periods of stress may depress metabolic function. Information about metabolic changes which occur under stress can therefore be measured indirectly by examining changes in oxygen consumption (Knops *et al.,*2001). To date, few BEWS have used respiration rates as a characteristic to be monitored. Several BEWS have used measurements of the ventilation rate of fish and other organisms to make assumptions about the respiration rate of organisms; however, the use of oxygen-level monitoring is much less used (Martins *et al.,* 2007a). A major focus of this project will be the automation of respiration rate monitoring and its incorporation into a multi-species, early-warning biomonitoring technology which will simultaneously monitor the movement behaviour of test organisms.

1.5 Bioassay Organisms

1.5.1 *Daphnia magna*

Background

Daphnia magna (Figure 1) are small (0.5-5 mm in length) invertebrate crustaceans which have been widely used in a variety of ecotoxicology testing in the past (Ren *et al.*, 2007). *Daphnia* are considered to be highly sensitive to a variety of chemicals at low concentrations and are therefore used as model organisms for predicting the impacts of contaminants in the environment (Kieu *et al.*, 2001; Kiss *et al.*, 2003; Schmidt *et al.*,2005; Ren *et al.*, 2009). This is because gill systems, digestive tract, and overall body surface are constantly exposed to contaminants which are dissolved or suspended within the aquatic environment (Green *et al.*, 2003). *D. magna* also tend to bioaccumulate toxic compounds much more rapidly than larger organisms, and are more likely to show changes in behaviour and physiology when exposed to extremely low doses of a pollutant (Green *et al.*, 2003). *Daphnia* are easy and inexpensive to culture in a laboratory setting (Ren *et al.*, 2009) and have a short life-cycle which allows them to breed and mature quickly (Ren *et al.*,2007). Changes in life history, behaviour and morphology in response to contaminants have been extensively studied in *Daphnia* (Barber *et al.*, 1990; Goodrich and Lech, 1990; Arner and Koivisto, 1993; Dodson *et al.*, 1995; Paul *et al.*, 1997; Wollenberger *et al.*, 2000; Villegas-Navarro *et al.*, 2003; Flaherty and Dodson, 2005; Hoang *et al.*, 2006).

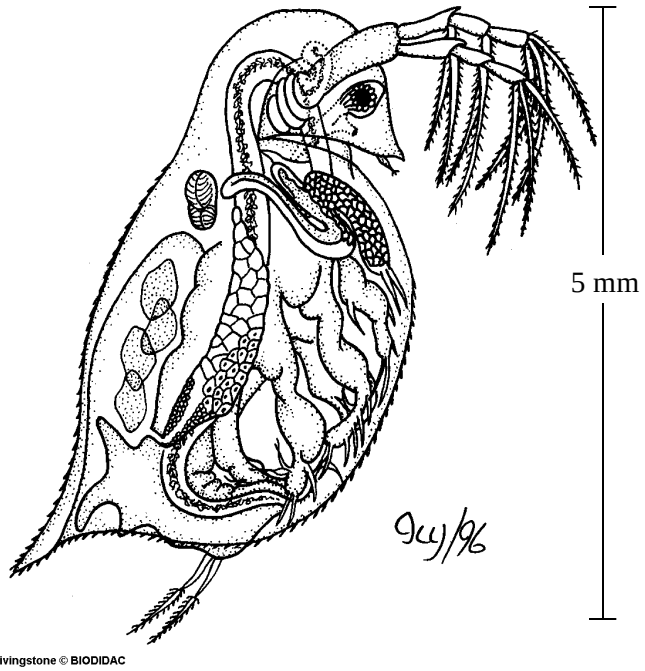

Figure 1: Illustration of *Daphnia magna* (BIODIDAC, 1996)

Daphnia are common in freshwater systems such as lakes, rivers, ponds and other surface waters throughout Canada and the United States, including the Great Lakes ecosystem (Dodson and Hanazato, 1995; Ryan and Dodson, 1998). In their natural habitats, *Daphnia* are often the dominant herbivores present and make a great contribution to water clarity as they consume large

amounts of phytoplankton (Dodson *et al.,* 1995; Fischer *et al.,* 2006). Daphnids are also an important component of the diets of many species of fish, amphibians, and larger zooplankton due to their rapid reproductive rate and large size, and act as a critical link in the foodweb between phytoplankton and large organisms (Dodson and Hanazato, 1995; Fischer *et al.,* 2006; Gerhardt *et al.,* 2006a).

Behaviour and Past Behaviour Bioassays

Daphnia are permanently swimming organisms and thus changes in their movement due to stress can have a detrimental impact on survival of the organisms (Schmidt *et al.,* 2005). Under normal conditions, daphnids move with powerful, smooth strokes of their secondary antennae in straight directional movements. *D. magna* swim in a saltatory or jumping swimming style and are often known as water fleas (Dodson and Hanazato, 1995). Their distinctive style of swimming allows them to find and maintain their position in patches of algae distributed throughout the height of the water column, as well as to group with other daphnids and to move away from predators (Ryan and Dodson, 1998; Christensen *et al.,* 2005). By swimming in straight lines, the organisms are able to travel to food patches more quickly and move rapidly away from predators (Ryan and Dodson, 1998). Daphnids also display escape responses in the presence of predators which consists of a short, very rapid burst of swimming and some spinning behaviour (Dodson *et al.,* 1995). Changes in swimming behaviour and displaying a different swimming style than other organisms may make individuals more visible to predators, thus increasing the likelihood of being preyed upon. *D. magna* have a characteristic swimming speed and style which can be rapidly altered by the presence of toxic compounds, and thus observing this characteristic is a useful endpoint in ecotoxicological research (Baillieul and Scheunders, 1998). Changes in swimming behaviour can be caused by attempts to avoid the toxin or by impairment of the ability to swim following exposure to toxic compounds (Green *et al.,* 2003).

One method which has been used in past studies to examine changes in *Daphnia* swimming is digital image analysis. Digital image analysis involves constant monitoring of *D. magna* using a digital video camera and computer set-up. Water is allowed to enter a flow-through chamber in which the *D. magna* are kept and onto which the camera is focused (Bailleul and Scheunders, 1998). The camera is attached to a frame grabber which digitizes the images and enters them into a computer program which is able to perform trajectory analyses for each

daphnid in the chamber (Bailleul and Scheunders, 1998). The computer traces the movement of the center of each daphnid and provides a black and white vector image for each organism based on a simple geometrical model which describes the displacement of objects, as well as a graphical and tabular output (Bailleul and Scheunders, 1998; Lechelt *et al.,* 2000). Up to 25 organisms can be analyzed at once and the computer is able to determine several parameters associated with swimming (Bailleul and Scheunders, 1998). These parameters include average velocity, fractal dimension (measure of turning and circling by daphnids) of the organisms, a V-class index (places organisms into velocity ranges) comparing the various treatments, the average height in the water column of each organism, the distance between each organism, and the overall number of daphnids moving (Lechelt *et al.,* 2000; Green *et al.,* 2003). The computer program is able to determine baseline values for these parameters under normal water conditions and once a significant change in one or more of the characteristics is detected, an alarm is sounded and further testing of water quality can be performed in order to determine the nature of the problem (Lechelt *et al.,* 2000; Green *et al.,* 2003). Several digital imaging analysis systems are available commercially, the most commonly used for *D. magna* biomonitoring being the BBE Moldaenke Daphnia Toximeter (Lechelt *et al.,* 2000; Watson *et al.,* 2007). Several studies have been conducted which show how chemical contaminants can be rapidly and effectively detected using digital image analysis of daphnid swimming behaviour. Green and colleagues (2003) found a significant change in the swimming velocity and other motion characteristics was observed using the BBE Moldaenke Daphnia Toximeter in response to sub-lethal concentrations of five different chemical nerve agents within two hours of initial exposure to the toxins (Green *et al.,* 2003). This has important applications to drinking water protection with respect to terrorism prevention and demonstrates that since the detection time was so short, it would be possible to shut down water delivery systems in the event of a terrorist attack or environmental disaster (Green *et al.,* 2003). Lechelt and fellow researchers (2000) demonstrated similar rapid results for the detection of the insecticide trichlorofon by *D. magna.* After 2 hours exposure to sub-lethal concentrations of trichlorofon, daphnids showed a stress reaction of increased swimming velocity, lower swimming depth, closer groupings of the organisms, and extreme speed classes (either very fast or very slow swimming) and an overall decrease in movement (Lechelt *et al.,* 2000). Although changes in swimming behaviour will vary depending on the type and concentration of contaminant, this study clearly shows the rapid detection time available

using digital image analysis systems. Digital image analysis of daphnid movement can also be used to detect taste- and odour-causing compounds which need to be treated for at water purification facilities. Watson and colleagues (2007) found that following exposure to the taste and odour compounds B-cyclocitral and 2(E),4(E),7(Z)-decantrienal, daphnid swimming velocity increased, velocity class increased, height from the bottom of the chamber decreased and fractal dimension decreased indicating a more directional movement pattern, likely away from the offensive compounds, in less than one hour following exposure. In addition to being able to monitor toxic chemicals entering water treatment plants, digital image analysis of daphnid behaviour has the potential to detect other compounds which may need to be treated at the plant, illustrating its many possible applications to water-quality monitoring. Digital image analysis has excellent potential for use in multi-species, early-warning biomonitoring technology due to its rapid detection rate and continuous automated online monitoring of water quality.

The locomotion of daphnids has many components, all of which are important to examine in order to determine how a chemical is affecting behaviour overall. Individual characteristics of swimming behaviour should be easily measured and sensitive to low levels of contaminants (Dodson *et al.,* 1995). Ren and colleagues (2009) have performed bioassays with a range of contaminants evaluating changes in overall behavioural strength, defined as a "measure of intensity of behavioural parameters representing motility", including swimming velocity, behavioural frequency, and movement extent (Ren *et al.,* 2007; Ren *et al.,* 2008). Using the Multispecies Freshwater Biomonitor, in a laboratory setting, it was found that exposure to various concentrations of the pesticides deltamethrin, chlorothalonil and nitrofen produced significant decreases in the overall behavioural strength of adult daphnids after a 48-hour exposure period (Ren *et al.,* 2009). All treatments caused a decrease in overall swimming activity and parameters associated with swimming. Responses were time- and concentration-dependent with responses seen more quickly and having greater severity in higher concentrations (Ren *et al.,* 2009). A similar study found that three organophosphorous pesticides (dipterex, malathion, and parathion) caused significant decreases in the behavioural strength of exposed daphnids at concentrations of 0.045 µg/L, 0.38 µg/L and 0.125 µg/L respectively over 24 hours (Ren *et al.,* 2007). This decrease signifies a decrease in the activity level of the organisms overall. Behavioural changes were again time- and concentration-dependent, with higher

concentrations causing a more marked change in behaviour over a shorter exposure time (Ren *et al.*, 2007).

The MFB can be used to evaluate the behaviour of a variety of organisms; however, the *D. magna* is one of the most commonly used organisms during water quality testing with the MFB. Typical movement behaviours which can be observed in *D. magna* using the MFB include locomotion (big movements using antennae) and ventilation (small movements using the phylopods within the carapace) (Gerhardt *et al.*, 2003). In laboratory studies, a decrease in water pH from 7.5 to 3.4 due to a pulse injection of acid resulted in a significant decrease in both ventilation and locomotion observed by the MFB after 3 hours of exposure (Gerhardt *et al.*, 2003). Immediately following initiation of exposure to the acid, an increase in both activities was detected, likely indicating a stress escape response (Gerhardt *et al.*, 2003).

Dodson and colleagues (1995) also support the idea that multiple characteristics should be studied when examining swimming behaviour. Using digital video analysis, changes in net angle of swimming, average turning angle, sinuosity (the variance in change in direction between video frames related to smoothness of swimming), upward and downward movement angles, curvature co-efficient (calculation of variance from straight line swimming), vertical variance, spinning, hopping and velocity of swimming were studied in response to the addition of the pesticide carbaryl and predator kairomones into the water for 24 hours (Dodson *et al.*, 1995). Within 1 hour of exposure, significant decreases in velocity and turning angle were noted in the highest concentrations of carbaryl. After 24 hours, changes were seen in multiple parameters in all concentrations over 1 ppb (Dodson *et al.*, 1995). This indicates that *Daphnia* are very sensitive to low concentrations of contaminants and that evaluating changes in swimming behaviour in the organisms is a useful mechanism for detecting these contaminants.

Changes in the general activity level and swimming speed of the organisms have also been examined in response to environmental contaminants such as metals. Using digital video analysis, the total amount of time the organisms were active and the swimming velocity at several time periods over a 24 hour exposure to a range of copper (0-30 ppb) concentrations was examined (Untersteiner *et al.*, 2003). Daphnids displayed a significant decrease in the overall time spent on active motion and in mean swimming velocity in 10, 20 and 30 ppb copper. Responses were dependent on time and concentration, with higher concentrations causing a more

severe reaction in a shorter time span (Untersteiner *et al.,* 2003). The use of secondary antennae responsible for propulsion in *Daphnia magna* also showed a decreased beat frequency with exposure to increasing copper concentrations (Untersteiner *et al.,* 2003).

In addition to examining the effect of contaminants on the swimming behaviour of daphnids, research has been conducted to examine how the organisms may change their behaviour in the presence of chemicals given off by their predators. Using a digital video analysis system called ExperVision, Szulkin and colleagues (2006) examined changes of swimming behaviour in daphnids in the presence of kairomones produced by a predatory fish and an insect. A decrease in swimming speed, hop rate, and sink rate and a shift to more horizontal swimming was observed in both treatments (Szulkin *et al.,* 2006).

Another movement based parameter which has been known to change in daphnids following exposure to chemical contamination is phototactic response. Phototaxis is defined as an oriented reaction to a light source (Gerhardt *et al.,* 2006a). Phototaxis can be positive, with directed movement toward a light source, or negative, with movement away from a light source (Kieu *et al.,* 2001; Gerhardt *et al.,* 2006a; Martins *et al.,* 2007a). Different strains of *D. magna* will have different responses to light stimulation; however, the response of each strain is considered to be a stable and repeatable behaviour under control conditions (Kieu *et al.,* 2001). Phototaxis is related to the diel vertical migration many daphnids undergo in natural conditions and consists of active swimming in response to light changes (Cushing, 1951; Gerhardt *et al.,* 2006a). In natural settings, most daphnids respond to light with negative phototaxis during the day, resulting in daphnids grouping near the bottom of the waterbody (Cushing, 1951; Gerhardt *et al.,* 2006a). This is explained by the idea that during the day daphnid predators such as fish may be able to easily see the daphnids in the well lit upper portion of the water column (Cushing, 1951; Gerhardt *et al.,* 2006a). Daphnids remain at the bottom of the water column during the day and migrate to the top of the column at night to graze when the light source is no longer present (Cushing, 1951; Gerhardt *et al.,* 2006a). Higher rates of survival, resource exploitation, and niche separation are seen in strains of daphnids which display these migration patterns (Goodrich and Lech, 1990). Most ecotoxicological studies focus on positively phototactic strains of *D. magna* which react to the presence of light by moving towards the top of the water column or towards an artificial light source (Kieu *et al.,* 2001). Phototactic response is a result of the

integration action of the nervous and muscular system in daphnids and can be altered by the presence of toxic compounds (Kieu *et al.,* 2001; Martins *et al.,* 2007a). Several studies have demonstrated that alteration of this behaviour is a relevant characteristic to be evaluated when using BEWS (Gerhardt *et al.,* 2006a). Most phototactic response studies involve creating an artificial water column with a light source located at the top of the column. The tube is divided into sections by height and the distribution of daphnids following exposure to the light source for a given period of time is determined using a Phototactic Behaviour Index. Index values are found by dividing the number of daphnids present in the upper section of the column by the total number of daphnids in the column (Martins *et al.,* 2007a). Values can range from 1, where all are present in the upper section, to 0, where all are present in the lower sections (Martins *et al.,* 2007a). The Phototactic Behaviour Index value is a relative value in which values from daphnids exposed to contaminants are compared to index values from control groups (Martins *et al.,* 2007a). If the experimental index value is lower than the control value, response to light stimuli has been reduced due to the presence of the contaminant (Michels *et al.,* 1999). If the difference between index values is significant, the contaminant is considered to have an impact on the phototactic behaviour of the daphnids. Phototactic behaviour can be monitored experimentally by hand or automatically using digital image analyses similar to described above (Van Hoof *et al.,* 1994). Using digital image analysis, the number of *D. magna* moving in each section of the column during light exposure periods is monitored. If this value differs significantly from the control value an alarm is sounded (Van Hoof *et al.,* 1994).

Several studies support the use of phototactic response in BEWS. Kieu and fellow researchers (2001) found that there was a significant change in the phototactic response of daphnids in the presence of both copper and PCP. At 33% of the LC_{50} of PCP and 37% of the LC_{50} of copper, a significant decline in phototactic response was seen with less than 3 hours of exposure (Kieu *et al.,* 2001). Michels and colleagues (1999) found a similarly negative effect on phototactic behaviour upon exposure to copper. At one-fifth of the LC_{50} value of copper, a significant change in phototactic response was seen with less than 4 hours of exposure (Michels *et al.,* 1999). Martins and colleagues (2007a) found that eight common compounds found in European waterways could be detected by changes in phototactic behaviour in daphnids at concentrations 16-32 times lower than reported LC_{50} and EC_{50} values. This study showed rapid changes in behaviour, as responses took place between 0.25-3.5 hrs of exposure (Martins *et al.,*

2007a). Changes in phototactic response were also measured in response to a variety of compounds including heavy metals, pesticides, hydrocarbons, and other common environmental pollutants, indicating that this method could be used in a multi-organism, early warning biomonitoring technology where a variety of compounds could be found (Martins *et al.*, 2007a). Results from previous studies indicate that changes in phototactic behaviour in *D. magna* as a response to chemical contaminants is a rapid and sensitive parameter and should potentially be considered for use in multi-organism, early warning biomonitoring technology at water treatment plants.

Respiration and Past Respiration Bioassays

In addition to examining changes in behaviour, several researchers have examined the changes in *Daphnia magna* respiration rates in response to contaminant exposure and have proposed using this parameter as part of a multi-organism, early warning biomonitoring technology (Geiger and Buikema, 1981). One study examined the effects of long-term exposure to varying concentrations of three different contaminants, CTAB (cetyltrimethylammonium bromide – a commonly used surfactant), copper, and cadmium. Daphnids were exposed to the contaminants for 8 days during their juvenile period and respiration was monitored continuously using a Micro-Oxymax respirometer to determine the overall rate of oxygen consumption per unit of organism biomass (Knops *et al.*, 2001). Over the extended exposure period, no biomass related changes in oxygen consumption were found for any of the treatments indicating that overall metabolic rate of the organisms was not affected by chemical exposure (Knops *et al.*, 2001). Other studies have found similar results using shorter exposure periods. In a study examining the effects of naphthalene, phenanthrene, fuel oil, and creosote on the respiration rates of *Daphnia pulex*, Geiger and Buikema (1981) used modified Winkler titrations to monitor the dissolved oxygen content of test vessels containing *Daphnia* exposed for 24 hours. No significant differences in any of the chemical treatments were seen when compared to the control respiration rate of 0.10 µL oxygen/*Daphnia*/day (Geiger and Buikema, 1981). Sigmon (1979) also found that exposure to concentrations of 1 and 3 ppm of the pesticide 2,4 – D had no effect on overall oxygen consumption, which was measured continuously by a respirometer over a 9 hour exposure period.

Other short-term studies examining *Daphnia* respiration have found contradictory results. When exposed to 1 and 3 ppm of the pesticide 2,4,5 – T, *Daphnia pulex* showed a significant decrease in oxygen consumption in the 1 ppm treatments and a significant increase in the 3 ppm treatments (Sigmon, 1979). A study exploring the effects of UV radiation on oxygen consumption in *Daphnia catawba* found that there was there was a significant increase (31.8%) in oxygen consumption when test organisms were exposed to 2.08 kJ UVB radiation for 12 hours (Fischer *et al.,* 2006). However, when radiation was increased to 4.16 kJ UVB radiation, there was a significant decrease (70.6%) in oxygen consumption during the same period. No change was seen at intermediate levels of UVB exposure (Fischer *et al.,* 2006). In this study, respiration was stimulated at the lowest stress level but depressed at the highest stress level while no significant differences were noted at intermediate exposures.

Rationale for Use of *Daphnia magna* as Bioassay Organisms

Daphnia magna have been shown to be highly sensitive to a variety of common environmental contaminants. The organisms are of ecological significance in Canadian waterways and further study of their responses to stressors will provide a better idea of impacts from contaminants on populations in natural habitats. *Daphnia* demonstrate several defined and quantifiable behavioural parameters which have been examined using digital video analysis and their use in BEWS and sub-acute bioassays have already been established. Additional work examining the effects of TBT and atrazine, the two contaminants examined extensively in this thesis, will contribute to an existing body of knowledge about the impacts of contaminants on daphnid behavioural patterns. An extensive literature review has revealed limited past research on the impacts of these two contaminants on daphnid movement, suggesting that further research should be conducted in order to help create a dose-response model in order to detect specific concentrations of the contaminants. *Daphnia* have also been used in respiration bioassays; however, varying results have been obtained suggesting that the organisms may not be suitable to use for direct measurements of oxygen consumption. Further study in this project will aim to determine if direct measure of the oxygen consumption of daphnids is a suitable measure of stress and whether it should be incorporated into a multi-species, early-warning biomontoring technology.

1.5.2 *Hyalella azteca*
Background

The second test organism used for laboratory bioassays in this project is *Hyalella azteca* (Figure 2), a freshwater amphipod which is highly sensitive to aquatic contamination (Wang *et al.,* 2004). *Hyalella* are one of the most commonly-used organisms in aquatic toxicity testing and have been most used in sediment toxicology testing due to their close contact with sediments (Collyard *et al.,* 1994; Borgmann *et al.,* 1996; Hatch and Burton, 1999). *Hyalella* are useful test organisms in a laboratory setting as they are easily cultured and mature quickly (Wang *et al.,* 2004). *Hyalella* can survive in water with a wide range of dissolved oxygen content, alkalinity, sediment size, and organic matter content, making them easy to care for in a controlled setting (Wang *et al.,* 2004). Organisms can grow to be up to 5 mm in length and a variety of ages and sizes have been used in past toxicity testing (Collyard *et al.,* 1994).

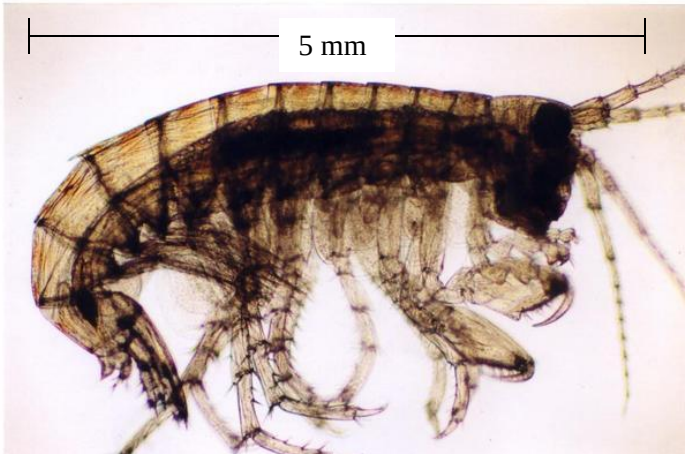

Figure 2: Image of *Hyalella azteca* (American Society of Limnology and Oceanography, 2004)

Hyalella are widespread throughout North and South America, and are present in the Great Lakes region (Blockwell *et al.,* 1998; Wang *et al.,* 2004). They are the most common freshwater amphipod in North America and play an important role in the ecosystems in which they are present (Borgmann *et al.,* 1996). In their natural habitat, these organisms are omnivorous detritivores feeding on algae, leaf litter, freshly-killed animals, small isopods, bacteria and aquatic plants, thus helping to recycle nutrients and maintain water clarity (Blockwell *et al.,*1998; Wang *et al.,* 2004). These organisms prefer a solid substrate and often live in algae mats, rocky habitats or in the sediment (Wang *et al.,* 2004). *Hyalella* are exposed to contaminants from a variety of sources including the water column, sediments while foraging for

food, and from food particles which have bound contaminants (Wang *et al.,* 2004). *Hyalella* also are an important food source for higher organisms including fish, waterfowl, wading birds, and larger macroinvertebrates (Borgmann *et al.,* 1996; Blockwell *et al.,*1998).

Behaviour and Past Behaviour Bioassays

One behavioural parameter that has been studied in the past in *Hyalella* is burrowing. As these organisms are closely associated with the sediment phase of the aquatic environment, it seems logical that these organisms may burrow into sediment for a variety of reasons, including predator avoidance and foraging for food. There is some question as to whether burrowing is a normal behaviour and whether or not the amount of time spent burrowing by these organisms varies under stress conditions (Wang *et al.,* 2004). In nature, it has been noted that *Hyalella* are generally found in the top 1-2 cm of sediment, with over 70% of the organisms in the top 0.5 cm (Wang *et al.,* 2004). These results were observed through sediment core sampling and may not reflect an accurate picture of true burrowing behaviour due to the disruptive nature of the sampling technique which may have caused *Hyalella* to burrow more deeply (Wang *et al.,* 2004). Wang and colleagues (2004) suggest that the organisms should be found at the interface between water and sediment where oxygen levels are higher and algae are much more abundant than within the sediment, and that there is no need for the organisms to continually burrow in order to collect food. Under control conditions, it has been noted that *Hyalella* spend the majority of their time at the sediment-water interface and moved along the sediment by crawling and through the water column using a "sideswimming" movement (Wang *et al.,* 2004). Burrowing only occurred in response to water perturbation or when the organisms were frightened (Wang *et al.,* 2004). When burrowing did occur, the organisms did not remain permanently within the sediment and were often seen to emerge after varying periods of time (Wang *et al.,* 2004). It is believed that burrowing behaviour in *Hyalella azteca* is a type of avoidance behaviour and that in the presence of contaminants, the organisms are more likely to burrow in an attempt to escape hazardous substances (Hatch and Burton, 1999). Hatch and Burton (1999) showed that adult *Hyalella* spent significantly more time burrowed in sand and soil sediments when exposed to various concentrations of the poly-aromatic hydrocarbon fluoranthene than organisms in clean water. As concentrations of the contaminant increased from 6.25-25 µg/L, the amount of time and the number of organisms which had formed burrows increased (Hatch and Burton, 1999). In the

same study, the grouping behaviour of *Hyalella* was also examined in the presence of fluoranthene. Grouping occurred when two or more *Hyalella* aggregated together within the test vessel and was considered to be a stress response in the organisms (Hatch and Burton, 1999). As concentrations of the contaminant increased, a significant increase in the number of the organisms grouping was seen compared to the control treatments (Hatch and Burton, 1999).

Several studies have shown that the majority of contaminant exposure in *Hyalella* comes from food sources and from the water column, rather than from sediment exposure (Suedel and Rodgers, 1996; Wang *et al.,* 2004; Moore *et al.,* 2006). When exposed to chronic, sub-lethal concentrations of cadmium, an increase in accumulation in body tissues is seen with increased levels of cadmium found in the water column but not when in the contaminant is present in increased concentrations the sediment (Wang *et al.,* 2004). This is likely because either water or food particles in the water column are the major source of contaminant exposure, as opposed to sediment (Wang *et al.,* 2004). Moore and colleagues (2006) found that *Hyalella* growth rates were significantly reduced in the presence of several pesticides when chronically exposed to the contaminants in the water column. The contamination of sediments with the equivalent levels of pesticides caused no change in the overall growth rates of the organisms (Moore *et al.,* 2006). Given that water is likely the major contaminant source for *Hyalella*, it follows that if the organisms are able to detect changes in the water column, they may attempt to avoid contaminants by burrowing into clean sediment in order to decrease their exposure to harmful compounds (Hatch and Burton, 1999). Changes in burrowing behaviour, the amount of time spent burrowing, and the numbers of the organisms in a population burrowing after a period of exposure are therefore potential factors to be considered for use in a multi-species early-warning biomonitoring system. Changes in other locomotory behaviour may also be induced in response to stress, validating the study of changes in swimming and crawling behaviour in the presence of increasing concentrations of contaminants (Wang *et al.,* 2004). Finally, it has been seen that the tendency of *Hyalella azteca* to form groups increases when the organisms are stressed (Hatch and Burton, 1999), indicating that this may be an important parameter to examine and potentially incorporate in our technology.

Respiration and Past Respiration Bioassays

It has been noted that in addition to changes in behaviour in response to contaminants, many invertebrates also exhibit increased or decreased physiological and metabolic functioning, which is evident through changes in their overall oxygen consumption. Not a great deal of work has been published examining the effects of aquatic contaminants on the respiration of *Hyalella azteca*. However, the effect of temperature stress on respiration has been studied in this organism. Changes in oxygen consumption in response to temperature stress were examined by Oberlin and Blinn (1997) using the closely related species, *Hyalella montezuma*. Oxygen measurements were made using an oxygen electrode after adult *Hyalella montezuma* were exposed for 30 minutes to treatments of 20, 25 and 30 degrees Celsius (Oberlin and Blinn, 1997). As temperatures increased, a significant increase in oxygen consumption per unit mass was noted as well as an increase in associated metabolic enzyme function (Oberlin and Blinn, 1997). This increase in respiration and metabolism was attributed to an overall increase in organism movement and activity in response to increased temperatures (Oberlin and Blinn, 1997). Results of this study indicate oxygen consumption measurements are indeed possible using *Hyalella* and should be adapted for use when examining different stressors in the environment and validate the use of adult *Hyalella* for this type of bioassay.

Rationale for Use of *Hyalella azteca* as Bioassay Organisms

Hyalella azteca have been shown to be highly sensitive to a variety of common environmental contaminants and their use has been long established in toxicity testing. The organisms are ecologically significant in Canadian waterways and further study of their responses to stressors will provide a better idea of impacts from contaminants on populations in natural habitats. *Hyalella* demonstrate several defined and quantifiable behavioural parameters and which have been monitored using digital video analysis and could potentially be evaluated in a multi-organism, early-warning biomonitoring technology. To date, few studies have been conducted examining changes in behaviour in a sub-acute time period and this project will help to determine if this organism can be used for rapid detection of contaminants in less than 24 hours. Information about respiration rate changes in response to chemical contamination in *Hyalella* is also lacking, especially at a sub-acute level. Experiments conducted as part of this project will help to determine if *Hyalella* are suitable to use for this type of toxicity testing.

1.5.3 *Lumbriculus variegatus*
Background

Lumbriculus variegatus (Figure 3) (also known as the blackworm or mudworm) are a species of freshwater oligichaete worm commonly used in aquatic toxicity testing (Drewes and Cain, 1999; O'Gara *et al.*, 2004; Gerhardt, 2007a; Gerhardt, 2009). *Lumbriculus* are easy to raise in a laboratory setting, maintain and handle (Leppanen and Kukkonen, 1998; Ding *et al.*, 2001). *Lumbriculus* are commonly used in bioaccumulation studies, in helping to determine the trophic status of lakes, in sediment toxicity testing due to their close contact with aquatic sediment, and in metabolic rate and toxicokinetic studies (Leppanen and Kukkonen, 1998; O'Gara *et al.*, 2004; Gerhardt, 2007a; Gerhardt, 2009). These organisms also have the potential to be used in toxicity tests determining mortality, reproduction, metabolic changes and behavioural changes in response to contaminants (Leppanen and Kukkonen, 1998; Gerhardt, 2007a). *Lumbriculus* have also been used in microcalorimetry tests where the heat given off by the organisms was measured and used to determine if changes in metabolism occurred in response to contaminants (Leppanen and Kukkonen, 1998).

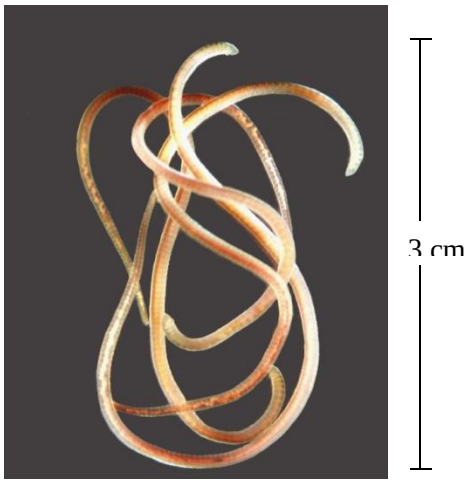

Figure 3: Image of *Lumbriculus variegatus* (University of Iowa, 2009).

Lumbriculus are more useful in aquatic toxicity testing compared to other worm species (e.g. *Tubifex tubifex)* as these organisms live in the sediment and water column at the same time (Drewes and Fourtner, 1989; Gerhardt, 2007a). This allows for exposure to contaminants in both compartments of the aquatic environment. *Lumbriculus* are also more sensitive to low concentrations of certain contaminants and have a defined set of behaviours which can easily be monitored (Gerhardt, 2007a). *Lumbriculus* also demonstrate free swimming and crawling behaviour which is not seen in *Tubifex* (Drewes and Cain, 1999; Drewes, 1999).

Lumbriculus variegatus are universally distributed in North America and Europe, including the Great Lakes ecosystem (Ding *et al.*, 2001; O'Gara *et al.*, 2004; Gerhardt, 2007a). They have also been introduced to Africa, Australia, and New Zealand (Ding *et al.*, 2001;

O'Gara *et al.*, 2004). *Lumbriculus* are benthic and often are commonly found in the shallow areas of freshwater ponds, lakes, slow flowing rivers, and marshes (Putzer *et al.*, 1990; O'Gara *et al.*, 2004; Gerhardt, 2007a). The organisms are tolerant of a wide range of oxygen concentrations in their environment as they are subject to such fluctuations with changes in seasons (Putzer *et al.*, 1990). The worms play an important role in aquatic communities as they aid in the decomposition of organic matter in the sediment and act as a food source for animals higher in the foodweb (O'Gara *et al.*, 2004). Worms feed on decaying vegetation, micro-organisms, and sediment (Gerhardt, 2007a).

In the laboratory, the worms reproduce primarily by asexual morphollaxis, where the body fragments into two or more pieces from which new organisms are able to grow (Drewes and Fourtner, 1989; Gerhardt, 2007a). Regeneration of new worms takes a few weeks following complete separation (Drewes and Fourtner, 1989). In the wild, the organisms have also been observed to reproduce sexually (Drewes and Fourtner, 1989).

Several types of behaviour under normal conditions have been determined and described for *Lumbriculus.* In a natural habitat, when sediment material is present, worms will burrow their anterior ends in the sediment and extend the tips of their tails into the water column to allow for gas exchange via the dorsal blood vessel (Drewes and Fourtner, 1989; Ding *et al.*, 2001; O'Gara *et al.*, 2004; Gerhardt, 2007a). *Lumbriculus* in this position will respond to tactile stimulation or shadow by withdrawing their posteriors into the sediment (Drewes and Fourtner, 1989; Ding *et al.*, 2001). Worms have photosensors and chemoreceptors on their posterior segments which allow them to detect changes in light and water quality and rapidly respond to avoid danger (Drewes and Fourtner, 1989). On smoother surfaces, the organisms move along the substrate using a crawling motion (Ding *et al.*, 2001). Finally, in open spaces, the organisms respond to head stimulation by performing a body reversal and to tail stimulation by swimming in a helical pattern, described in greater detail below (Ding *et al.*, 2001). These behaviours are well studied and easy to monitor, making them an ideal organism to use in a multi-species, early-warning biomonitoring technology based on organism movement and other behavioural patterns.

Behaviour and Past Behaviour Bioassays

Lumbriculus variegatus display a number of characteristic movement behaviours which are easily monitored and are subject to change under stress conditions. In response to tactile or other stimulation to its tail region worms display what is known as helical swimming (Drewes, 1999; Ding *et al.,* 2001; O'Gara *et al.,* 2004). This behaviour is characterized by rapid shortening of the body and rhythmic waves of helical body bending which alternate between the left and right sides of the organism's body. Movement progresses forward from head to tail, rapidly moving the worm forward (Drewe, 1999; Ding *et al.,* 2001; O'Gara *et al.,* 2004). The organisms appear to "corkscrew" through the water to a safer location away from the stimulus and often follows a helical swimming event with continued crawling (O'Gara *et al.,* 2004). As *Lumbriculus* live with their tails extended into the water column, leaving them vulnerable to predation, this movement style has developed to allow for rapid escape when threatened (O'Gara *et al.,* 2004). Helical swimming events usually last less than 1 second following stimulation and are often followed by crawling behaviour (Drewes, 1999). Swimming is advantageous in open spaces where worms may have little traction to perform crawling and it allows the organisms to move more quickly than normal crawling (Drewes and Cain, 1999).

Lumbriculus also display a characteristic locomotion response when stimulated in the head region. When the anterior 1/3 of the worm is touched, it rapidly withdraws in what is known as a reversal behaviour (Drewes, 1999). Reversal behaviour begins with a rapid shortening of the body mediated by longitudinal muscles along the length of the body. The worm will then bend the posterior part of its body into a "J" shape and flip the anterior end around so that it is pointed away from the source of stimulation (Drewes, 1999). This type of behaviour allows the worm to reposition its head away from the threat, as they are unable to swim backwards (Drewes and Cain, 1999). When the worm has reversed its body, it is then able to swim rapidly away from the threat. Worms often aggregate around a food source in nature and when they are disturbed, they will reverse rapidly and swim away from the pack. This likely confuses predators and improves the individual chances of survival for each worm (Drewes and Cain, 1999; Drewes, 1999). Both swimming and body reversal provide the organisms with a faster way to escape predators than typical crawling behaviour and allows for rapid relocation to a safe habitat.

On smooth substrate, worms also possess a characteristic crawling behaviour which can be easily observed. Crawling consists of a series of rhythmic peristaltic contractions beginning at the anterior end of the worm and moving towards the tail (Ding *et al.*, 2001). Crawling is performed using contractions of circular and longitudinal muscles along the length of the worm's body and is assisted by chaetae located on each segment of the body (Drewes and Cain, 1999). Movements are slower and do not display the helical bends seen in helical swimming.

Several studies have looked at the changes of behaviour which occur in the stereotyped *Lumbriculus* movement described above in response to various chemical contaminants. Ding and colleagues (2001) examined the effects that sub-lethal concentrations of the insecticide ivermectin had on the swimming and reversal behaviour and crawling rate of the organisms after 0, 1, 3 and 8 hours of exposure using digital video analysis. The study found that exposure to the insecticide resulted in changes to helical swimming and body reversal in a time- and concentration-dependent manner (Ding *et al.*, 2001). At the highest concentration of 300 nM, worms were paralyzed after only 1 hour of exposure and displayed no swimming, reversal or crawling behaviour (Ding *et al.,* 2001). The effects of lower concentrations of the chemical were similar, but less extreme and required longer exposure periods for complete reaction (Ding *et al.,* 2001). For example, changes in helical wave patterns were noted with waves beginning in the middle of the body rather than the anterior end of the worm (Ding *et al.,* 2001). IC_{50} values for 3 hours of exposure were calculated for helical swimming, reversal and crawling and were found to be 1.1, 16 and 90 nM ivermectin respectively (Ding *et al.,* 2001). Results of this study indicate that changes in *Lumbriculus* behaviour are a very sensitive parameter and that responses are seen at very low concentrations of contaminant indicating their suitability for use in a multi-species, early-warning biomonitoring technology.

Further studies on the impacts of contaminants on the helical swimming and body reversal of *Lumbriculus* were performed by O'Gara and colleagues (2004). The ability of the organisms to respond to head and tail stimulus after 0,1,3, and 8 hours of exposure to copper concentrations ranging from 12.5-102 µg/L was examined using digital video analysis (O'Gara *et al.,* 2004). Immediately following initial exposure, the organisms were observed to be hyperactive with a great deal of writhing and coiling of bodies observed, but quickly became lethargic and non-responsive to tactile stimulation (O'Gara *et al.,* 2004). Shortening of body

length was also observed in all concentrations following 3 hours of exposure (O'Gara *et al.*, 2004). The time of immobilization was concentration-related, as were changes in swimming and reversal behaviour (O'Gara *et al.*, 2004). Over the 8-hour exposure period, all concentrations of copper significantly affected the ability of worms to move in response to stimulation, indicating the organisms ability to escape from predators would be impaired (O'Gara *et al.*, 2004).

In addition to locomotory behaviour, avoidance behaviour has been studied in *Lumbriculus variegatus*. *Lumbriculus* inhabit sediment and are able to burrow into sediment to avoid predators and in theory to avoid chemical contaminants in the water column (Gerhardt, 2007a). Gerhardt (2007a) examined the spontaneous locomotory activity of *Lumbriculus* when exposed to clean water/contaminated sediment and contaminated water/clean sediment, and monitored the avoidance behaviour of the organisms by examining the amount of time spent moving in total and between the two compartments. The study was conducted using the Multispecies Freshwater Biomonitor (MFB) for a period of 24 hours with exposures to concentrations of lead ranging from 0.1-10 mg/L (Gerhardt, 2007a). In all treatments of lead with both contaminated water and sediment, a significant change in the amount of time performing locomotion was noted. An increase in activity was seen after initial exposure followed by a significant decrease or no movement near the end of the exposure period (Gerhardt, 2007a). It was also observed that the test organisms were more active when exposed to contaminated water than contaminated sediments, indicating that burrowing occurred in response to the addition of contaminants in the water column (Gerhardt, 2007a). This indicates that burrowing is a useful potential parameter to monitor and utilize in a multi-species, early-warning biomonitoring technology.

Respiration and Past Respiration Bioassays

Some work has been conducted to examine the impact of contaminants on respiration and overall metabolism of *Lumbriculus variegatus*; however, a great deal still needs to be done. Penttinen and Kukkonen (2000) examined the effects of a range of concentrations of PCP (0.05-1 nM) on the metabolism of *Lumbriculus variegatus* using microcalorimetry over a 72-hour period. Microcalorimety measures changes in water temperature and associates these changes with an increase or decrease in overall metabolism. An increase or decrease in metabolism can then be associated with an increased or decreased oxygen demand (Penttinen and Kukkonen,

2000). Results showed that all concentrations of PCP raised the metabolic rate of the population of worms, thus indicating increased aerobic respiration (Penttinen and Kukkonen, 2000). However, it was found that worms needed to be exposed to the contaminant for at least 24 hours to allow for accumulation of the toxicant in the tissues before any change in metabolism was seen (Penttinen and Kukkonen, 2000).

Rationale for Use of *Lumbriculus variegatus* as Bioassay Organisms

Lumbriculus variegatus have been shown to be highly sensitive to a variety of common organic and inorganic environmental contaminants and are commonly used in ecotoxicological testing. The organisms are ecologically-significant in Canadian waterways and further study of their responses to stressors will provide a better idea of impacts from contaminants on populations in natural habitats. *Lumbriculus* demonstrate several defined and quantifiable behavioural parameters which have been examined using digital video analysis and their sub-acute bioassays have already been established. Studying the effects of TBT and atrazine on the behaviour of these organisms will determine if they are suitable species for use in multi-species, early-warning biomonitoring technology and whether they are able to detect and react to the two environmental contaminants in exposure periods of less than 24 hours. Some research has used *Lumbriculus* to indirectly examine changes in respiration; however, little work has been done with respect to direct measurements of oxygen consumption and whether this is a suitable parameter to use with these organisms in a multi-species, early-warning biomonitoring technology. This study aims to determine if direct oxygen consumption measurements with *Lumbriculus* are appropriate for incorporation into a multi-species, early-warning biomonitoring technology.

1.6 Bioassay Contaminants

1.6.1 Tributyltin

Background

Tributyltin (TBT) (Figure 4) is a trisubstituted organotin which is highly persistent in the aquatic environment and is toxic at the nanogram per litre level to many organisms (Alzieu, 1998; Horry *et al.,* 2004). Tributyltin is hydrophobic and has an octonal-water partitioning co-efficient which ranges from 3.21 to 3.85 depending on the species of the chemical and the

temperature and pH of the water (Alzieu, 1998). The compound comes in many forms including oxides, chlorides, fluorides, and acetate, each of which are slightly soluble in both freshwater and seawater (Alzieu, 1998). Solubility values range from 1-10 mg/L for tributyltin oxide, depending on the composition of the water, and under 20 mg/L for other types of the compound (Alzieu, 1998).

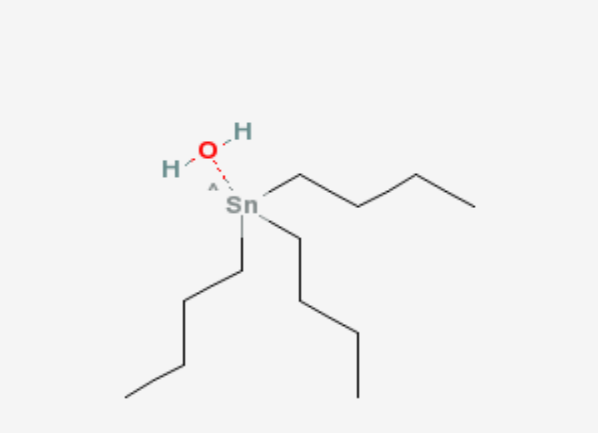

Figure 4: Chemical structure of TBT hydroxide (National Center for Biotechnology Information PubChem (2009a)

The major source of TBT in the aquatic environment is from leaching of anti-fouling paints from boats and cargo ships (Fent and Looser, 1995; Borgmann *et al.*, 1996; Chau *et al.*, 1997; Alzieu, 1998; Konstantinou and Albanis, 2004). This represents over 70% of the usage of TBT (Alzieu, 1998). TBT is applied as a paint additive to the submerged section of boats and other floating structures such as oil rigs, buoys, and fish cages (Alzieu, 1998; Konstantinou and Albanis, 2004). The compound acts as a biocide which prevents the growth of living organisms on surfaces which may slow ships, increase fuel usage, cause corrosion, and increase the weight of floating structures (Alzieu, 1998). Ship paints also often include solvents which aid in application and make TBT compounds more soluble in water (Alzieu, 1998). It is estimated that the daily leaching rate of TBT is between 1 and 10 µg/L TBT per cm^2 of application area (Alzieu, 1998). This results in the addition to the environment of between 0.2 and 2 grams of TBT per day for a small sailboat and between 50 and 500 grams of TBT per day for a large commercial vessel (Alzieu, 1998). TBT concentrations are highest in areas of high shipping and boating traffic, but the compound has also been detected in freshwaters removed from harbours and shipping areas (Borgmann *et al.*, 1996). The use of TBT in shipping has been regulated in Canada since 1990 (regulation history described below); however, the compound is still found in measureable quantities in many ecosystems causing concern about the impacts it may have on aquatic life (Borgmann *et al.*, 1996; Konstantinou and Albanis, 2004). Pulse introductions of TBT into the environment are also of concern as large ships move through waterways (Fent, 1996). Environment Canada has established interim water quality guidelines of 3.3 ng tin/L of water in order to protect aquatic organisms (Chau *et al.*, 1997). This value was derived by

determining the lowest reported chronic exposure effect in literature and applying a safety factor of 10 (Chau *et al.*, 1997). Health Canada has no drinking water quality guidelines for either TBT or tin in general (Canadian Drinking Water Quality Guidelines, 2008).

Other significant sources of TBT include remobilization from contaminated sediments and suspended particles in the water (Fent and Looser, 1995; Chau *et al.*, 1997; Alzieu, 1998). It is also used as a slimicide at nuclear power plants and as an industrial and agricultural pesticide, resulting in runoff into aquatic environments (Borgmann *et al.*,1996; Chau *et al.,* 1997). TBT can also be used as a PVC stabilizer, resulting in leaching from PVC piping into waterways (Chau *et al.*,1997; Borgmann *et al.,* 1998). The compound is also used as a wood preservative and in some factories as an industrial catalyst (Fent and Looser, 1995; Borgmann *et al.*,1996). Many common industrial and urban uses result in a build-up of TBT in wastewater, which is eventually released into the aquatic environment from wastewater treatment plants (Fent and Looser, 1995; Alzieu, 1998).

The current method for detecting TBT is using either gas chromatography/mass spectrometry or inductively coupled plasma mass spectrometry, processes which are very expensive and do not provide rapid results (Horry *et al.,* 2004). A biosensor which uses genetically-altered strains of *E. coli* which fluoresce in response to low levels of TBT and its metabolite dibutyltin (DBT) have been developed (Horry *et al.,* 2004). The sensor is able to detect concentrations of TBT less than 26 µg/L and DBT under 0.03 µg/L (Horry *et al.,* 2004). The need for the development of alternative means of detecting the chemical is needed. The development of a dose response model for TBT which could be incorporated in a multi-species, early-warning biomonitoring system would therefore be highly beneficial in order to help water treatment plant managers detect the presence and concentration of the organotin contaminant.

Tributyltin Ban

Due to its toxic nature, TBT has been banned for use as an anti-fouling agent on small boats in Canada, the United States, Japan, and Western Europe (Fent and Looser, 1995). The use of TBT as a paint additive began in the 1960s and in the early 1980s, several countries began to restrict its use due to toxic effects observed in several aquatic species (Alzieu and Heral, 1984; Alzieu *et al.,* 1986; Fent, 1996; Chau *et al.,* 1997). In 1982, France was the first country to

restrict the use of the compound in boats less than 25 metres in length and with a mean leaching rate of more than 4 ug/cm^2/day (Chau *et al.,* 1997; Alzieu, 1998). Bans in the United Kingdom (1987), the United States (1988), Australia (1989), the Netherlands, Hong Kong and Japan (1992) soon followed (Chau *et al.,* 1997). Canada first introduced restrictions in 1989 under the Pest Control Products Act which restricted the use of TBT as an anti-fouling agent on boats under 25 m in length (Borgmann *et al.,*1996; Chau *et al.,* 1997; Alzieu, 1998). In 2001, the European Council included TBT as a priority substance in its policy on water quality and all countries within the European Union (EU) were required to restrict the use of the substance (Alzieu, 1998; Horry *et al.,* 2004). An international ban on the use of TBT was introduced in 2003 by the International Maritime Organization (IMO) and the Marine Environment Protection Committee; however, many countries which export goods are not signatories to the convention and large boats still may use the compound (Konstantinou and Albanis, 2004).

In general, reductions in concentrations of TBT in freshwater environments and the recovery of populations of organisms affected by the contaminant have been seen since the bans were enacted in Canada and throughout the world. In Canada, TBT, DBT and MBT are found in freshwater much less frequently and in lower concentrations with lower mean ranges than prior to the restrictions started in the 1980s (Chau *et al.,*1997). However, reductions are often not seen in areas of high shipping traffic and with large ships not subject to the restrictions or in areas where boats are present which may have been painted prior to 1989 (Fent, 1996; Chau *et al.,* 1997). In some areas, TBT concentrations still exceed the Environment Canada interim water quality guidelines for the protection of aquatic life (Chau *et al.,* 1997). Because of the persistence of the contaminant, the introduction of more TBT from boats not restricted by international bans and because of its potential to be resuspended in the water column, TBT remains an important environmental contaminant whose effect on organism behaviour and physiology should be examined for potential detection using a BEWS.

Human Health Concerns

Contact exposure to TBT can cause irritations in the skin and eyes, which may lead to severe dermatitis (Alzieu, 1998). No cases of poisoning via ingestion have ever been reported in humans (Alzieu, 1998).

Environmental Concerns

TBT is highly lypophillic due to its three alkyl groups and low solubility in water (Maguire, 1987). This can lead to bioaccumulation of the contaminant in the fatty tissues of living organisms and biomagnifications within the aquatic foodweb (Maguire, 1987). TBT exerts its toxic properties at a cellular level by causing malformations of the mitochondrial membrane (Alzieu, 1998). At extremely low concentrations in the body, TBT stimulates the production of ATP and inhibits its conversion to ADP, leading to cellular malformations and decreased metabolic output (Fent, 1996). TBT has also been linked to endocrine disruption in several organisms at sub-lethal concentrations (Horry *et al.*, 2004).

Concerns were first raised in the 1970s about the potential toxicity of TBT and its metabolites. A decline in shellfish populations had been noted in the Archachon Bay region of France, with abnormal reproduction, shell calcification problems and decreases in overall population numbers being noted in primarily in the mollusc *Crassostrea gigas* (Alzieu and Heral, 1984; Alzieu *et al.*, 1986; Maguire, 1987). The population changes were most commonly seen in harbour areas, leading researchers to believe that TBT may be the cause (Alzieu and Heral, 1984; Maguire, 1987). The decline in mollusc populations negatively affected the economically important shellfish industry in the Atlantic region of France, and lead to that country's TBT ban introduced in 1982 (Alzieu and Heral, 1984; Fent, 1996). Molluscs have been shown to be particularly sensitive to endocrine disruption caused by TBT, often with parts per trillion (ppt) concentrations causing significant health effects (Alzieu, 1998). Concentrations under 1 ng/L cause imposex (the appearance of male characteristics in female organisms) in many species of gastropods (Alzieu, 1998). This can lead to sterility in organisms and a decline in overall success of the population (Alzieu, 1998). Concentrations of 2 ng/L have caused increased shell calcification in the oyster *Crassostrea gigas*, while concentrations around 20 ng/L have caused a decline in reproduction in other bivalve molluscs (Alzieu, 1998).

Other organisms are less sensitive to the presence of TBT in their environment, but still show a reaction in low concentrations of the compound. Concentrations between 1 and 10 µg/L affect the reproduction of most species of fish studied, whereas concentrations between 1-1000 µg/L affects the swimming behaviour of several species of fish (Alzieu, 1998). Several species of crustaceans have also demonstrated reduced reproduction, as well as reduced neonate and

juvenile growth rates when exposed to sub-lethal concentrations of TBT (Schmidt *et al.,* 2005). LC_{50} values for organisms are very low as well, with 10-day LC_{50} values in amphipods ranging from 1.5-32 µg/L depending on species and 4-day LC_{50} values for rainbow trout and lake trout of 1.4 and 5.2 µg/L respectively (Borgmann *et al.,* 1998).

Tributyltin in Aquatic Ecosystems

Although the use of TBT has been banned, it is still a contaminant of significant concern in the aquatic environment in Canada and throughout the world. Worldwide chronic concentrations exceeding 100 ng/L have been found in both fresh and marine environments following the ban (Fent and Looser, 1995). Prior to the TBT ban in North America concentrations in freshwater averaged between 50 and 500 ng/L, with a highest recorded chronic value of 1 µg/L in several heavily-travelled harbours (Alzieu, 1998). In a 1994 study following the introduction of the TBT ban in Canada, several freshwater areas still had concentrations of TBT exceeding the interim limit to prevent damage to aquatic life (Chau *et al.,* 1997). In this study, 12 of 89 tested sites had detectable levels of TBT in freshwater, with concentrations up to 17.8 ng/L (Chau *et al.,* 1997). Of these 12 sites, 9 had concentrations which exceeded the guidelines to protect freshwater aquatic life (Chau *et al.,* 1997). Within the sediment, 42 of 89 samples had detectable levels of TBT, with the maximum recorded concentration being 975 ng tin/g sediment (Chau *et al.,* 1997). Concentrations in industrial effluent have been recorded as high as 61.8 µg/L in Germany, indicating that industrial emissions may be a significant source of pulses of TBT into the environment (Schmidt *et al.,* 2005). Although concentrations have generally declined since the TBT ban, its presence is still seen in heavily-travelled shipping areas and harbours in concentrations which could potentially affect aquatic organisms.

TBT is very persistent in the aquatic environment and its environmental chemistry and fate in the environment are not completely understood (Fent and Looser, 1995; Fent, 1996; Alzieu, 1998; Horry *et al.,* 2004). The compound is present in all components of the aquatic environment, including water, sediments, bound particles, and in living organisms (Alzieu, 1998). In the water column, the half-life of the compound will vary depending on temperature, pH, turbidity and light conditions, but generally ranges from a few days to several weeks (Fent, 1996; Alzieu, 1998). TBT degrades much more slowly in sediment, with a half-life of several years (Borgmann *et al.,*1996; Alzieu, 1998). TBT in sediments has the potential to be

resuspended in the water column and thus poses a continued threat to water quality (Fent, 1996; Chau *et al.*,1997). Approximately 5% of TBT introduced to the aquatic ecosystem is found adsorbed to suspended particles in the water column where they are available to filter-feeding organisms (Alzieu, 1998). TBT is broken down by pH-dependent hydrolysis to the less toxic metabolites dibutyltin (DBT) and monobutyltin (MBT) (Fent and Looser, 1995). The bond between the tin and carbon molecules can also be fractured by UV photolysis and the action of some micro-organisms, again breaking down to DBT and MBT (Alzieu, 1998).

Tributyltin and Past Bioassays with Study Organisms

Several bioassays have been performed in the past using study organisms explored in this study. Fent and Looser (1995) examined the uptake and bioaccumulation of TBT in *Daphnia magna* with respect to changes in pH in the water. It was found that *Daphnia* take up and accumulate significantly more TBT, DBT and MBT in water with a pH of 8.0 than in water with pH 6.0 after 72 hours of exposure to sub-lethal concentrations of the contaminant (Fent and Looser, 1995). It is known that in higher pH conditions, TBT is present in the TBT-OH form, rather than the TBT^{+} form found at lower pH (Fent and Looser, 1995). TBT-OH is more lipophilic than the charged species and is more able to cross biological membranes (Fent and Looser, 1995). This indicates that water conditions may impact the uptake and overall toxicity of TBT to study organisms.

Bioassays examining the changes in swimming behaviour of *Daphnia magna* in response to TBT have also been performed. Schmidt and colleagues (2005) used the BehavioQuant video imaging system to monitor changes in swimming speed, swimming depth and secondary antennae use after 21 days of exposure to 6.6 µg/L of TBT in adult organisms. A significant decrease in mean velocity was noted after 19 days of exposure and a significant decrease in swimming depth and antennae movement was observed after 10 days of exposure (Schmidt *et al.*, 2005). A 21-day LC_{50} value of 2.5 µg/L was determined for organisms used in this experiment (Schmidt *et al.*, 2005). Also noted was a 35% decrease in reproduction for organisms over the 21-day experiment, with an NOEC concentration of 0.16 µg/L found for reproduction over 21 days (Schmidt *et al.*,2005).

Mortality bioassays for *Daphnia magna* have determined a variety of 48-hour LC_{50} values. LC_{50} values vary based on the species of TBT examined and are generally between 2.3 and 70 µg/L (Schmidt *et al.,* 2005). NOEC values for mortality have also been determined for *Daphnia,* at 1.2 µg/L and 5.5 µg/L after 96 and 24 hours respectively (Schmidt *et al.,* 1995).

Bioassays examining the effects of TBT on *Hyalella azteca* have also been conducted. Borgmann *et al.* (1996) conducted short (1 week) and long (4 week) exposure bioassays to determine LC_{50} values and to examine the relationship between body size and accumulation of TBT within the tissues. TBT concentrations of 0.56, 1.0, 1.8, 3.2, 5.6 and 10 µg/L TBT were all shown to cause bioaccumulation in the organisms and the concentrations in tissues increased rapidly in the first 3-4 days of exposure before reaching a peak concentration after 1 week (Borgmann *et al.,* 1996). Rapid equilibration with TBT concentrations in water occurred in all concentrations and final concentrations in tissue did not vary among treatments (Borgmann *et al.,* 1996). Accumulation was not dependent on body size, indicating adult or juvenile organisms were suitable for bioassays (Borgmann *et al.,* 1996). A one-week LC_{50} of 2.3 µg/L and a 4-week LC_{50} value of 0.58 µg/L was found for *Hyalella* (Borgmann *et al.,* 1996).

Several bioassays have been done examining the effects of TBT on proposed study organisms; however, these studies were conducted with exposure periods ranging from 1-3 weeks. Little or no studies have been conducted using this particular contaminant to examine impacts on a sub-acute (fewer than 24 hours) timescale. Observations of behavioural changes in response to TBT are also lacking, making it an ideal contaminant to determine a behavioural response pattern in the study organisms.

Rationale for Use

TBT is highly toxic and persistent at very low levels in the aquatic environment. It is an environmentally-relevant toxicant in Canadian waterways and the potential exists for pulse introductions of TBT to enter drinking water supplies. As it is not specifically tested for at drinking water intakes due to the cost and time involved, the use of a BEWS to detect the substance would be appropriate. To date, very few sub-acute (under 24 hour) bioassays examining behaviour and respiration responses to the contaminant have been conducted using the test organisms *Daphnia magna, Hyalella azteca* and *Lumbriculus variegatus*. Testing is

therefore required to determine if these organisms are responsive to low levels and pulse levels of the contaminant and if it could potentially be detected by a BEWS using these organisms.

1.6.2 Atrazine

Background

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is a chloro-N-diakyl substituted triazine herbicide which has a chemical formula $C_8H_{14}ClN_5$ (Figure 5) (Health Canada, 1993; Detenbeck *et al.,* 1996). The chemical has a molecular weight of 215.7, a melting range of 175-177 degrees Celsius and a boiling point of 279 degrees Celsius (USEPA, 2003; Health Canada, 1993). Atrazine has a low water solubility of approximately 33 mg/L at 25 degrees Celsius (USEPA, 2003). In comparison a highly soluble compound, salt (NaCl), has a solubility of 360 g/L (Alfa Aesar, 2007). Atrazine has an octanol-water partition co-efficient of 2.82 and a hydrolysis half-life of over 1000 days (USEPA, 2003). Its chemical properties make it a persistent and toxic contaminant in aquatic environments.

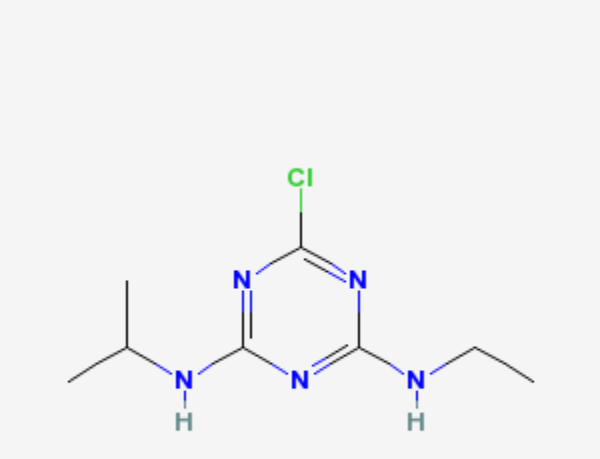

Figure 5: Chemical structure of atrazine (National Center for Biotechnology Information PubChem, 2009b)

Atrazine was first introduced during the 1950s and is used most commonly in Canada as a pre- and post-emergence weed control agent (Detenbeck *et al.,* 1996; Anderson and Zhu, 2004). Atrazine is applied to vegetables and grain fields, vines, fruit orchards, citrus groves, and sugar cane fields to control weeds which may impact the crop growth (Anderson and Zhu, 2004). Atrazine functions by inhibiting photosystem II of the chloroplasts of plants, preventing energy transfer required for plants to perform photosynthesis (DeNoyelles *et al.,* 1982; Anderson and Lydy, 2002). Atrazine is the most heavily used pesticide in North America, with between 70,000 and 90,000 tonnes applied to croplands each year (Graymore *et al.,* 2001). Over 20,000 tonnes are sold in Canada, with 70% used in Ontario (Health Canada, 1993). It is most commonly used to spray corn and rapeseed fields, as well as other crops mentioned above, and enters aquatic ecosystems via leaching and run-off from rain or irrigation (DeNoyelles *et al.,* 1982; Waring and

Moore, 2004). Atrazine can also adsorb to soil particles which are then eroded into running water or can drift via air into water immediately following spraying (Health Canada, 1993). Because of this, atrazine is the most frequently detected pesticide in surface and well water in Canada and the United States (Health Canada, 1993). Well and surface water contamination by atrazine has been reported in British Columbia, Nova Scotia, Quebec, Saskatchewan, Ontario, and Prince Edward Island, with higher concentrations reported in spring due to increased run-off from fields (Health Canada, 1993). Health Canada regulates the maximum acceptable concentration in drinking water to be 5 µg/L, representing a total of pure atrazine and its metabolites (Health Canada, 1993), whereas the USEPA recommends that concentrations in drinking water not exceed 3 µg/L (USEPA, 2003). The Canadian Water Quality Guidelines stipulate that concentrations of atrazine not be over 2 µg/L for the protection of aquatic life (Canadian Water Quality Guidelines, 2008). However, studies have shown that concentrations in both drinking water and surface water frequently exceed these recommended values. Concentrations in drinking water have been reported up to 81 µg/L in Canada and surface water concentrations have been known to reach as high as 108 µg/L in the United States following spring application (Graymore *et al.,* 2001; USEPA, 2003).

The detection of atrazine in water samples is performed using gas chromatography with either flame ionization, electron capture, or mass spectrometry (Health Canada, 1993). These are all costly and labour-intensive methods which do not provide rapid results. As it is possible to remove up to 91% of atrazine from drinking water using granular activated carbon, powdered activated carbon, ion exchange, ozone oxidation or, UV radiation, it is important to know when the compound is present so that proper treatment of drinking water can occur (Jiang *et al.,* 2006). Therefore, determining a set of predictive behavioural patterns for several relevant study organisms to detect atrazine in a multi-species, early warning biomonitoring system is very important.

Human Health Concerns

Humans are most likely to be exposed to atrazine through consumption of contaminated drinking water rather than through contact or inhalation (Health Canada, 1993). Contact through air is highly unlikely due to the compound's low volatility, except during or immediately after application (Health Canada, 1993). It is also unlikely that consumption from food products

sprayed with the chemical will occur because residues on food are low or non-existent (IARC, 1999). When it enters the body through drinking water consumption, 93-100% of atrazine is absorbed across the gastro-intestinal system and taken into cells where it is broken down into metabolites by cytochrome P-450 (Health Canada, 1993).

Exposure via drinking water has been linked to a number of health issues in humans. Immediately after consumption of contaminated water, patients often complain of nausea and dizziness (Health Canada, 1993). The real risk occurs with chronic exposure to low concentrations of atrazine. An increased risk of ovarian, uterine and breast malignancies, as well as non-Hodgkin's lymphoma have been associated with chronic atrazine exposure in workplace and rural settings (Donna *et al.*, 1984; Hoar *et al.*, 1988; Health Canada, 1993). The increased risk of reproductive system tumours is likely tied to the disruptive effect that atrazine has on hormone regulatory systems. Atrazine has been demonstrated to act on the pituitary-gonadal system which is responsible for the regulation of several hormones (Health Canada, 1993). Exposure to atrazine has been shown to increase follicle stimulating hormone (FSH) and luteinizing hormone (LH) and to cause abnormalities in the body's ability to properly metabolize testosterone (Health Canada, 1993). No conclusive findings about atrazine's role as a carcinogen have been determined and it is therefore classified as a Group 3 Carcinogen by Health Canada (possibly carcinogenic to humans) (Health Canada, 1993). The International Agency for Research on Cancer (IARC) also classifies atrazine as a possible human carcinogen (IARC, 1999). Both Health Canada and the World Health Organization (WHO) recommend that human intake not exceed 0.5 mg atrazine per kilogram body weight per day in order to reduce the risks associated with atrazine intake (Health Canada, 1993). As atrazine is a potential human carcinogen, it is important to detect and subsequently remove it from drinking water. The development of a system that monitors changes in behavioural parameters of organisms in the presence of atrazine would greatly help in this goal.

Environmental Concerns

Many studies have also looked at the health effects of atrazine on aquatic organisms and larger land mammals. Several studies have been conducted using rats as models for the effects of atrazine in humans. In rat studies, dose-related increases in mammary gland and lymph system tumours were reported (IARC, 1999), as well as increased embryonic and foetal deaths,

decreased foetal weights, and retarded skeletal development in young following two years of chronic low level exposure to 20-40 mg/kg atrazine in food (Health Canada, 1993). In an environmental context, this is an extremely high level of exposure, unlikely to ever be seen at an equivalent level in an aquatic setting.

Studies of uptake in aquatic organisms exposed to the contaminant have also been performed. When exposed to atrazine concentrations of 230 µg/L and higher, the aquatic insect *Chironomus tentans* had reduced hatching success, abnormal larvae development, and a reduction in the number of organisms which reached the pupae life stage (Dewey, 1986). Fish and larval tadpoles are also common test species used during atrazine exposures. After exposure to 120 µg/L atrazine, brook trout experienced a significant reduction in growth rate while both zebrafish and rainbow trout experienced changes in swimming behaviour and motility when exposed to concentrations of 6 and 80 µg/L respectively (Dewey, 1986; Steinberg *et al.*,1995). *Rana catesbiana* tadpoles exposed to 20 µg/L of atrazine for 80 days showed a significant decrease in biomass compared to controls, and have an LC_{50} of 410 µg/L (Detenbeck *et al.*,1996). Abnormal gonadal development such as feminization, hermaphroditism, and reduced laryngeal muscle size in *Xenopus laevis* tadpoles have been reported following prolonged exposure to concentrations as low as 1 µg/L (USEPA, 2003). This is likely connected to endocrine disruption caused by the compound (USEPA, 2003).

Atrazine in Aquatic Ecosystems

Atrazine is highly persistent and has a half-life of 12 weeks in acidic water conditions, and up to 2 years or more in neutral or basic waters (Health Canada, 1993; Detenbeck *et al.*, 1996). Concentrations of up to 1000 µg/L have been reported in streams and rivers directly next to fields where atrazine has been applied (Denoyelles *et al.*, 1982). Many coastal and estuarine areas have reported detectable levels of atrazine, but due to dilution concentrations are often much lower than freshwater bodies located near farm areas (Graymore *et al.*, 2001). As mentioned before, concentrations of atrazine in freshwater varies depending on season, with spring and summer months showing increased aquatic deposits due to sprayings during the growing season and high runoff from summer storms (Graymore *et al.*, 2001; Anderson and Lydy, 2002; USEPA, 2003). Ambient concentrations in many lakes and rivers vary between 1 and 10 µg/L, depending on the time of year and the size of the water body (USEPA, 2003).

Aquatic environments are thus constantly exposed to chronic low levels of atrazine, as well as short-term pulses of high concentrations of the pesticide (Detenbeck *et al.,*1996; USEPA, 2003).

The breakdown of atrazine in the water column is accomplished through a combination of biological and chemical mechanisms (Winkelman and Klaine, 1990; Graymore *et al.,* 2001; USEPA, 2003). The compound is broken down into two main metabolites, 2- and 4-dihydroxy derivatives (Hamilton *et al.,* 1989; Winkelman and Klaine, 1990; Graymore *et al.,*2001). Bacteria and fungi in the water column complete the first step of degradation by splitting the ethyl groups from the triazine ring to use as an energy source. Chemical hydrolysis then removes the chloride ions from the molecule. A series of dealkylation and hydroxylation reactions follows, reducing the remaining molecule to the primary metabolites (Hamilton *et al.,* 1989; Winkelman and Klaine, 1990). Atrazine can also be broken down through photochemical processes; however, this mechanism is less important and takes a much longer period of time to occur than other mechanisms (Graymore *et al.,*2001).

As atrazine acts as a herbicide, the aquatic populations first affected are often algae and aquatic macrophytes (Graymore *et al.,* 2001; USEPA, 2003). Reduction in algal biomass and abundance have been seen with exposure to atrazine concentrations as low as 20 µg/L (Graymore *et al.,* 2001). In concentrations less than 10 µg/L, a decrease in photosynthesis in phytoplankton and periphyton communities has been seen (Graymore *et al.,* 2001). At concentrations over 500 µg/L, photosynthesis, carbon uptake and biomass are reduced by 95% in under two days (Graymore *et al.,*2001). These results are particularly alarming as concentrations such as these are often found in aquatic waterbodies located near sprayed fields and decreases in aquatic plant life can affect the entire food web due to their use as food sources for animals within the aquatic system (Denoyelles *et al.,* 1982).

Atrazine and Past Bioassays with Study Organisms

Several bioassays have been conducted in the past examining the effects of atrazine on the survival of *Daphnia magna, Hyalella azteca,* and *Lumbriculus variegatus*. LC_{50} values for various periods of time have been reported for all three organisms. The USEPA (2003) reports that *Daphnia magna* have a 48-hour LC_{50} value of 49 mg/L atrazine, that *Hyalella azteca* have 48-hour LC_{50} value of 14.7 mg/L and *Lumbriculus variegatus* have a 48-hour LC_{50} value of 37.1

mg/L. An 18-hour LC_{50} for *Hyalella* has also been determined to be 2 mg/L (USEPA, 2003) and a 72-hour LC_{50} for *Daphnia* has been reported to be 72 mg/L (Wan *et al.*, 2006). Reproductive bioassays have also been conducted using *Daphnia magna*. At concentrations of 250 µg/L and higher, a significant reduction in the number of offspring produced was observed (Dewey, 1986). Several bioassays have been done examining the effects of atrazine on proposed study organisms; however, these studies were conducted with exposure periods ranging days to weeks with mortality as an endpoint. Few studies have been conducted using this particular contaminant to examine impacts on a sub-acute (fewer than 24 hours) timescale. Observations of behavioural changes in response to atrazine are also lacking, making it an ideal contaminant to determine a behavioural response pattern for in the study organisms.

Rationale for Use of Atrazine

Atrazine is chronically toxic to many organisms at very low levels and very persistent in the environment. The chemical is constantly found at low levels in the aquatic environment and pulse inputs of very high concentrations have been noted after application and from agricultural run-off. Current detection methods are expensive and time-consuming, and detection of the contaminant is important due to human health risks and the potential for drinking water treatment facilities to remove it during treatment. A multi-species, early-warning biomonitoring technology may greatly contribute to the detection and removal efficiency of atrazine from drinking water. To date, very few sub-acute (under 24 hour) bioassays examining behaviour and respiration responses to the contaminant have been conducted using the test organisms *Daphnia magna*, *Hyalella azteca*, and *Lumbriculus variegatus*. Testing is therefore required to determine if these organisms are responsive to low levels and pulse levels of the contaminant and if it could potentially be detected by a multi-species, early-warning biomonitoring technology using these organisms.

1.6.3 Dimethyl Sulfoxide (DMSO)

Dimethysulfoxide (DMSO) is a commonly used organic solvent in toxicity testing. DMSO (Figure 6) was used in the following study to make stock solutions of tributyltin and atrazine, which have a low solubility in water. DMSO helps to solubilise the contaminant and makes sure that it is distributed evenly throughout the water column (Bowman *et al.*, 1981). Some concerns have been raised about the toxicity of the compound and the idea that it may impact the test

organisms. At low concentrations on a per volume basis, DMSO is less toxic than other commonly used solvents such as methanol, ethanol, acetone and acetonile (Bowman *et al.*, 1981). In this study, an overall concentration of 0.1% v/v DMSO was used for all bioassays. This concentration has been shown to be a safe working concentration that has no impact on behaviour or mortality in test organisms (Ura *et al.*, 2002; Hutchison *et al.*, 2006; Hallare *et al.*, 2006; Martins *et al.*, 2007b; Ren *et al.*, 2008; Ren *et al.*, 2009).

Figure 6: Chemical structure of dimethylsulfoxide (DMSO) (NCBI PubChem, 2009c)

1.7 Thesis Rationale and Objectives

The area of behavioural ecotoxicology is rapidly expanding, as more interest is taken in rapidly detecting and mitigating environmental damage and protecting limited drinking water resources. However, as many contaminants exist in aquatic environments, a great deal remains unknown about the impacts of individual chemicals at an organismal level. The objectives of this study are as follows:

- To perform laboratory behavioural bioassays, monitored using digital video analysis, in order to determine if environmentally-relevant concentrations of tributyltin (TBT) and atrazine have a significant impact on the several behaviour characteristics in the three study organisms. Information gained from digital video analysis can be used in the future in a video-based biomonitoring technology, or can be used to determine relevant characteristics to monitor using an impedance-based technology, such as the MFB
- To determine effective concentration (EC) values for TBT and atrazine for various behavioural parameters in the three study organisms
- To determine if *Daphnia magna*, *Hyalella azteca*, and *Lumbriculus variegatus* can be reliably utilized in sub-acute bioassays and to determine what parameters of locomotory behaviour in the three organisms are most useful for incorporation into a model to help determine classes and exact concentrations of contaminants

- To determine if respiration measured via direct oxygen consumption is a suitable bioassay parameter to be used in a multi-species, early-warning biomonitoring technology and if changes in respiration rates are observed in response to environmentally relevant concentrations of TBT and atrazine

This project will contribute to an existing body of knowledge regarding behavioural and physiological ecotoxicology and will help to determine appropriate organisms and responses to be monitored *in situ* using a fully automated multi-species, early-warning biomonitoring technology.

2.0 Materials and Methods

2.1 Bioassay Organism Culturing

2.1.1 *Daphnia magna*

Culturing procedures were developed based on protocols from Environment Canada (1996), the USEPA (2002) and the OECD (2004). Four cultures of *Daphnia magna* were maintained in the laboratory for use in the evaluation of changes in cladoceran behaviour and physiology in response to the addition of contaminants. Cultures were raised in four separate 9-L glass aquaria. An overlay of 7 L of natural spring water (Selection brand, Hillsburgh, ON) (OECD, 2004) was added to each tank to allow for an appropriate depth of water (approximately 15 cm) for the *D. magna*. This water was aerated for 2 days prior to initial usage to ensure at least 80% oxygen saturation of the water (approximately 8.5 mg/L). Water temperature in the aquaria was maintained at room temperature (18-20 degrees Celsius) throughout the culturing and experimental period. Dissolved oxygen (DO) levels were maintained using an aquarium bubbler. Aquaria were kept in a laminar-flow hood (Canadian Cabinets, model number H4-MW-97- C-30) to prevent dust and other debris from entering the culture tanks and negatively impacting the organisms. A 16hr : 8hr light: dark cycle was maintained using a timer and fluorescent lights, with a light intensity of approximately 500 lux (Environment Canada, 1998), under the laminar flow hood. The 16hr : 8hr light: dark cycle was used during all experiments with *D. magna*, in order to prevent changes in behaviour associated with differing light conditions.

D. magna were fed Mondays, Wednesdays, and Friday a mixture of Roti-Rich Invertebrate Food (Ward's Natural Science, 88V5910) and *Selenastrum capricornutum* algae. Each tank was provided with 5 mL of the invertebrate food and 20 mL of the algae (1.5×10^6 cells/mL) during each feeding period. Water clarity was monitored visually to determine if algae was being consumed and feeding volumes were adjusted based on the amount of algae consumed by the daphnids between feeding periods. Excess food and other debris were removed during every feeding day using a sterile 1.5-mL transfer pipette. New spring water was also added to the aquaria on Monday, Wednesday and Friday to replace any culture water which may have been lost due to evaporation. A 4 L container of natural spring water was aerated continuously for the

purpose of replenishing culture tanks. As daphnid populations grew to more than 200 daphnids/aquaria, neonates were transferred to new tanks using sterile transfer pipettes to prevent overcrowding and to reduce stress amongst the organisms. Water used in bioassays was the same as in culture tanks in order to minimize disruptions in behaviour which could potentially be caused by changes in water temperature.

2.1.2 *Hyalella azteca*

Culturing procedures were developed based on protocols established by Environment Canada (1997) and the USEPA (2000). Three cultures of *Hyalella azteca* were maintained in the laboratory for use in the evaluation of the amphipod swimming behaviour and respiratory rate changes in response to the addition of contaminants. Cultures were raised in three separate 9-L glass aquaria. An overlay of 7-L of natural spring water (Selection brand, Hillsburgh, ON) (USEPA, 2000) was present in each tank to allow for an appropriate depth of water (approximately 15 cm) for the *H. azteca*. This water was aerated for 2 days prior to initial usage to ensure at least 80% oxygen saturation (approximately 8.5 mg/L). Water temperature in the aquaria was maintained at room temperature (18-20 degrees Celsius) throughout the culturing and experimental period. Dissolved oxygen levels were maintained using an aquarium bubbler. Aquaria were located underneath a hood with laminar flow to prevent dust and other debris from entering the culture tanks and negatively impacting the organisms. A 16hr : 8hr light: dark cycle was maintained using a timer and fluorescent lights, with a light intensity of approximately 500 lux (USEPA, 2002), under the laminar flow hood. The 16hr : 8hr light: dark cycle was used during all experiments with the *Hyalella*, in order to prevent changes in behaviour associated with differing light conditions. Water used in bioassays was the same as in culture tanks in order to minimize disruptions in behaviour which could potentially be caused by changes in water temperature.

The organisms were fed Mondays, Wednesdays and Fridays when three to four crushed flakes of TetraMin fish food were added to the aquaria. Excess food and other debris were removed every feeding day using a sterile 1.5-mL transfer pipette. *Hyalella* were also provided with sterile cotton gauze for use as a substrate. Gauze was soaked for 24 hours in a beaker of natural spring water prior to introduction to the aquaria and was replaced when more than 50% of the previously added material had been shredded by the organisms. Water was also added to

the aquaria on Mondays, Wednesdays and Fridays to replace any culture water which may have been lost due to evaporation.

2.1.3 *Lumbriculus variegatus*

Culturing procedures were developed based on protocols established by the USEPA (2000). Cultures were raised in two 9-L glass aquaria which were sealed at the corners with a non-toxic silica sealant. A 7-L overlay of dechlorinated tap water was provided in each tank (USEPA, 2000). *Lumbriculus variegatus* can be cultured in a variety of waters, including reconstituted water, dechlorinated tap water, and spring water (USEPA, 2002). The type of water used is therefore at the discretion of the researcher. Preliminary culturing experiments indicated that our organisms were most successful in dechlorinated tap water. Water was aerated vigorously for 48 hours and then filtered using an activated carbon aquarium filter prior to addition to the tank to ensure at least 80% oxygen saturation (approximately 8.5 mg/L) and dechlorination. Water temperature in the aquaria was maintained at room temperature (18-20 degrees Celsius) throughout the culturing and experimental period. Dissolved oxygen levels were maintained using an aquarium bubbler. A 16hr : 8hr light: dark cycle was maintained using fluorescent lights, with a light intensity of approximately 500 lux (USEPA, 1998), in the laboratory. The 16hr : 8hr light: dark cycle, was used during all experiments with *Lumbriculus variegatus*, in order to prevent changes in behaviour associated with differing light conditions. Water used in bioassays was the same as in culture tanks in order to minimize disruptions in behaviour which could potentially be caused by changes in water temperature.

Lumbriculus cultures were fed a diet of crushed TetraMin fish food three times a week. A substrate of unbleached paper towel was also provided to the organisms. Paper towel was hand-torn into 1-2 cm strips and a 1-2 cm layer of paper towel was allowed to accumulate at the bottom of the aquaria before the addition of the organisms. Shredded paper towel was soaked for a minimum of 24 hours prior to introduction to the tanks and was replaced as needed. Water in the aquaria was also replaced on an as-needed basis using dechlorinated, filtered tap water.

2.2 Washing Procedures

Prior to use, all glassware, aquaria, and other reusable pieces of lab equipment were washed thoroughly to ensure that any traces of chemicals from prior use were removed and did

not affect test organisms. Washing procedures were based on those described by Environment Canada (1996).

Glassware was first washed using acetone three times, and then rinsed with tap water. Glassware was then soaked in an Extran soap solution for 15 minutes, and scrubbed to remove any residue. The soap was then rinsed off using tap water. Finally, glassware was washed in 10% v/v hydrochloric acid, and rinsed three times using distilled water. Glassware was placed in an inverted position to dry prior to being used.

2.3 Dilutions

All dilutions were made from stock solutions of 100 mg/L tributyltin (TBT) in DMSO and 100 mg/L atrazine in DMSO. Dilution calculations are presented in Appendix A. Test solutions all had a final concentration of 0.1% DMSO, a value which has been used in past bioassays and is not considered to have an impact on aquatic organisms (Ura *et al.,* 2002; Hutchison *et al.,* 2006; Hallare *et al.,* 2006; Martins *et al.,* 2007b; Ren *et al.,* 2008; Ren *et al.,* 2009). In all bioassays, reference and 0.1% DMSO control treatments were performed at the same time as the TBT and atrazine treatments to examine normal behaviour and to ensure that the DMSO was not contributing any toxicity.

2.4 Behaviour Bioassays

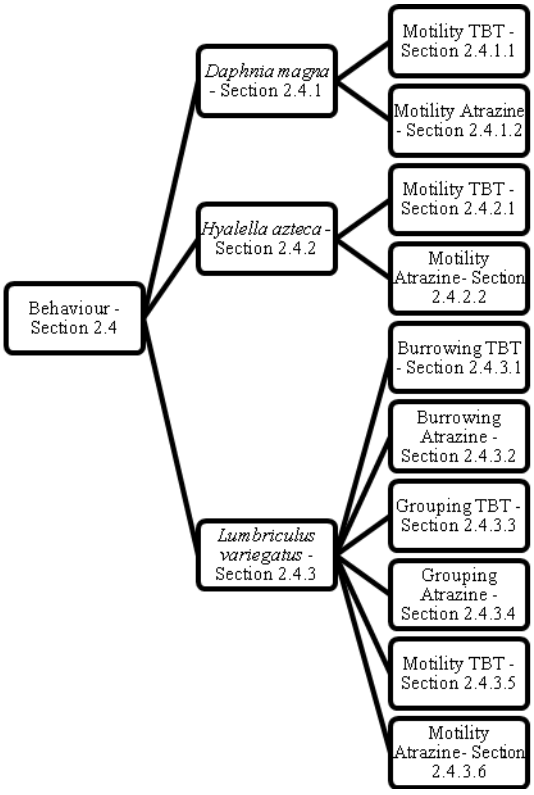

Figure 7: Outline of behavioural bioassays performed.

2.4.1 *Daphnia magna* Behaviour

2.4.1.1 Daphnia magna Motility TBT (0 – 24 Hours Inclusive)

Swimming behaviour bioassays using *Daphnia magna* exposed to varying concentrations of TBT were performed using digital video analysis. Square glass containers with a total volume of 200 mL with 150 mL of each test solution were used as bioassay vessels in this experiment. Three different concentrations of TBT were tested in this bioassay (10, 50 and 100 µg/L). Since no prior research has been conducted examining changes in *Daphnia* movement in response to TBT, initial experiments were performed prior to this bioassay, which showed concentrations of TBT ≤ 10 µg/L had no effect on *Daphnia magna* movement behaviours. Concentrations were therefore increased in this bioassay in order to determine if elevated concentrations would impact the swimming behaviour of the organisms. A reference (aerated natural spring water) and a solution with 0.1% DMSO were also evaluated in order to examine behaviour under normal conditions and when exposed to the carrier alone. Vessels containing the five different treatments were randomly arranged in order to control for variation caused by lighting, temperature, and

other abiotic conditions within the laboratory. Five daphnids were present in each test vessel. Three replicates of the bioassay were performed.

Changes in swimming behaviour were evaluated using digital video and personal observations made while the experiment was being conducted. Videos and personal observations were made after 0, 1, 2, 3, 4, 5, 6, 12, and 24 hours of exposure to the test solutions. Video was taken from above each test vessel using a Canon S515 digital camera. The camera was positioned above each test vessel using a tripod and five minute segments of video were taken of each treatment vessel for each replicate during each hour of exposure.

Behavioural patterns which were observed were based on past bioassays conducted by Dodson *et al.* (1995); Untersteiner *et al.* (2003); Schmidt *et al.* (2005) and Szulkin *et al.* (2006) and on personal observations made by the researcher during initial behavioural monitoring. Characteristics which were observed included changes in the organisms' swimming height in the water column (Dodson *et al.,* 1995; Schmidt *et al.,* 2005), movement style and fluidity (Dodson *et al.,* 1995; Szulkin *et al.,* 2006), the presence of looping or twirling behaviour (Dodson *et al.,* 1995), the overall activity level of the organisms (Untersteiner *et al.,* 2003; Schmidt *et al.,* 2005), immobility of the organisms (personal observation), the use of secondary antennae (Untersteiner *et al.,* 2003; Schmidt *et al.,* 2005), and the body position of the organisms (upright or on their sides) (personal observation). Observations of behavioural patterns were made by the experimenter during the bioassay and by viewing the videos following the experiment. Specific evaluations were made of: i) swimming height in the water column, ii) spinning, iii) body orientation, iv) immobilization, v) secondary antennae use, and vi) swimming style.

Changes in individual behaviours over time were evaluated by determining the percent of the organisms which displayed altered behaviour in each replicate. Qualitative observations of general trends of behavioural changes were noted and discussed for use in ranking of the importance of the various parameters. A non-parametric Kruskal-Wallis test was then performed to determine if the different treatments had an effect on the individual movement parameters when compared to reference treatments over the 24-hour experimental period. Statistical differences were not analyzed between concentrations or temporally since experiments were conducted for ranking the importance of the various behaviour parameters, rather than

determining specific impact concentrations. Further statistical analyses and modelling, which is beyond the scope of this project, is required to determine exact dose-response relationships.

Daphnids were examined for mortality after 72 hours of exposure to the TBT test solutions. Any organisms not moving were gently prodded with a sterile transfer pipette to induce movement or gently picked up with the transfer pipette to be more closely examined for signs of movement and life. Organisms were considered dead if no movement occurred during these examinations. The number of dead organisms for each concentration was determined and the percentage of dead organisms per treatment was compared using a non-parametric Kruskal-Wallis test to see if treatment had an impact on the number of mortalities over 72 hours. Examining organisms for mortality was a secondary impact of the contaminant and was not included in the study objectives; however, observations of TBT concentrations that caused death were worth noting.

2.4.1.2 Daphnia magna Motility Atrazine (0 – 24 Hours Inclusive)

This bioassay was set-up in a similar manner to the set-up for the TBT bioassay described above in Section 2.4.1.1 with the following modifications. Three different concentrations of atrazine were used, 5, 50, and 100 µg/L. Three replicates of the bioassay were again performed. The same changes in swimming behaviour were examined using digital video and personal observations while the experiment was being conducted. Videos and personal observations were made after 0, 1, 2, 3, 4, 5, 6, 10, and 24 hours of exposure to the test solutions. Evaluations of changes in behaviour were made as described above.

2.4.2 *Hyalella azteca* Behaviour

2.4.2.1 Hyalella azteca Motility TBT (0-24 Hours Inclusive)

The effects of three different concentrations of TBT on the movement behaviour of the amphipod was evaluated using digital video analysis. Bioassay vessels used in this experiment were 200 mL square glass containers containing 150 mL of each test solution. Organisms were also provided with an overlay of 0.5 cm of silica sand to use as a substrate. The silica sand substrate was rinsed 3 times in deionized water in order to remove any dust or other debris which may have been present and then autoclaved.

Three different concentrations of TBT were tested in this bioassay (10, 50, and 100 µg/L). Preliminary experiments using lower concentrations of the contaminant showed that

concentrations ≤ 10 µg/L TBT had no effect on the locomotion of the *Hyalella*, thus necessitating increased concentrations in this bioassay to determine what, if any, concentration of the contaminant would negatively affect the organisms. A reference (aerated spring water) and a solution with 0.1% DMSO were also evaluated in order to examine behaviour under normal conditions and when exposed to the carrier alone. Five organisms were present in each vessel. Three replicates of the bioassay were performed. Vessels containing the five different treatments were randomly arranged in order to control for variation caused by lighting, temperature, and other abiotic conditions within the laboratory.

Changes in swimming and movement behaviour were evaluated using digital video and personal observations while the experiment was being conducted. Videos and personal observations were made after 0, 1, 2, 3, 4, 5, 6, 12, and 24 hours of exposure to the test solutions. Video was taken from above each test vessel using a Canon S515 digital camera. The camera was positioned above each test vessel using a tripod and five minute segments of video were taken of each treatment vessel for each replicate during each hour of exposure.

Behavioural patterns which were observed were based on past bioassays conducted by Hatch and Burton (1999) and Wang *et al.* (2004) and on personal observations made by the researcher during initial behavioural monitoring**.** Characteristics which were observed included general activity of the test organisms (personal observation), immobilization of organisms (personal observation), the performance of swimming events (Wang *et al.*, 2004), organisms crawling on the substrate (Wang *et al.*, 2004), burrowing and grouping by organisms (Hatch and Burton, 1999; Wang *et al.*, 2004), and body positioning including shortening of body length (personal observation), and the ability of organisms to maintain an upright position on the substrate (personal observation). Observations of behavioural patterns were made by the experimenter during the bioassay and by viewing the videos following the experiment. Specific evaluations were made of : i) swimming events, ii) substrate crawling, iii) immobilization, iv) burrowing, v) grouping, vi) body length, and vii) body orientation.

Changes in individual behaviours over time were evaluated by determining the percent of organisms which displayed altered behaviour in each replicate. Qualitative observations of general trends of behavioural changes were noted and discussed for use in ranking of the importance of the various parameters. A non-parametric Kruskal-Wallis test was then performed

to determine if the different treatments had an effect on the individual movement parameters when compared to reference treatments. Statistical differences were not analyzed between concentrations or temporally since experiments were conducted for ranking the importance of the various behaviour parameters, rather than determining specific impact concentrations. Further statistical analyses and modelling, which is beyond the scope of this project, is required to determine exact dose-response relationships. After 72 hours, the *Hyalella* were examined for mortality as described above for the *Daphnia magna.* No deaths were seen, and thus this information is not presented in the Results and Discussion section.

2.4.2.2 Hyalella azteca Motility Atrazine (0-24 Hours Inclusive)

The effects of three different concentrations of the pesticide atrazine on the movement behaviour of the amphipod *Hyalella azteca* was evaluated using digital video analysis. This bioassay was set-up in a similar manner to the set-up for the TBT bioassay described above in Section 2.4.2.1 with the following modifications. Three different concentrations of atrazine were tested in this bioassay, 5, 50, and 100 µg/L. The same changes in swimming and movement behaviour were examined using digital video and personal observations while the experiment was being conducted. Videos and personal observations were made after 0, 1, 2, 3, 4, 5, 6, 10, and 24 hours of exposure to the test solutions. Evaluations of behaviour were made as described above in Section 2.4.2.1.

2.4.3 *Lumbriculus variegatus* Behaviour

2.4.3.1 Lumbriculus variegatus Burrowing TBT (0 – 4.5 Hours Inclusive)

The experiment was designed to test the effect of 3 different concentrations of tributyltin (TBT) on the burrowing behaviour of *Lumbriculus variegatus*. This evaluation was performed using digital time-lapse photography.

Bioassay vessels used in this experiment were 300 mL glass beakers. Five replicates of the experiment were performed in a single day, using a reference, DMSO reference (0.5% DMSO) and three different concentrations of TBT. The concentrations of TBT tested were 0.1, 1.0, and 10.0 µg/L TBT in water.

Five rows of five beakers were set-up on the white paper. Labels which identified the concentration and replicate number were created and taped to the corresponding beaker, so that

they were visible when examining the pictures during the analysis phase of the experiment. Beakers were placed in five by five rows in random order.

In each of the beakers, inert silica sand was used as a substrate for the *Lumbriculus* to burrow. Approximately 1 cm of silica sand was added to the bottom of each beaker to provide adequate depth for burrow formation. A 150-mL overlay of each treatment was added to each beaker. Beakers were covered with cheese-cloth to prevent dust and other material from entering and sand which was resuspended during the addition of water was allowed to settle for 1 hour.

Prior to setting up the experimental beakers, 250 adult *Lumbriculus* were separated from the culture colonies. Ten adult worms were placed in each beaker and a photograph was taken as described below marking time zero and the beginning of the experiment. After the *Lumbriculus* were placed in the experimental vessels, their burrowing behaviour was monitored for 4.5 hours using time-lapse photography. A Canon S515 digital camera was used in this experiment. One photograph was taken of each beaker every 15 minutes. A timer was used and photographs of each replicate were taken in the same order each time to ensure consistency of timing. The camera was held 10-15 cm above each beaker and a photograph was taken looking down into the vessel. The camera was allowed to auto-focus to ensure photo quality. Each round of 25 pictures took approximately 1 minute to complete.

Images were uploaded to a PC laptop from the camera's digital memory card. Images were arranged in folders in order of the time they were taken and by replicate. Images of each concentration and each replicate were examined and the number of organisms which were burrowed below the surface of the silica sand was counted. An organism was considered to be burrowed if no part of its body was extended to the surface of the silica sand. If any part of the organism was visible, that organism was not considered to be burrowed. The number of organisms burrowed at each time point for each replicate and concentration was recorded in table format. The average number of organisms burrowed in each treatment at each time point was determined and the average rate at which organisms burrowed was graphed. Trends in burrowing in the reference, 0.1% DMSO, and three TBT treatments were qualitatively analyzed to see if the contaminant had an overall effect on burrowing.

2.4.3.2 Lumbriculus variegatus Burrowing Atrazine (0 – 6 Hours Inclusive)

A bioassay examining the effects of three different concentrations of atrazine on the burrowing behaviour of *Lumbriculus variegatus* was performed over a 6-hour test period. Bioassay set-up was similar to the set-up for the TBT bioassay described in section 2.4.3.1, but with the following modifications. Three different concentrations of atrazine were tested in this bioassay, 5, 50, and 100 µg/L. After the *Lumbriculus* were placed in the experimental vessels, their burrowing behaviour was monitored for 6 hours using time-lapse photography. One photograph was then taken every 30 minutes for the 6-hour duration of the experiment. Burrowing behaviour was evaluated as described above in Section 2.4.3.1.

2.4.3.3 Lumbriculus variegatus Grouping TBT (0 – 48 Hours Inclusive)

This experiment was designed to test the effect of three different concentrations of tributyltin (TBT) on the grouping behaviour of *Lumbriculus variegatus*. This evaluation was performed using digital time-lapse photography. The experimental set-up and digital image analysis was performed as described above for the *Lumbriculus variegatus* burrowing bioassay but with the following modifications. No substrate was provided to the organisms. White paper was placed on the experimental set-up bench to provide good contrast of the organisms in the digital photographs. Labels were placed underneath the beakers to identify the concentrations and replicate number when examining the pictures during the analysis phase of the experiment. TBT concentrations of 10 µg/L, 50 µg/L and 100 µg/L were tested to provide a range of environmentally-relevant concentrations. Initial grouping behaviour evaluations indicated that no response was seen to concentrations of TBT below 10 µg/L, necessitating modifications to experimental procedures including increased contaminant concentrations and longer observation periods. Four replicates of the experiment were performed. Photographs were then taken every 30 minutes for the first 6 hours of the experiment, as well as 9, 24, 30, 42 and 48 hours after the introduction of the organisms to the test solutions.

During the image analysis, pictures from the experiment were examined and the number of organisms involved in a colony group was counted. A colony was considered to be a group if two or more organisms were tightly coiled together. The number of organisms involved in a group at each time point for each replicate was found. The effect of the contaminant on grouping behaviour was then evaluated qualitatively by examining the average rate at which organisms moved in and out of colonies.

2.4.3.4 Lumbriculus variegatus Grouping Atrazine (0 – 6 Hours Inclusive)

The bioassay to examine *Lumbriculus variegatus* grouping behaviour after exposure to atrazine was performed with a similar bioassay set-up as described above in Section 2.4.3.3, with the following modifications. Concentrations of atrazine used were 5, 50, and 100 µg/L. Photographs were taken every 30 minutes for the 6 hour duration of the experiment. Evaluations of grouping behaviour were performed as described above.

2.4.3.5 Lumbriculus variegatus Motility TBT (0 – 24 Hours Inclusive)

Bioassays assessing the effect of three different concentrations of TBT on the movement behaviour of *Lumbriculus variegatus* were performed over a 24-hour test period. Twenty 400-mL glass beakers were used as bioassay vessels in this experiment. A 150-mL overlay of each test solution was added to each beaker. Beakers were then randomly placed on white paper to provide good contrast of the organisms in the digital photographs. Four rows of 5 beakers were set-up on the white paper and were labelled as previously described. Three different concentrations of TBT were tested in this bioassay, 10, 50, and 100 µg/L. A reference (dechlorinated, filtered tap water) and a solution with 0.1% DMSO were also evaluated in order to examine behaviour under normal conditions and when exposed to the carrier alone. Vessels containing the five different treatments were randomly arranged in order to control for variation caused by lighting, temperature and other abiotic conditions within the laboratory. In total 4 replicates of the bioassay were performed simultaneously. The behaviour of 10 organisms was examined in each treatment beaker.

Movement behaviour was evaluated by using personal observations of the experimenter conducted after 0, 1, 3, 5, and 24 hours of exposure to the various treatments. Observations were based upon behavioural parameters of *Lumbriculus* noted by Drewes (1999), Drewes and Cain (1999), Ding *et al.* (2001), and O'Gara *et al.* (2004), and on personal observations made by the researcher during initial behavioural monitoring. Movement characteristics observed included positioning within the test vessel (personal observation), locomotion style (Drewes, 1999; Drewes and Cain, 1999; Ding *et al.,* 2001; O'Gara *et al.,* 2004), general activity level of organisms (Drewes, 1999; Drewes and Cain, 1999; Ding *et al.,* 2001; O'Gara *et al.,* 2004), immobility of organisms (O'Gara *et al.,* 2004), body length (O'Gara *et al.,* 2004), straightness of organisms' bodies (personal observation), and movement in, out and within groups of organisms (personal observation).

Changes in individual behaviours over time were evaluated by determining the percent of organisms which displayed altered behaviour in each replicate. Qualitative observations of general trends of behavioural changes were noted and discussed for use in ranking of the importance of the various parameters. A non-parametric Kruskal-Wallis test was then performed to determine if the different treatments had an effect on the individual movement parameters when compared to reference treatments. For parameters involving grouping, only organisms involved in a group were considered. For parameters involving locomotion, only individual organisms were considered. Statistical differences were not analyzed between concentrations or temporally since experiments were conducted for ranking the importance of the various behaviour parameters, rather than determining specific impact concentrations. Further statistical analyses and modelling, which is beyond the scope of this project, is required to determine exact dose-response relationships. Parameters specifically evaluated were: i) position in the bioassay vessel, ii) locomotion style, iii) immobilization, iv) body length, v) body orientation, and vi) group movement.

The organisms were examined for mortality after 72 hours of exposure to the test solutions. Any worms not moving were gently prodded with a sterile transfer pipette to induce movement or gently picked up with the transfer pipette to be more closely examined for signs of movement and life. Worms were considered dead if no movement occurred during these examinations. The number of dead *Lumbriculus* for each concentration was determined and the percentage of dead organisms per treatment was compared using a non-parametric Kruskal-Wallis test to see if treatment had an impact on mortality over 72 hours. Examining organisms for mortality was a secondary impact of the contaminant and was not included in the study objectives; however, observations of TBT concentrations that caused death were worth noting.

2.4.3.6 Lumbriculus variegatus Motility (0 – 24 Hours Inclusive)

Bioassays assessing the effect of three different concentrations of atrazine on the movement behaviour of *Lumbriculus variegatus* were performed over a 24-hour test period. Bioassay set-up was similar to that used in the TBT bioassay described above in Section 2.4.3.4, but with the following modifications. Three different concentrations of atrazine were tested in this bioassay, 5, 50 and 100 µg/L. Movement behaviour was evaluated by using personal observations of the experimenter conducted after 1, 3, 6, 9, and 24 of exposure to the various

treatments. The same behavioural characteristics were examined and similar evaluations of change were made as described above.

2.5 Respiration Bioassays

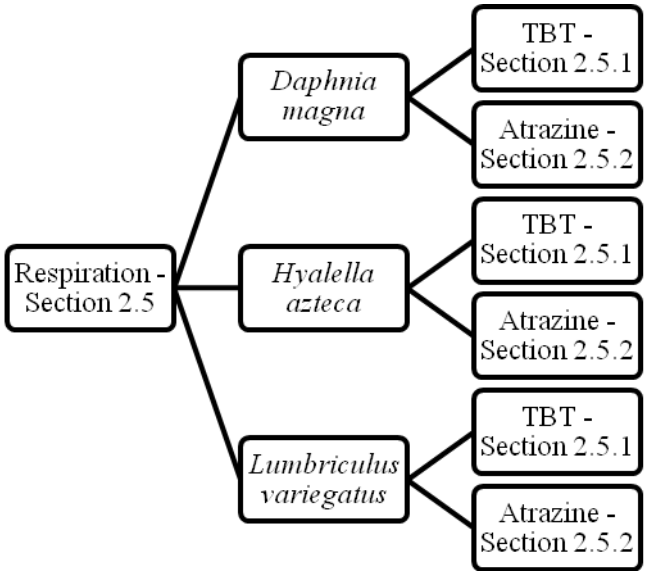

Figure 8: Outline of respiration bioassays performed.

2.5.1 *Daphnia magna, Hyalella azteca* and *Lumbriculus variegatus* Respiration in TBT

The effect of three different concentrations of TBT on the respiration rates of *Daphnia magna, Hyalella azteca,* and *Lumbriculus variegatus* were tested in three separate bioassays. Bioassay vessels used in these experiments were 25-mL scintillation vials with tight-fitting conical lids which displaced water when closing (ensuring no headspace) and prevented gas exchange with the surrounding environment. Lids were polypropylene and the rate at which oxygen could diffuse through the lid was negligible relative to oxygen consumption rates by respiration. Prior to beginning, the bioassay vials were half-filled with aerated natural spring water (culture media for *Daphnia magna* and *Hyalella azteca*) or dechlorinated, filtered tap water (*Lumbriculus* culture media). In the *Daphnia* bioassay, eight adult *Daphnia magna* were randomly selected from the culture tank and placed into each vial using a sterile transfer pipette. *Daphnia* were visually inspected prior to bioassay use to ensure they were not pregnant. Similarly in the *Hyalella* bioassay, three organisms were randomly selected and placed in the vials. Finally, in the *Lumbriculus* bioassay, ten organisms were randomly selected and placed in the vessels. Vials were then filled to the top with water and the appropriate amount and

concentration of TBT in 0.1% DMSO (vol/vol, final concentration in test vessels) was added to each vial using a micropipette.

TBT concentrations of 10 µg/L, 50 µg/L, and 100 µg/L were tested in each bioassay to provide a range of values. A 0.1% DMSO reference was also used to examine the effects of the carrier on respiration separate from the contaminant. A control of natural spring water or dechlorinated, filtered tap water was also used to provide a baseline for normal respiration behaviour. Bioassays were performed in triplicate for statistical significance.

Dissolved oxygen measurements were performed using a modified Winkler titration method (Appendix B) (MIT, 2006). For the *Daphnia magna* and *Lumbriculus variegatus,* respiration bioassay measurements were performed at the start of the bioassay (time zero) and at the beginning of every hour for the next six hours, and after 12 and 24 hours of exposure to the contaminant. For the *Hyalella azteca,* bioassay measurements were made at the start of the experiment and after 2, 4, 6, 10, and 24 hours of exposure. After 24 hours, all samples were titrated and respiration rates were determined based on replicated regression analysis of oxygen concentration versus time (Systat, version 11). Rate calculation was restricted to the interval over which oxygen consumption was linear. For cross-treatment comparisons, rates of respiration normalized for biomass (described below) were compared using t-tests for comparison of slope.

Separately, five groups of eight daphnids, three *Hyalella* and ten *Lumbriculus* were randomly taken from the culture tanks and wet weights were found using an analytical balance. The average weight of the groups of organisms was found and used to determine respiration rates per unit biomass (nanomole O_2/mg body mass/hour). Weight values from individual groups and average values are found in Appendix V.

2.5.2 *Daphnia magna, Hyalella azteca* and *Lumbriculus variegatus* Respiration in Atrazine

The effects of three different concentrations of atrazine on the respiration rates of populations of *Daphnia magna, Hyalella azteca* and *Lumbriculus variegatus* were observed using a Clark-type oxygen microelectrode (Unisense, Denmark). This electrode has a membrane diameter of 25 µm and can measure dissolved oxygen concentrations with high precision while minimizing oxygen consumption by the electrode. The size of the membrane also makes this probe very insensitive to stirring, and, hence, very stable. In a multi-species, early-warning

biomonitoring technologies, all monitoring must be automated. The use of Winkler titration, while highly accurate, is very time consuming and require a lab technician to perform. The microelectrode probe is much easier to automate and include in a field study when developing our multi-species, early-warning biomonitoring technology.

Bioassay vessels used in this experiment were 25-mL glass scintillation vials with tight-fitting conical lids which prevented gas exchange with the surrounding environment. Prior to beginning the bioassay, vials were half-filled with aerated natural spring water (culture media for *Daphnia magna* and *Hyalella azteca*) or dechlorinated, filtered tap water (*Lumbriculus* culture water). Fifteen adult *Daphnia magna,* eight adult *Hyalella* or twenty adult *Lumbriculus* were then randomly selected from their culture tanks and placed in each vial using a sterile transfer pipette. Vials were then filled to the top with water and the appropriate amount and concentration of atrazine was added to each vial using a micropipette.

A range of atrazine concentrations was tested (5, 50, and 100 µg/L). A reference (aerated natural spring water) and a solution with 0.1% DMSO were also evaluated in order to examine behaviour under normal conditions and when exposed to the carrier alone. Each vial was labelled with the treatment and replicate number and randomly placed in a test tube rack for the duration of the experiment. Vessels containing the five different treatments were randomly arranged in order to control for variation caused by lighting, temperature and other abiotic conditions within the laboratory. In total 3 replicates of the bioassay were performed simultaneously. The dissolved oxygen content of 3 replicates of blank solution containing no organisms or contaminants was also measured to ensure that there was no change in DO content over time without test conditions present.

Dissolved oxygen content in the vials was monitored for a total of 24 hours. Dissolved oxygen content in the vials was measured using a Unisense oxygen microelectrode attached to a PA2000 picoammeter (Unisense, 2009). A detailed description of the functioning of the microelectrode is presented below in Appendix C. Sample readings were taken after 0, 2, 4, 6, 10, and 24 hours of exposure to each treatment. Readings were also taken in a sample of water saturated with oxygen and from a water sample saturated with nitrogen. These readings were used to establish an oxygen content calibration curve. Picoampere readings from each vial at the

different time periods were then used to determine dissolved oxygen concentrations based on a calibration curve.

The respiration rates for each replicate vial were found by determining the slope produced when looking at the change in oxygen rate over time, and regression was restricted to the interval when oxygen decrease was linear with time. Separately, 5 groups of 15 daphnids, 8 *Hyalella,* and 20 *Lumbriculus* were randomly taken from the culture tanks and weighed using an analytical balance. The average mass of the groups of organisms was found and used to determine respiration rates per amount of biomass. Masses from individual groups and average values are found in Appendix V. The three individual respiration rates for each treatment were compared to the three respiration rates for the reference samples using a One-Way ANOVA, with post-hoc pairwise comparisons using a Tukey's HSD test.

3.0 RESULTS AND DISCUSSION

3.1 *Daphnia magna* Behaviour

3.1.1 *Daphnia magna* Motility

Several aspects of *Daphnia magna* swimming behaviour were evaluated during these bioassays and were found to change in the presence of varying concentrations of the contaminants TBT and atrazine. Changes in response to the contaminants are summarized below with a discussion of the ecological impacts of the change. Qualitative observations of general trends of behavioural changes were made to aid in the ranking of the importance of the various parameters. A discussion of the utility of the various parameters for evaluation in a multi-species, early-warning biomonitoring technology is also included. The following characteristics of *Daphnia magna* motility were examined: i) swimming height in the water column, ii) spinning, iii) body orientation, iv) immobilization, v) secondary antennae use, and vi) swimming style.

i) Swimming Height in the Water Column

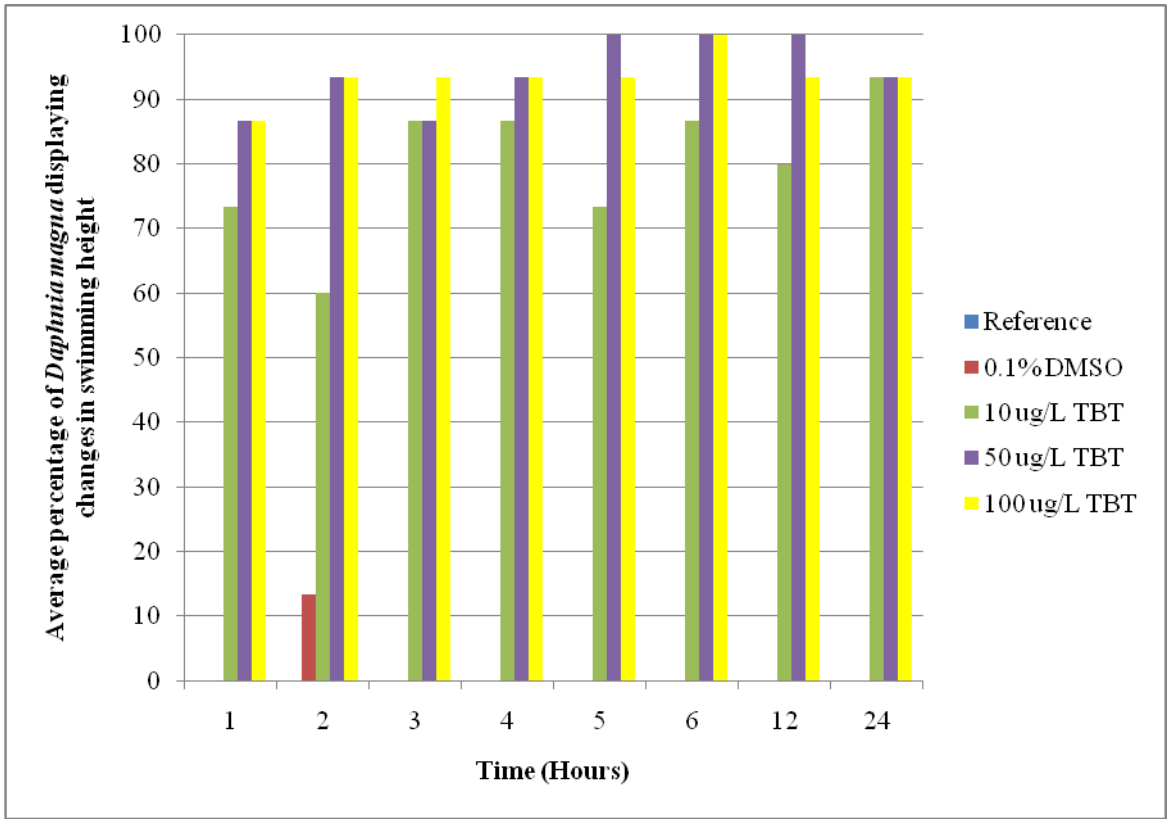

Figure 9: Average percentage of *Daphnia magna* displaying changes in swimming height in three concentrations of TBT over a 24 hour experimental period.

In the TBT bioassay, there was no significant difference in the swimming heights of the daphnids in the reference and 0.1% DMSO treatments (p = 0.317). There was a significant change in the swimming heights of *Daphnia* in the 10 µg/L TBT treatments (p = 0.000), the 50 µg/L TBT treatments (p = 0.000) and the 100 µg/L TBT treatments (p = 0.000) when compared to the reference treatments over the 24 hour experimental period. All three concentrations of TBT affected the daphnids in a time and concentration dependent manner, with an increasing number of organisms being impacted as time the experiment continued (Figure 9). In general, a greater number of organisms were impacted at higher concentrations than at lower concentrations.

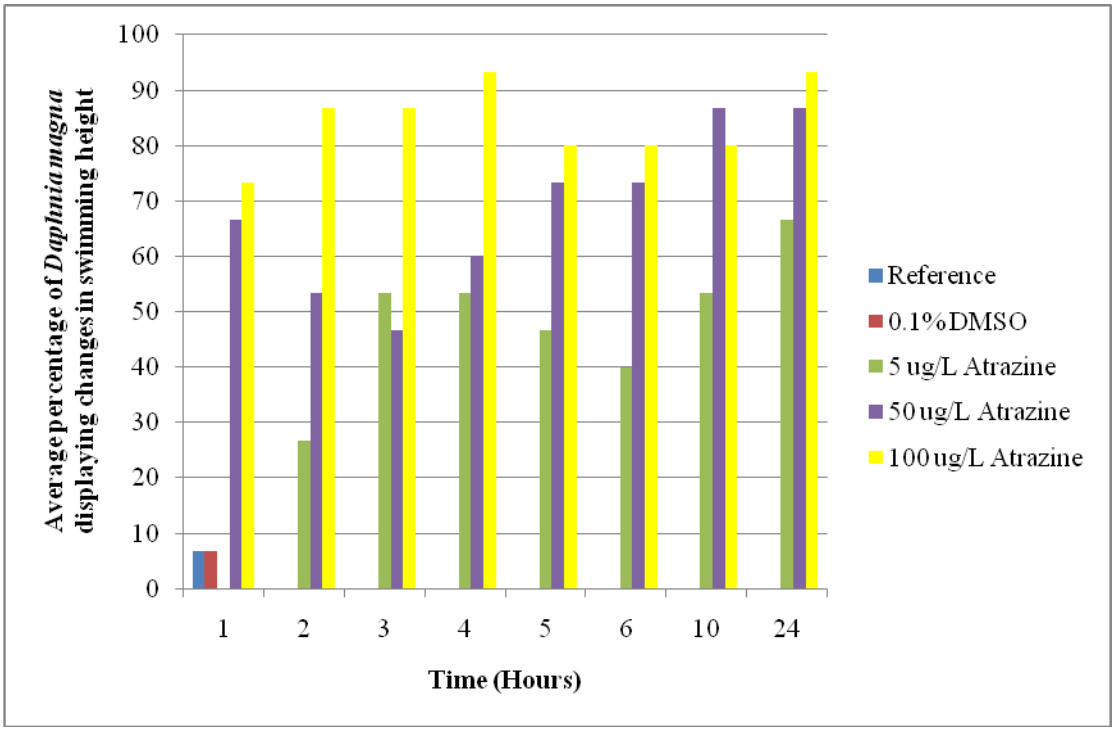

Figure 10: Average percentage of *Daphnia magna* displaying changes in swimming height in three concentrations of atrazine over a 24 hour experimental period.

In the atrazine bioassay, no significant differences in swimming height were seen when comparing the reference and 0.1% DMSO treatments (p = 1.000) over the 24 hour test period. Significant differences in the swimming height of organisms exposed to 5 µg/L atrazine (p =

0.000), 50 µg/L atrazine (p = 0.000), and 100 µg/L atrazine (p = 0.000) were seen when compared to the reference treatments. The 5 µg/L , 50 µg/L, and 100 µg/L atrazine treatments affected the swimming height of the organisms in a time and concentration dependent manner, with an increasing number of organisms displaying changes in swimming height as time progressed (Figure 10). In general, a greater percentage of the organisms displayed altered swimming height at higher concentrations than in the lower concentrations of contaminant.

In both bioassays, the organisms in the reference and 0.1% DMSO treatments moved throughout the height of the water column and did not spend extended periods of time at the surface of the water or resting on the bottom of the bioassay vessel. Similar behavioural responses were seen to the addition of both TBT and atrazine, with the majority of affected organisms remaining at the bottom of the bioassay vessel, with a few stuck at the surface of the water column seemingly unable to break through the surface tension. A decline in vertical movement was noted in all affected organisms. This parameter appears to be a sensitive and rapidly reached endpoint, and it is recommended that changes in swimming height of *Daphnia* be monitored in a multi-organism, early-warning biomonitoring technology in order to detect elevated levels of TBT and atrazine. Swimming height in the water column appears to be more sensitive to lower levels of TBT than atrazine (Figures 9 and 10). This indicates that changes in swimming height of daphnids may be useful for the detection of low levels of TBT and higher levels of atrazine. However, further analyses beyond the scope of this thesis are necessary to determine the exact time and concentration of exposure necessary to elicit the impacts seen, due to similar changes in behaviour seen in response to both contaminants.

The ability to swim up and down through the water column is closely related to diel vertical migration in daphnids. During the day under more intense light conditions, daphnids will spend the majority of their time near the bottom of the water column where they are less visible to visual predators. At night organisms will rise in the water column to forage and are less visible to predators due to the dark conditions (Cushing, 1951). If organisms are unable to rise throughout the water column or remain stuck at the surface of the water, they may be more vulnerable to predation by visual hunters. Daphnids must be able to move up and down in the water column in order to find food patches and to maintain their position in a patch of algae (Ryan and Dodson, 1998). Decreased foraging success will be seen if organisms are unable to

move from the sediment-water interface, as most algae is located higher in the water column where light is available. Increased predation and decreased foraging success may lead to a decline in the *Daphnia* population overall, which could potentially affect the entire aquatic foodweb (Schmidt *et al.*, 2005).

Similar changes in swimming height and loss of ability to perform vertical migration have been seen in studies performed by Kieu *et al.* (2001); Michels *et al.* (2001) and Martins *et al.* (2007a). *Daphnia* vertical migration has been shown to be affected by sub-lethal concentrations of copper, PCP, several pesticides, and various hydrocarbons within 4 hours of exposure (Kieu *et al.*,2001; Michels *et al.*, 2001 and Martins *et al.*, 2007a). These results support results obtained in the TBT and atrazine bioassays, and indicates that a lack of vertical migration is an abnormal swimming pattern and is indicative of chemical stress.

ii) Spinning

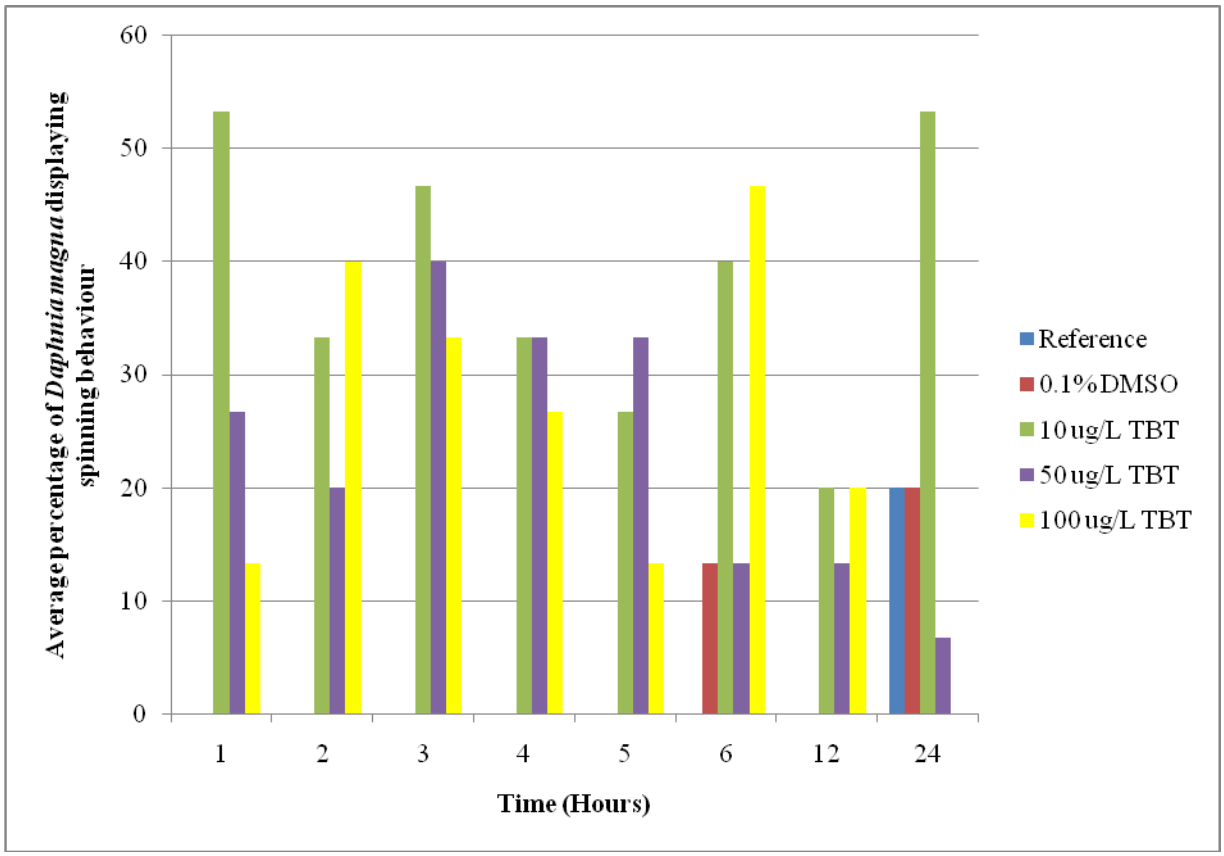

Figure 11: Average percentage of *Daphnia magna* displaying spinning behaviour in three treatments of TBT over a 24 hour experimental period.

In the TBT bioassay, no significant differences in the occurrence of spinning behaviour were seen when comparing the reference and 0.1% DMSO treatments ($p = 0.409$). A significant increase in the performance of spinning was seen when comparing the reference to the 10 µg/L TBT treatments ($p = 0.000$), the 50 µg/L TBT treatment ($p = 0.000$) and the 100 µg/L TBT treatments ($p = 0.000$) over the 24 hour experimental period. An increase in spinning behaviour was seen in all TBT treatments during the first 6 hours of the experiment. The average percentage of organisms performing swimming events fluctuated between timepoints and no consistent pattern of time or concentration dependence was seen with respect to the number organisms affected (Figure 11). In the two highest TBT concentrations, the number of organisms spinning decreased after 6 hours of exposure. This is related to the immobilization of organisms discussed below. As exposure continued, more and more organisms in the TBT treatments became immobilized and were thus unable to perform spinning behaviour. This indicates that spinning behaviour may be a good indicator of high concentrations of TBT following short-term exposure but not exposure periods over 6 hours, where immobilization may be a more sensitive indicator of stress.

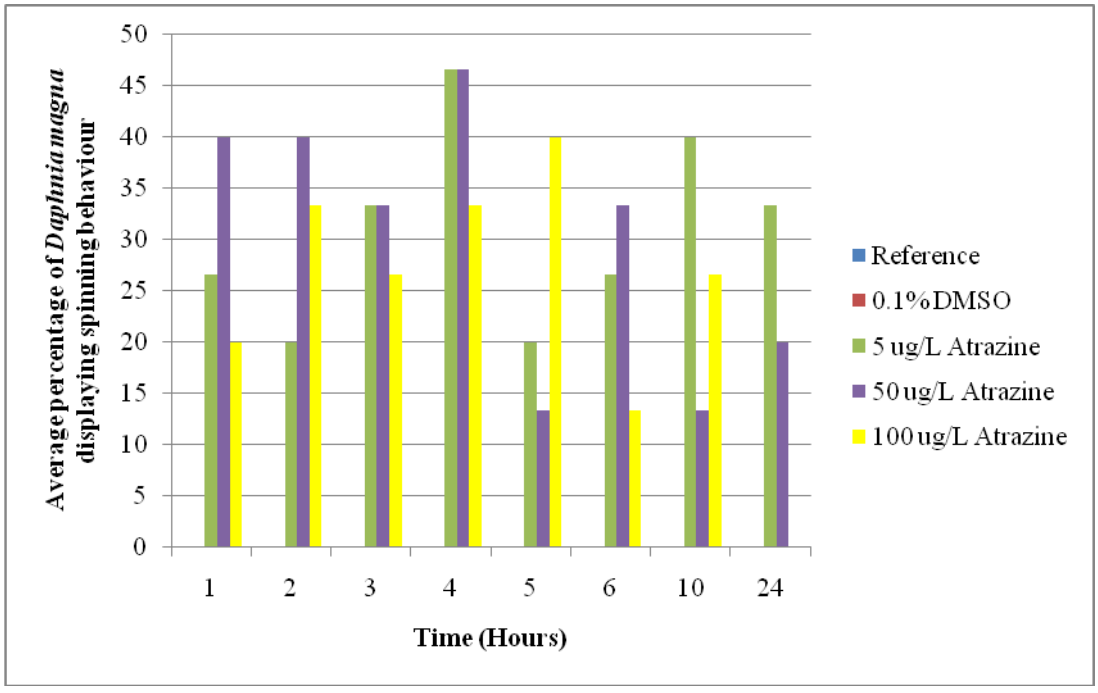

Figure 12: Average percentage of *Daphnia magna* displaying spinning behaviour in three treatments of atrazine over a 24 hour experimental period.

In the atrazine bioassay, no significant differences in the occurrence of spinning behaviour were seen when comparing the reference and 0.1% DMSO treatments ($p = 1.000$). A significant increase in spinning behaviour was seen in the 5 µg/L atrazine treatments ($p = 0.000$), the 50 µg/L atrazine treatments ($p = 0.000$), and the 100 µg/L atrazine treatments ($p = 0.000$) when compared to the reference treatments over the 24 hour period. The organisms in all three concentrations displayed spinning behaviour after 1 hour of exposure to the chemical. No pattern of concentration or time dependency was seen was seen and the average number of organisms performing swimming behaviour fluctuating at each time interval (Figure 12). The majority of spinning behaviour was seen between 2 and 6 hours of exposure and generally decreased after 10 hours of exposure to the chemical in all three treatments. This is closely related to the immobilization of the organisms. As the experimental period continued, a greater number of organisms became immobilized and were unable to perform spinning behaviour. This indicates that spinning behaviour may be a good indicator of atrazine contaminantion following short-term exposure, whereas immobilization may be a more sensitive indicator of stress after longer periods.

Increases in spinning behaviour occurred rapidly in response to both contaminants when compared to reference conditions. However, no time or concentration dependence was noted for either substance, making discrimination between the two contaminants difficult. As no clear pattern of response was noted in any concentration, it may also be difficult to create models able to determine the exact concentration of contaminant present. Spinning behaviour is clearly a rapidly induced stress response in *Daphnia magna*, and should be monitored in a multi-organism early-warning biomonitoring technology. However, due to the lack of a consistent pattern of response, changes in spinning behaviour should be considered less important than other parameters when attempting to detect substances such as TBT and atrazine.

Spinning is characterized by large increases in velocity and rapid changes in direction and occasional flips in the water. It is mostly performed at low levels in the water column and not while moving up and down through the water column (Dodson *et al.*, 1995). Spinning is believed to be a type of escape behaviour and has often been seen in response to fish attacks and rapid changes in light conditions (Dodson *et al.*,1995). It is therefore likely that increased swimming behaviour in response to TBT and atrazine represents the daphnids attempts to escape

the chemical when first exposed. Spinning behaviour is estimated to use approximately 400 times as much energy as normal swimming behaviour (Dodson *et al.*,1995). Decreases in spinning behaviour at the end of the experiment period suggest that the organism's energy resources have been depleted, likely due to contaminant exposure and use for escape behaviours. Spinning during swimming may have many ecological consequences for daphnids. Predation risk is likely to be increased. The behaviour is likely to attract the attention of visual predators due to its erratic nature and to tactile predators due to the increased vibrations in the water column from erratic movements (Dodson *et al.*, 1995). In a study by Dodson and colleagues (1995), spinning behaviour was induced in daphnids through exposure to high levels of carbaryl. It was found that bluegill sunfish will preferentially eat spinning organisms, even when presented with a normally swimming daphnid within closer proximity (Dodson *et al.*, 1995). Increases in this type of behaviour could therefore impact predation rates and cause an overall decrease in daphnid populations.

iii) Body Orientation

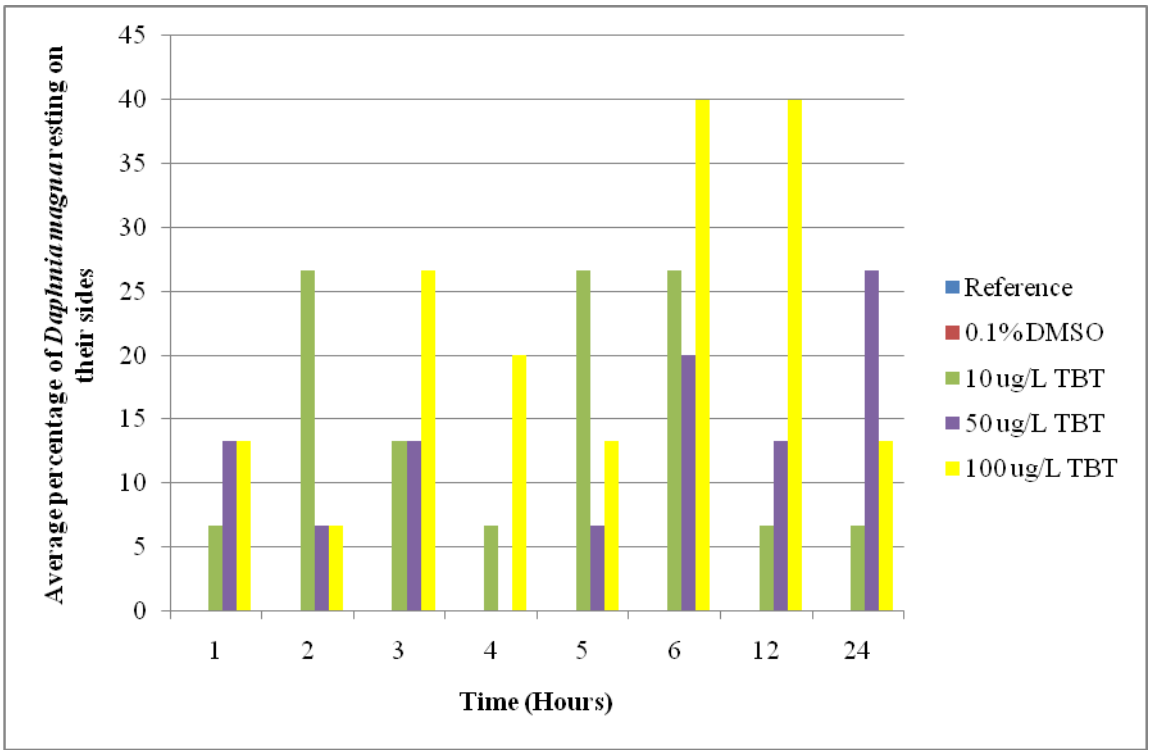

Figure 13: Average percentage of *Daphnia magna* displaying changes in body orientation in response to three concentrations of TBT over a 24 hour period.

In the TBT bioassay, there were no significant changes in the body orientation of the organisms when comparing the reference and the 0.1% DMSO treatments (p = 1.000). A significant increase in the number of organisms resting on their sides as opposed to swimming upright was seen in the 10 µg/L TBT treatment (p = 0.000), the 50 µg/L treatment (p = 0.000), and the 100 µg/L treatments (p = 0.000) when compared to the reference treatments over the 24 hour experimental period. No changes in body orientation were seen in the reference and DMSO treatments (Figure 13).

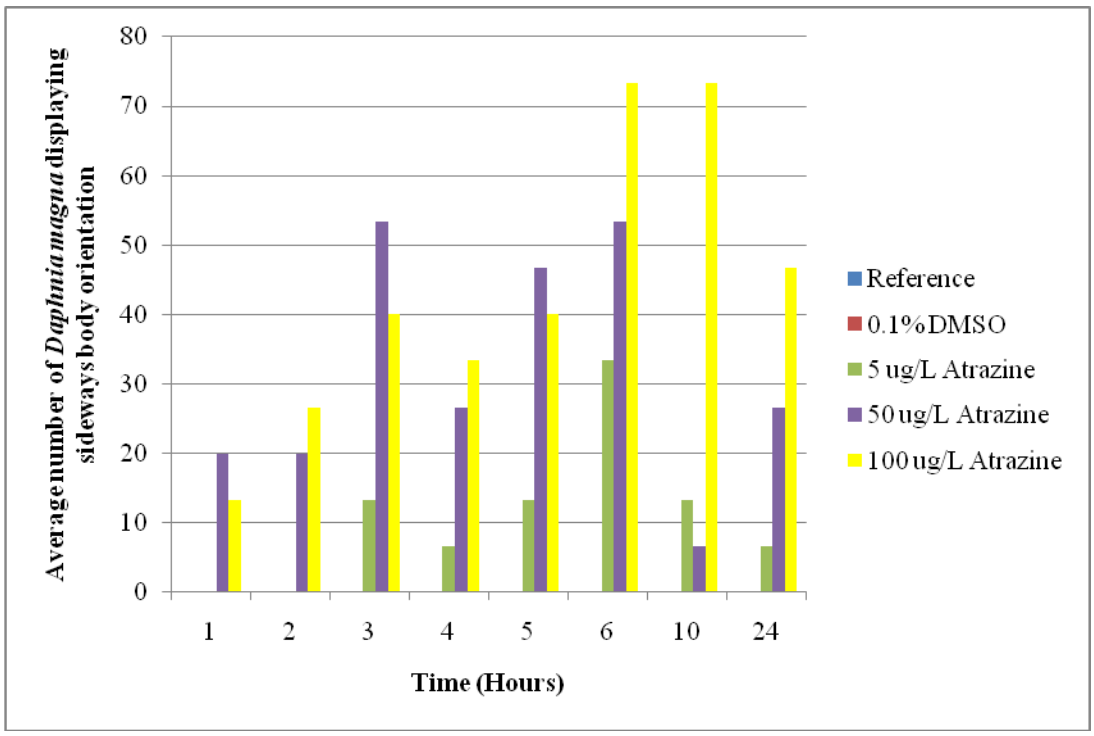

Figure 14: Average percentage of *Daphnia magna* displaying changes in body orientation in response to three concentrations of atrazine over a 24 hour period.

In the atrazine bioassay, there were no significant changes in the body orientation of the organisms when comparing the reference and the 0.1% DMSO treatments (p = 1.000). A significant increase in the number of organisms resting on their sides as opposed to swimming upright was seen in the 5 µg/L atrazine treatments (p = 0.002), the 50 µg/L atrazine treatments (p = 0.000), and the 100 µg/L atrazine treatments (p = 0.000) when compared to the reference treatments over the 24 hour experimental period.

A thorough literature review has revealed no past research which has been conducted to examine changes in body orientation of daphnids in response to chemical stress. Body orientation is related to other swimming parameters, as organisms must be upright in order to effectively propel themselves vertically through the water column. In both the TBT and atrazine bioassays, no changes in body orientation were seen in the reference and 0.1% DMSO treatments. Changes in body orientation were noted in all three concentrations of TBT and atrazine, but no pattern of time or concentration response was evident in response to either contaminant (Figures 13 and 14). Organisms were able to recover their body orientation and did not remain on their sides indefinitely, as seen by the fluctuation in numbers on their sides over time in all three concentrations of TBT and atrazine. Although a significant difference was seen when comparing all TBT and atrazine treatments to the reference, the lack of a consistent pattern of response suggests that this parameter may not be useful for modelling and incorporating into a multi-species, early-warning biomonitoring technology due to difficulties discriminating between concentrations.

iv) Immobilization

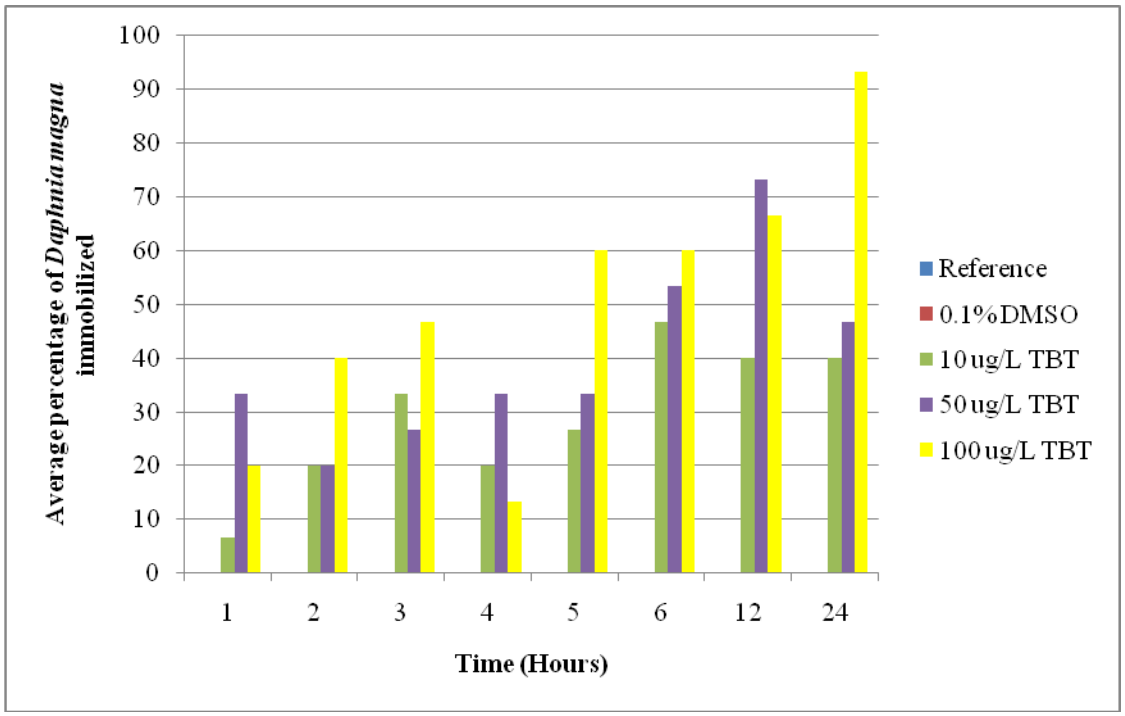

Figure 15: Average percentage of immobilized *Daphnia magna* in three treatments of TBT over a 24 hour period.

In the TBT bioassay, no significant difference was seen when comparing the number of organisms immobilized in the reference and 0.1% DMSO treatments (p = 1.000). A significant increase in the number of organisms immobilized was seen when comparing the reference and the 10 µg/L treatments (p = 0.000), the 50 µg/L treatments (p = 0.000), and the 100 µg/L treatments (p = 0.000). No changes in activity level were noted in the reference or 0.1% DMSO control organisms in the TBT bioassay (Figure 15). The organisms maintained a high activity level and no immobilizations were noted. Changes in activity level and organism immobilization were observed in response to exposure to all concentrations of TBT. Responses were concentration and time dependent. Higher concentrations of TBT immobilized a greater average number of organisms with shorter periods of exposure than lower concentrations.

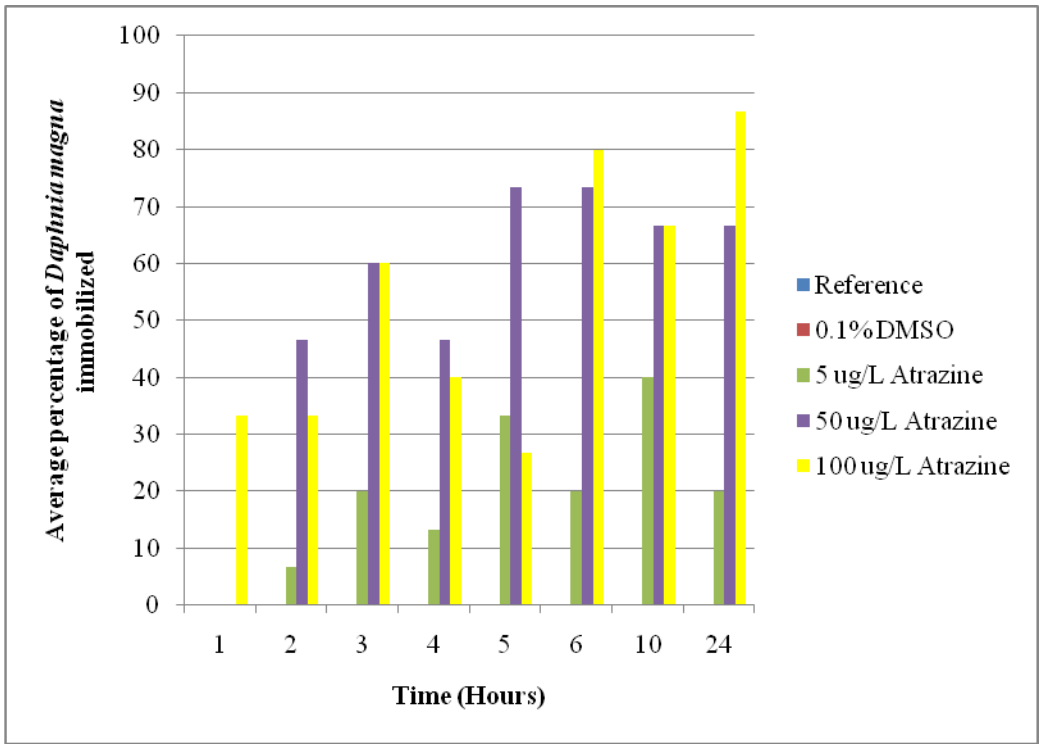

Figure 16: Average percentage of immobilized *Daphnia magna* in three treatments of atrazine over a 24 hour period.

In the atrazine bioassay, no significant difference in the number of organisms immobilized was seen when comparing the reference and the 0.1% DMSO treatments over the 24 hour experimental period (p = 1.000). A significant difference in the number of organisms immobilized was seen in the 5 µg/L atrazine treatment (p = 0.000), the 50 µg/L atrazine treatments, and the 100 µg/L atrazine (p = 0.000) treatments when compared to the reference

treatments. No changes in activity level were noted in the reference or 0.1% DMSO control organisms in the atrazine bioassay. Organisms maintained a high activity level and no immobilizations were noted (Figure 16). Changes in activity level and organism immobilization were observed in response to exposure to all concentrations of atrazine. Responses were generally concentration and time dependent, with a greater number of organisms displaying altered behaviour in higher concentrations and with shorter times to response.

Immobilization was seen in response to all three concentrations of TBT and atrazine. Both contaminants affected the activity level of the daphnids in a time and concentration dependent manner, indicating that this parameter may be highly appropriate for future modelling designed to discriminate between different concentrations of the TBT and atrazine based on behavioural responses of the daphnids. The average percentage of organisms immobilized at each time point varied between the two chemicals (Figures 15 and 16) suggesting that this parameter may be useful for distinguishing between the two contaminants, as well as determining contamination levels. Immobilization will generally lead to death and subsequent population level changes, as organisms are unable to move to find new sources of energy, to avoid predators or to perform any other life functions. Because of the potential impacts of this change in behaviour and the rapid and consistent onset in both TBT and atrazine, immobilization should be evaluated in a multi-organism, early warning biomonitoring technology.

There are several potential causes of organism immobilization. Swimming is a highly energetically demanding behaviour. Changes in the external environment of daphnids may induce a stress situation which impacts the metabolism and other internal functions of the organisms (Untersteiner *et al.,* 2003). A finite amount of energy is available to organisms and increasing energy use for one function may decrease the energy available for activities not directly necessary for survival, such as swimming (Untersteiner *et al.,* 2003; Schmidt *et al.,* 2005). Energy could also be rapidly depleted during escape attempts from an area of contamination (Dodson *et al.,* 1995). Organisms may thus try to conserve energy by falling to the bottom of the bioassay vessel and resting there in response to contamination. A second reason organisms may become immobilized is that the contaminant directly affects coordination, spontaneous muscle activity and other necessary internal processes required for movement (Untersteiner *et al.,* 2003; Schmidt *et al.,* 2005). Immobilization is often associated with

organism exhaustion and represents a point when organisms are unable to adapt their behaviour or internal physiology to withstand the impacts of a chemical (Ren *et al.*, 2008).

v) Secondary Antennae Use

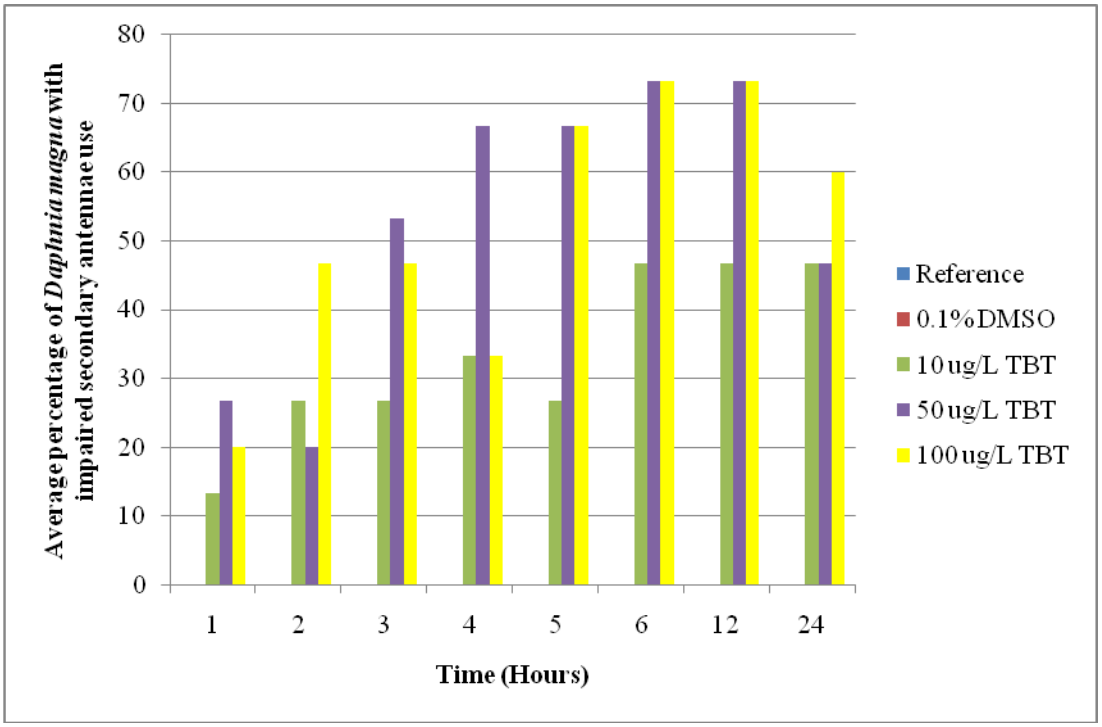

Figure 17: Average percentage of *Daphnia magna* displaying impaired secondary antennae use in three concentrations of TBT over a 24 hour period.

No significant difference in secondary antennae use was noted when comparing the reference and 0.1% DMSO treatments ($p = 1.000$). A significant increase in the impairment of secondary antennae was seen in the 10 µg/L TBT treatments ($p = 0.000$), the 50 µg/L treatments ($p = 0.000$), and the 100 µg/L treatments ($p = 0.000$) when compared to the reference treatments over the 24 hour exposure period. No changes in secondary antennae use was observed in organisms exposed to the reference and 0.1% DMSO treatments over 24 hours. Antennae use became impaired in organisms when exposed to all concentrations of TBT. The response was concentration and time dependent, with lower concentrations affecting a smaller proportion of the *Daphnia* population and taking a greater amount of time to affect the antennae use of the organisms (Figure 17).

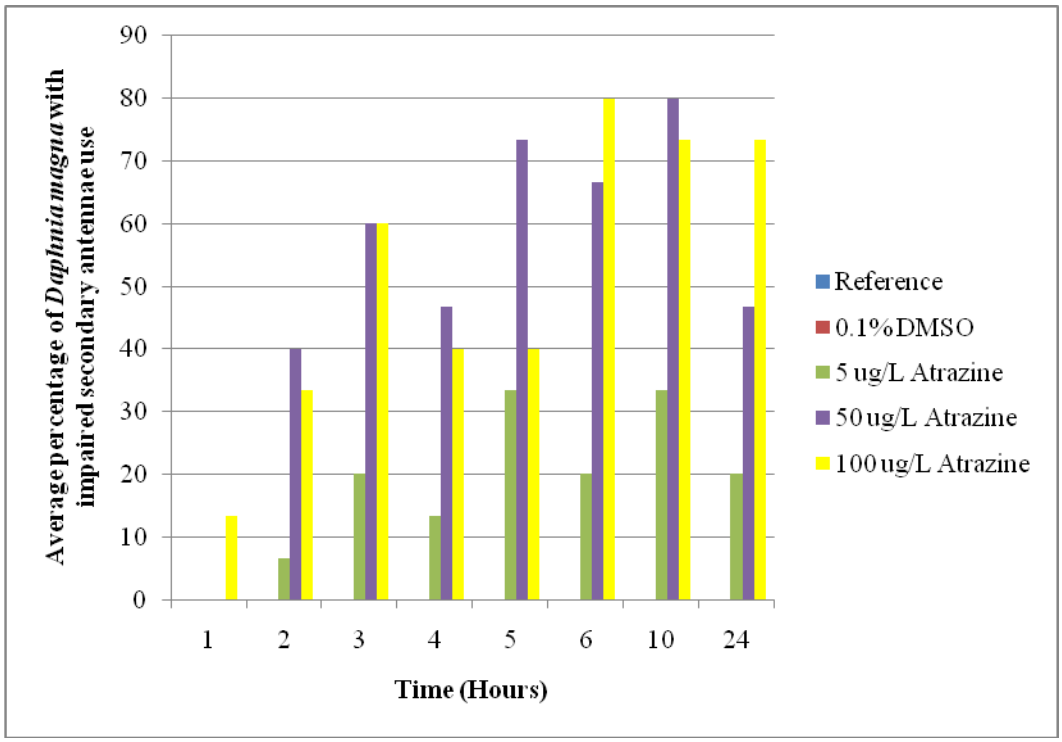

Figure 18: Average percentage of *Daphnia magna* displaying impaired secondary antennae use in three concentrations of atrazine over a 24 hour period.

No significant difference in secondary antennae use was noted when comparing the reference and 0.1% DMSO treatments ($p = 1.000$). A significant increase in the impairment of secondary antennae was seen in the 5 µg/L atrazine treatments ($p = 0.000$), the 50 µg/L atrazine treatments ($p = 0.000$), and the 100 µg/L atrazine treatments ($p = 0.000$) when compared to the reference treatments. No changes in secondary antennae use was observed in organisms exposed to the reference and 0.1% DMSO treatments over 24 hours. Antennae use became impaired in organisms in all atrazine concentrations. However, no consistent pattern of time or concentration dependence was seen (Figure 18), as the number of organisms displaying abnormal antennae use varied at each time point.

Impairment of secondary antennae was seen in response to both TBT and atrazine. A more consistent response with concentration and time dependency was seen when the organisms were exposed to all three concentrations of TBT. This suggests that changes in secondary antennae use may be a more sensitive parameter for detecting TBT and related organometallic compounds than for detecting herbicides such as atrazine. Changes in secondary antennae likely will be appropriate for developing a model to predict the exact concentration of TBT present in a

given water sample, due to the consistent response seen in the test daphnids. Changes in secondary antennae usage may be appropriate for detecting the overall presence of atrazine, but may be less useful for modelling purposes to determine specific concentrations, due to inconsistent responses. Other changes in swimming behaviour, such as swimming style and immobilization, are closely related to changes in secondary antennae use. Because of this and because of the rapid decrease of use in response to TBT and atrazine, evaluation of the frequency of use of secondary antennae should be incorporated into a multi-organism, early-warning biomonitoring technology.

Similar results have been observed in past bioassays. Bailleul and Blust (1999) found that secondary antennae beat frequency decreased significantly with increasing concentrations of copper due to a suspected neurological failure brought on by the contaminant. Impaired secondary antennae use was also observed with long-term exposure to both TBT and PCB, causing organisms to have a decreased swimming velocity and spend more time at the bottom of aquaria (Schmidt *et al.,* 2003). Secondary antennae are moved by muscles which may be negatively affected by a decrease in available energy reserves or which may be directly impacted by the contaminant acting upon them (Schmidt *et al.,* 2003; Untersteiner *et al.,* 2003).

vi) Swimming Style

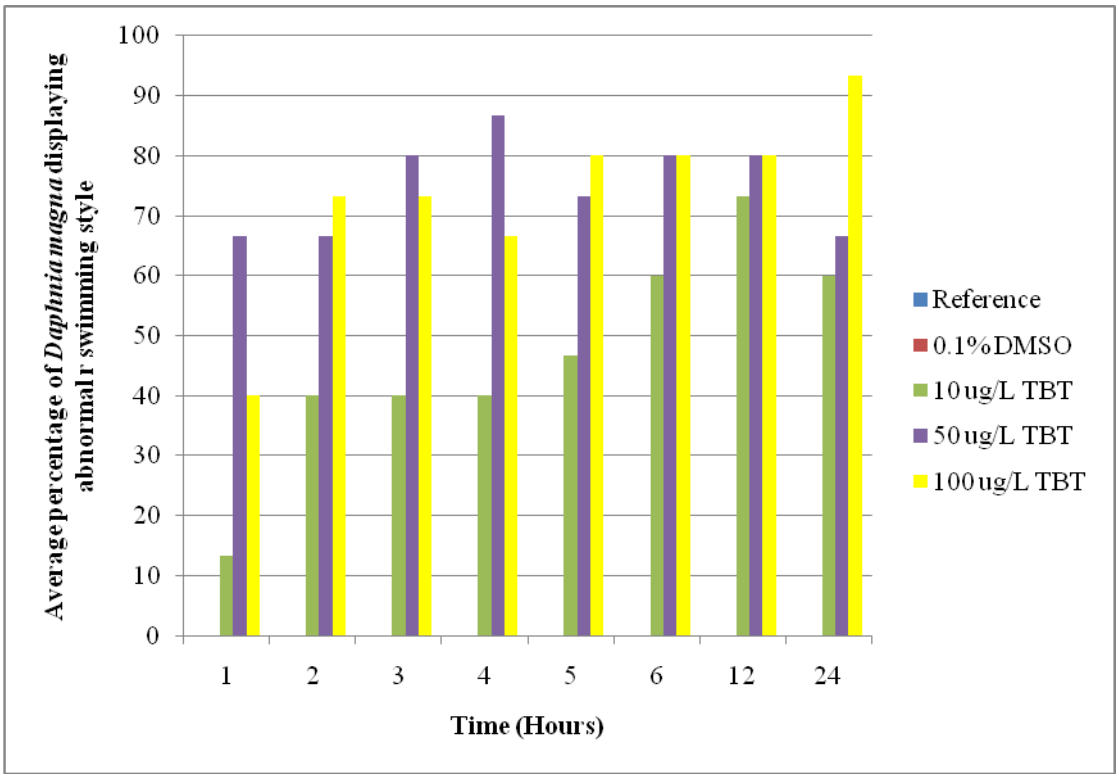

Figure 19: Average percentage of *Daphnia magna* displaying abnormal swimming style when exposed to 3 concentrations of TBT over a 24 hour period.

No significant changes in swimming style were seen when comparing the reference and 0.1% DMSO treatments (p = 1.000) over the 24 hour bioassay. A significant increase in the percentage of organisms displaying impaired swimming behaviour was seen in the 10 µg/L TBT treatments (p = 0.000), the 50 µg/L TBT treatments (p = 0.000), and the 100 µg/L TBT treatments (p = 0.000) when compared to the reference treatments. No changes in swimming style were observed in the organisms in the reference or 0.1% DMSO control samples in the TBT bioassay. All three concentrations of TBT affected the swimming style of *Daphnia* in a time and concentration dependent manner (Figure 19). The two highest concentrations of TBT affected the swimming style of organisms more rapidly and impacted a greater proportion of the population than the 5 µg/L treatments.

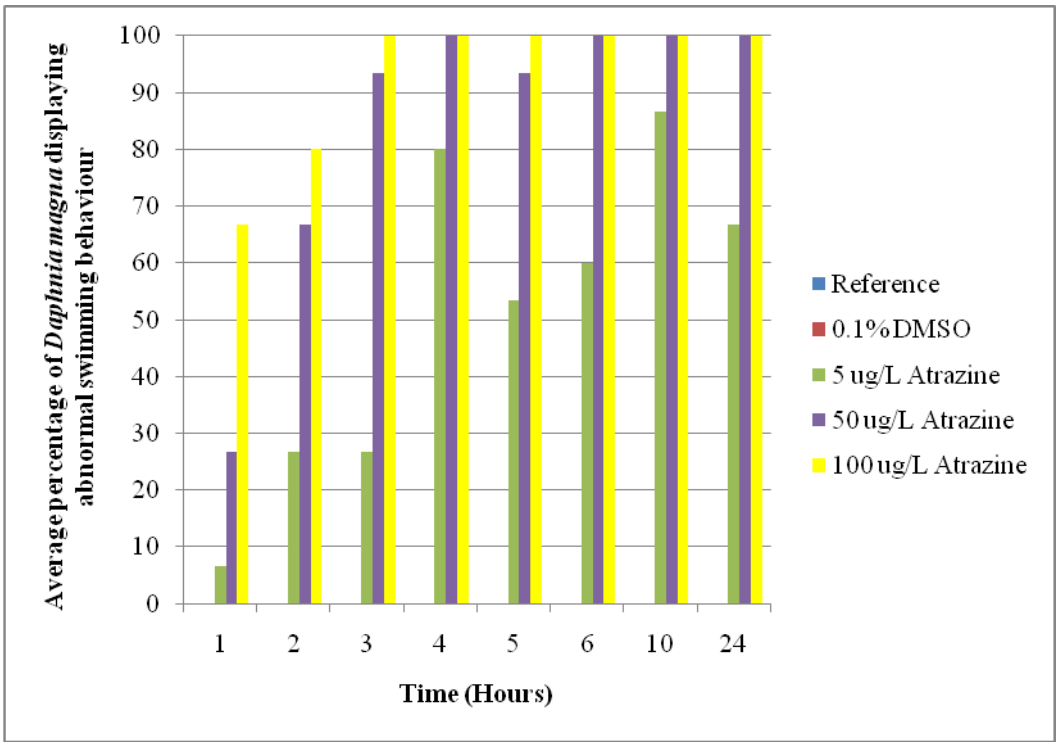

Figure 20: Average percentage of *Daphnia magna* displaying abnormal swimming style when exposed to 3 concentrations of atrazine over a 24 hour period.

No significant changes in movement style were seen when comparing the reference and 0.1% DMSO treatments ($p = 1.000$). Significant changes in movement style were seen in the 5 µg/L atrazine treatments ($p = 0.000$), the 50 µg/L atrazine treatments ($p = 0.000$), and the 100 µg/L atrazine treatments ($p = 0.000$) when compared to the reference over the 24 hour exposure period. No changes in swimming style were observed in the organisms in the reference or 0.1% DMSO control samples in the atrazine bioassay. All three concentrations of atrazine affected the swimming style of *Daphnia* in a both time and concentration dependent manner, with nearly all daphnids in the two highest concentrations displaying altered swimming behaviour after 3 hours of exposure (Figure 20). Changes in behaviour in the 5 µg/L treatments were less consistent, with fluctuation in the number of organisms displaying altered behaviour.

Similar movement abnormalities were seen in response to both TBT and atrazine. These included swimming using jerky, short strokes, using the bottom of the vessel for propulsion and immobilization of organisms. Responses to both contaminants were both concentration and time dependent, suggesting a high level of suitability for future modelling. This suggests that it may be possible discriminate between different concentrations of the TBT and atrazine based on the

overall proportion of daphnids displaying altered swimming behaviour. The average percentage of organisms immobilized at each time point varied between the two chemicals (Figures 19 and 20), suggesting that this parameter may be useful for distinguishing between the two contaminants, as well as determining contamination levels. Because of the rapid and consistent onset of changes in swimming style in *Daphnia magna* in response to TBT and atrazine, this parameter should be evaluated in a multi-organism early-warning biomonitoring technology.

Swimming style is very important to daphnids, as they are constantly swimming and changes in this behaviour can affect prey susceptibility, ability to find food and to form groups with other daphnids. By swimming normally in straight lines, organisms are able to travel to food patches more quickly and move rapidly away from predators (Ryan and Dodson, 1998). *Daphnia* with erratic swimming behaviour are more likely to attract the attention of visual predators and predators which use mechanoreception to hunt (Dodson *et al.,* 1995; Szulkin *et al.,* 2006). It has been shown that *Daphnia* with greater hop frequency and more erratic swimming behaviour are more likely to be eaten by bluegill sunfish (Ryan and Dodson, 1998). Irregularities in swimming behaviour are likely avoidance responses by the organisms in an attempt to move to a less contaminated area (Ren *et al.,* 2007). Normal escape responses to predators include a short burst of swimming with multiple direction changes and several large hops or spins (Dodson *et al.,* 1995). This could potentially explain the increase in jerky swimming behaviour in response to the two contaminants. Other studies have shown an increase in irregular swimming prior a decline in activity in *Daphnia* exposed to a variety of organophosphorous pesticides (Ren *et al.,* 2008).

Summary of Daphnia magna *Motility Bioassay*

The results of the *Daphnia magna* motility bioassay revealed that some movement behaviour parameters are more sensitive to chemical contaminants, specifically TBT and atrazine, than others and should therefore be evaluated in a multi-organism, early-warning biomonitoring technology. Since some parameters are more sensitive than others to the given contaminants, changes in these parameters should be considered more important stress indicators than other changes in behaviour and should be given higher priority when creating a model to detect types and concentrations of contaminants.

The response which should be given highest consideration is changes in vertical movement through the water column. Changes in this parameter were time and concentration dependent in all of the TBT and atrazine treatments, affected a high number of the organisms, and had a rapid response time for all treatments. The next most important response appeared to be changes in swimming style. This parameter also displayed affected a larger proportion of organisms, and was time and concentration dependent in both contaminants, but the onset of changes seemed to be generally slower than changes in vertical movement. The third most important parameter to be considered was immobilization, which also had a time and concentration dependent change in response to TBT and atrazine. The parameter seems less important than changes in vertical movement and swimming style due to a slower general onset and lower proportion of organisms affected. The reaction is also the most severe, and it is important to detect high levels of contaminant before such a reaction can occur. The parameter which should be given priority next is changes in secondary antennae use. Changes in this parameter occurred in all treatments of TBT and atrazine, but a time and concentration dependence was seen in only the TBT, indicating that the parameter may not be useful for atrazine detection. The changes in secondary antennae use also appeared to be less rapidly induced and consistent than some of the other parameters discussed above. The next most important parameter to be considered should be spinning behaviour. Spinning should be considered less important than the above parameters due to a lack of time and concentration dependence in both contaminants, an overall fluctuation in the number of organisms performing the behaviour and because it is not independent of immobility. Finally, the least important parameter, which should not be used in a multi-species, early-warning biomonitoring technology, is body orientation. Due to a lack of consistent time and concentration dependent response, a low percentage of organisms affected, the ability of organisms to recover their body positions, and a lack of ecological relevance, this parameter should not be evaluated.

Overall, the ranking of importance of the six parameters is: swimming height > swimming style > immobilization > secondary antennae use > spinning > body orientation. The first 5 parameters should be included for evaluation in a multi-species, early-warning biomonitoring technology, whereas body orientation should not.

3.1.2 *Daphnia magna* 72 Hour Mortality TBT

A 72 hour analysis of the average percentage of organism deaths which occurred with exposure to the five treatments was performed at the same time as the *Daphnia* swimming bioassay. The addition of the DMSO carrier had no effect on the mortality of *Daphnia magna* after 72 hours of exposure ($p = 0.317$) (Figure 21). All three concentrations of TBT resulted in 100% mortality for the exposed *Daphnia* after 72 hours and therefore the contaminant had a significant effect on the survival of *Daphnia* ($p = 0.025$).

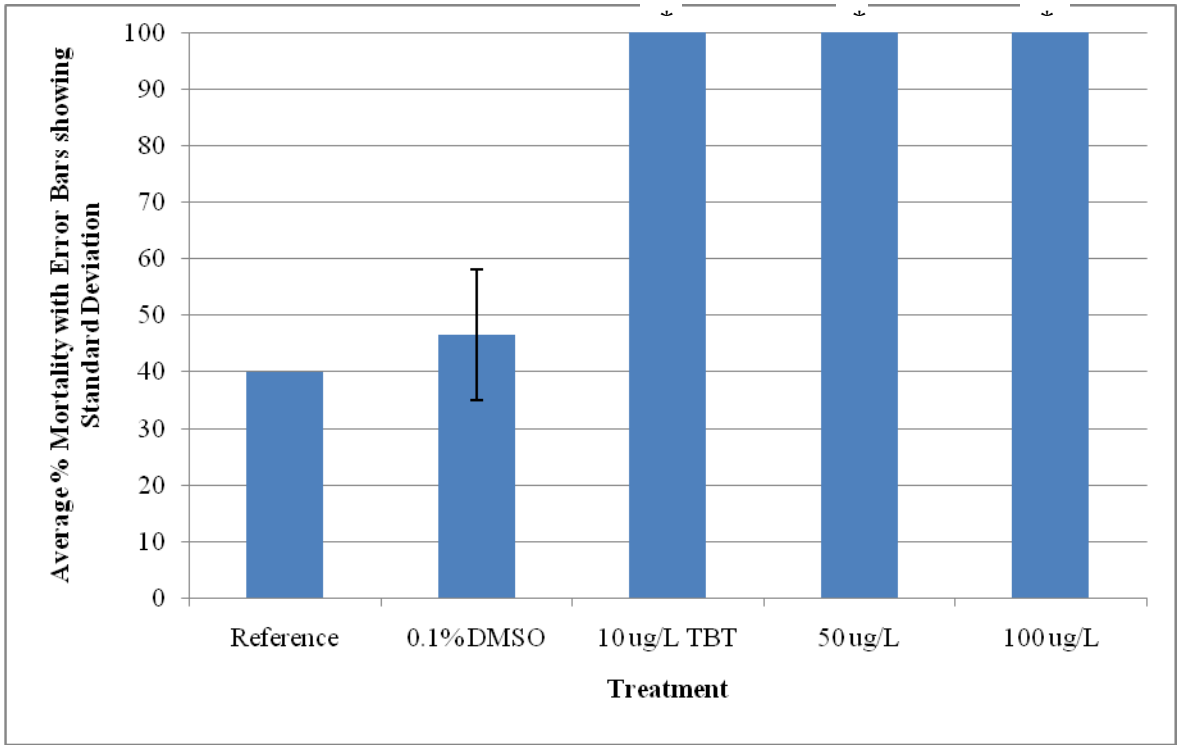

Figure 21: Average percent of *Daphnia magna* which died after 72 hours of exposure to varying concentrations of TBT. Treatments which have a significantly different ($p \leq 0.05$) average mortality than the reference are indicated with a *.

After 72 hours of exposure to all three concentrations of TBT, all organisms had died. There was a significant difference between the number of deaths which occurred between the reference and all three TBT treatments. This indicates that the concentrations which had sub-lethal effects on behaviour after one day of exposure were lethal to daphnids after 3 days of exposure.

3.2 *Hyalella azteca* Behaviour

3.2.1 *Hyalella azteca* Motility

Several aspects of *Hyalella azteca* motility behaviour were evaluated during these bioassays and were found to change in the presence of varying concentrations of the contaminants TBT and atrazine. Changes in response to the contaminants are summarized below with a discussion of the ecological impacts of the change. Qualitative observations of general trends of behavioural changes were made to aid in the ranking of the importance of the various parameters. A discussion of the utility of the various parameters for evaluation in a multi-organism early-warning biomonitoring system is also included. The movement characteristics evaluated included: i) swimming events, ii) substrate crawling, iii) immobilization, iv) burrowing, v) grouping, vi) body length, and vii) body orientation.

i) Swimming Events

In this bioassay, swimming events were monitored by observing whether or not individual organisms performed swimming events at the given time periods. No evaluation of the number of swimming events performed was made, only the occurrence of swimming events was evaluated.

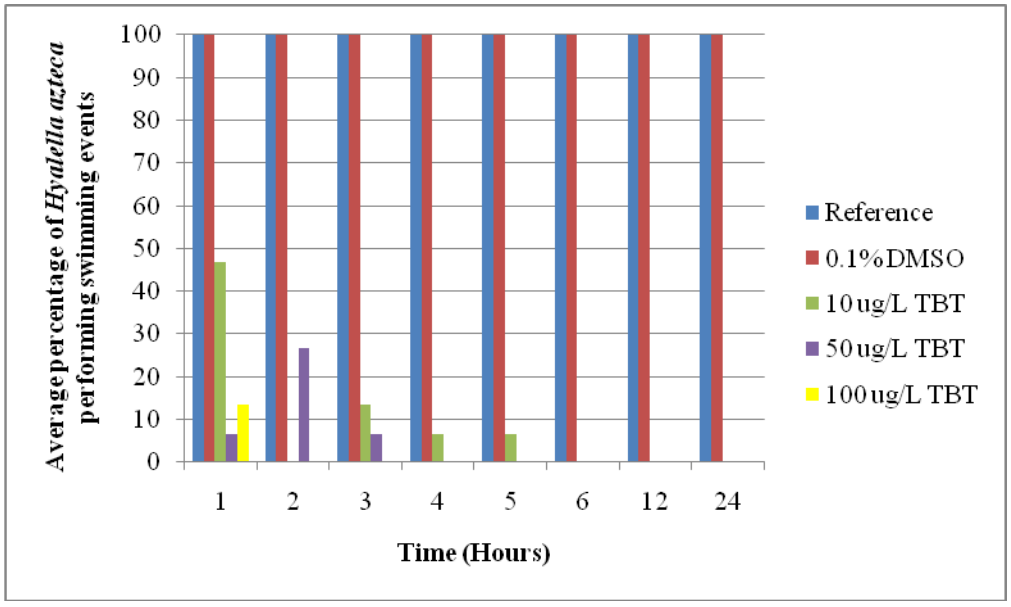

Figure 22: Average percentage of *Hyalella azteca* performing swimming events in three concentrations of TBT over a 24 hour period.

When comparing the reference and 0.1% DMSO treatments, no significant change in the number of the organisms performing swimming events is seen (p = 1.000) over the 24 hour experimental period. A significant decrease in the number of organisms performing swimming events is seen in the 10 µg/L TBT treatment (p = 0.000), the 50 µg/L TBT treatment (p = 0.000), and the 100 µg/L treatment (p = 0.000) when compared to the reference. No changes in the performance of swimming events were noted in the organisms in reference and 0.1% DMSO treatments over the 24 hour experimental period in the TBT bioassay. All organisms performed swimming events; however, the number of events seemed to decrease as the experiment went on. Changes in the performance of swimming events were seen in all 3 concentrations of TBT. No organisms in the two highest TBT concentrations performed swimming events after 4 hours of exposure and swimming events ceased in the 10 µg/L treatments after 6 hours of exposure (Figure 22). This indicates that the impacts of TBT on *Hyalella* swimming is concentration and time dependent and that higher concentrations exert impacts more quickly and have a greater overall impact.

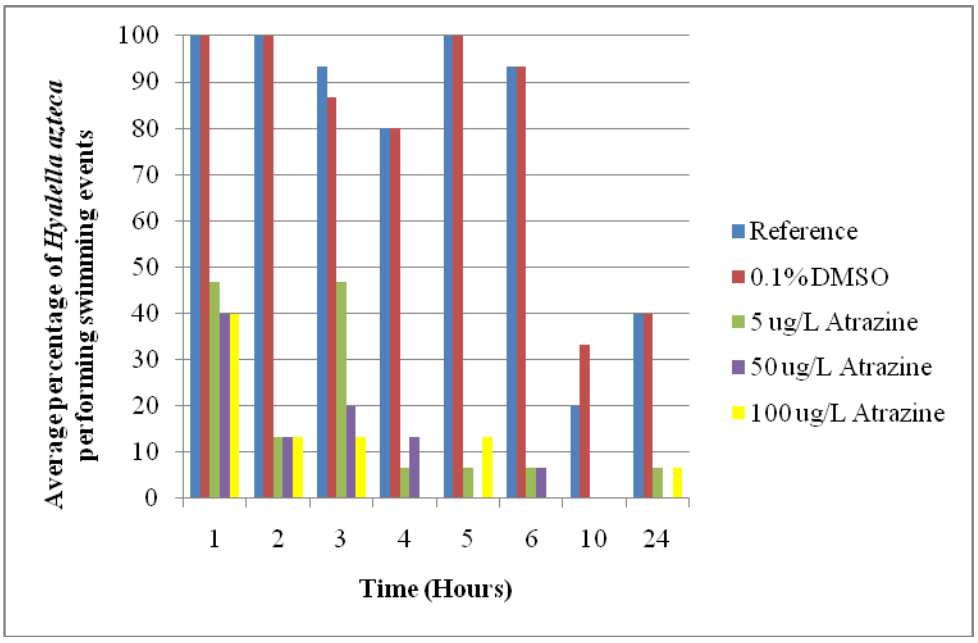

Figure 23: Average percentage of *Hyalella azteca* performing swimming events in three concentrations of atrazine over a 24 hour period.

When comparing the reference and 0.1% DMSO treatments, no significant change in the number of organisms performing swimming events is seen ($p = 0.982$) over the 24 hour experimental period. A significant decrease in the number of organisms performing swimming events is seen in the 5 µg/L atrazine treatments ($p = 0.000$), the 50 µg/L atrazine treatments ($p = 0.000$), and the 100 µg/L atrazine treatment ($p = 0.000$) when compared to the reference. Changes in the performance of swimming events also occurred in all three atrazine treatments, with the response being time and concentration dependent (Figure 23). A decrease in swimming events was seen in all three concentrations of atrazine after 1 hour of exposure.

Although a significant change in the performance of swimming events was observed in all concentrations of TBT and atrazine, the monitoring of the occurrence of swimming events may not be a useful parameter to evaluate using a multi-organism, early-warning biomonitoring technology. This is because a decrease in the average number of organisms performing swimming events was also seen in both the reference and DMSO control in the atrazine bioassay after 10 and 24 hours. A decrease in overall swimming performance in the reference and DMSO control were also seen in the TBT bioassay over time. This indicates that organisms may display altered swimming patterns in a multi-organism, early-warning biomonitoring technology after they are allowed to acclimate to the test chamber and could not be used reliably for an extended period of time. The complete lack of swimming events in all three concentrations of TBT after 6 hours of exposure may also make it difficult to discriminate between the different concentrations using a modelling approach.

Swimming behaviour allows for organisms to move more rapidly and over a greater distance than typical crawling on the sediment surface. Inhibition of swimming events may make *Hyalella* more vulnerable to predators as they cannot rapidly escape when threatened. *Hyalella* feeding activity may also be impacted, as organisms cannot reach algae blooms, large macrophytes, or other food sources which may be distributed above the sediment surface (Wang *et al.,* 2004). Organisms may also purposefully decrease their performance of swimming events. As *Hyalella* are exposed to contaminants from the water column (Wang *et al.,* 2004) remaining close to the sediment surface may be a response to detected contaminants and an attempt to reduce exposure to them.

ii) Substrate Crawling

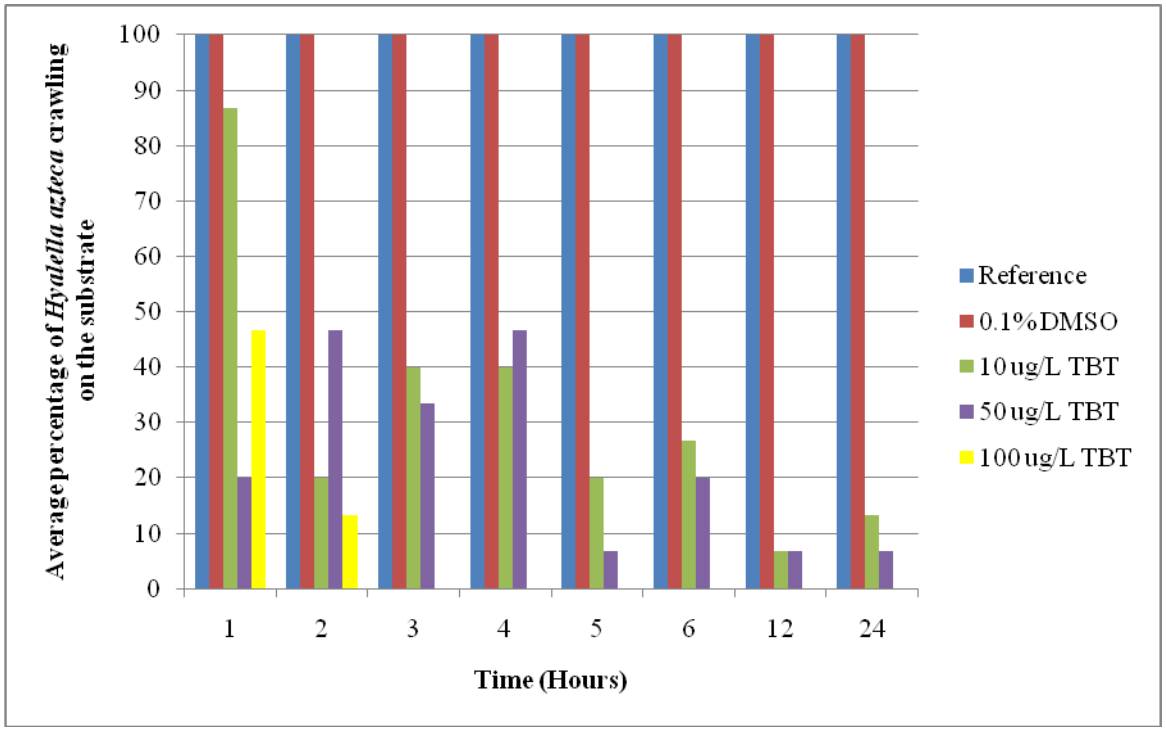

Figure 24: Average percentage of *Hyalella azteca* crawling on the substrate in three concentrations of TBT over a 24 hour period.

There was no significant difference in the number of organisms crawling on the substrate in the reference and 0.1% DMSO treatments over the 24 hour test period ($p = 1.000$). A significant decrease in the number of organisms crawling on the substrate was seen in the 10 µg/L TBT treatments ($p = 0.000$), the 50 µg/L treatments ($p = 0.000$), and the 100 µg/L TBT treatments ($p = 0.000$) when compared to the reference treatments. No changes in substrate crawling were observed in organisms exposed to reference and 0.1% DMSO treatments over the 24 hour experimental period. Significant decreases in substrate crawling were seen in all three concentrations of TBT (Figure 24). Changes in substrate crawling were concentration and time dependent, with higher concentrations exerting impacts more quickly and having a greater overall impact. After 3 hours of exposure to the 100 µg/L treatment, no organisms were observed crawling on the silica sand substrate, whereas there was still some movement on the substrate in the two lower concentrations.

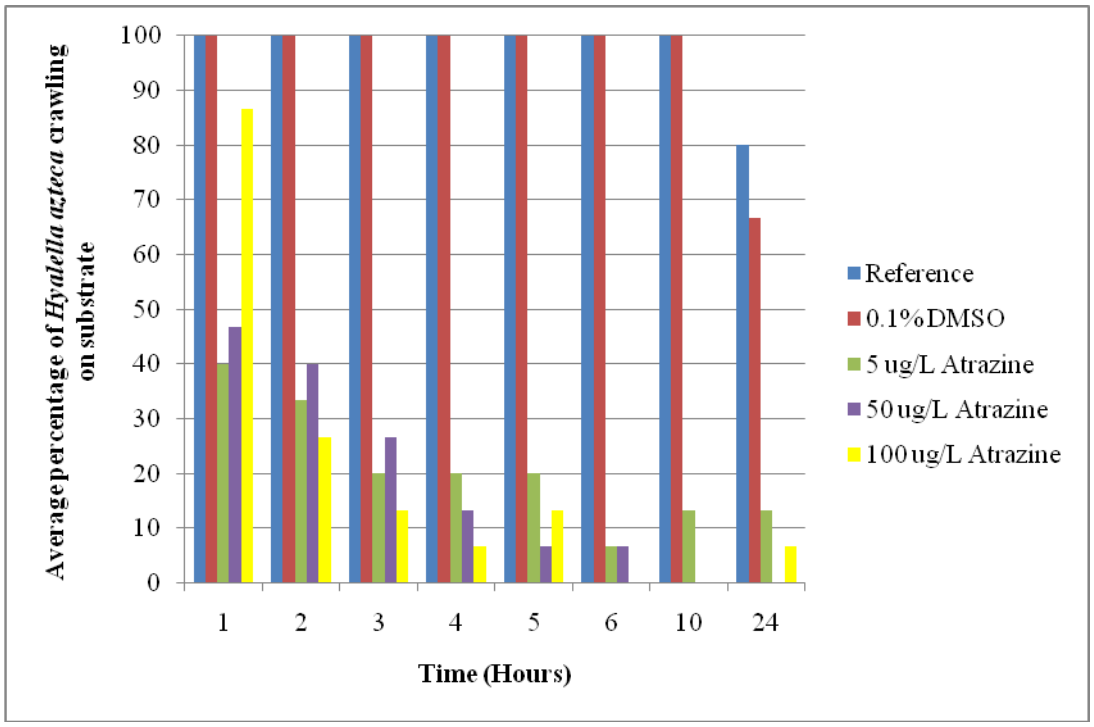

Figure 25: Average percentage of *Hyalella azteca* crawling on substrate in three concentrations of atrazine over a 24 hour period.

There was no significant difference in the number of organisms crawling on the substrate in the reference and 0.1% DMSO treatments over the 24 hour test period ($p = 0.976$). A significant decrease in the number of organisms crawling on the substrate was seen in the 5 µg/L atrazine treatments ($p = 0.000$), the 50 µg/L atrazine treatments ($p = 0.000$), and the 100 µg/L atrazine treatments ($p = 0.000$) when compared to the reference treatments. Decreases in crawling along the silica sand substrate were seen in all three concentrations of atrazine, but not in the reference or 0.1% DMSO treatments (Figure 25). Decreases in the performance of crawling by *Hyalella* were dependent on time but not concentration, as at several timepoints lower concentrations of the contaminant impacted a greater percentage of the organisms.

Decreases in crawling movements were noted in all three concentrations of both TBT and atrazine. Changes in crawling behaviour may be a more sensitive measure for the detection of sub-lethal concentrations of TBT, as a time and concentration dependent decrease in crawling movement was seen in all TBT treatments. This type of response can be easily incorporated into a model to help determine the exact type and concentration of a contaminant. On the other hand, changes in crawling behaviour may be a less useful parameter for detecting atrazine

contamination. Due to the variability between treatments and the lack of a concentration dependent response, it may be difficult to use changes in this parameter in a model to predict the precise concentration of atrazine in an incoming water sample. Crawling behaviour changes could be used to detect the presence of atrazine but not a specific concentration of the substance. Due to rapid changes in crawling behaviour on the sediment surface in response to two different classes of contaminants, this parameter should be incorporated into a multi-species, early-warning biomonitoring technology examining *Hyalella* movement, as crawling behaviour appears to be very sensitive to changes in water quality.

Crawling on the sediment is important for *Hyalella azteca* while performing foraging. *Hyalella* derive a great deal of their energy from algae growing at sediment-water interface and from other organic matter that has fallen to this surface (Wang *et al.*, 2004). Oxygen is also more plentiful at this level than within the sediment, so organisms may selectively spend time crawling on the sediment rather than burrowing in the hypoxic sediment (Wang *et al.*, 2004). Changes in crawling behaviour may therefore affect organism survival if maintained for an extended period of time.

iii) Immobilization

Hyalella were considered to be immobilized if no movement of any body parts occurred. This included not only locomotion associated with swimming events and substrate crawling, but also any movement in place on the substrate, movement associated with burrowing, and contractions of the body and movement of legs while the organisms were lying on their sides on the sediment.

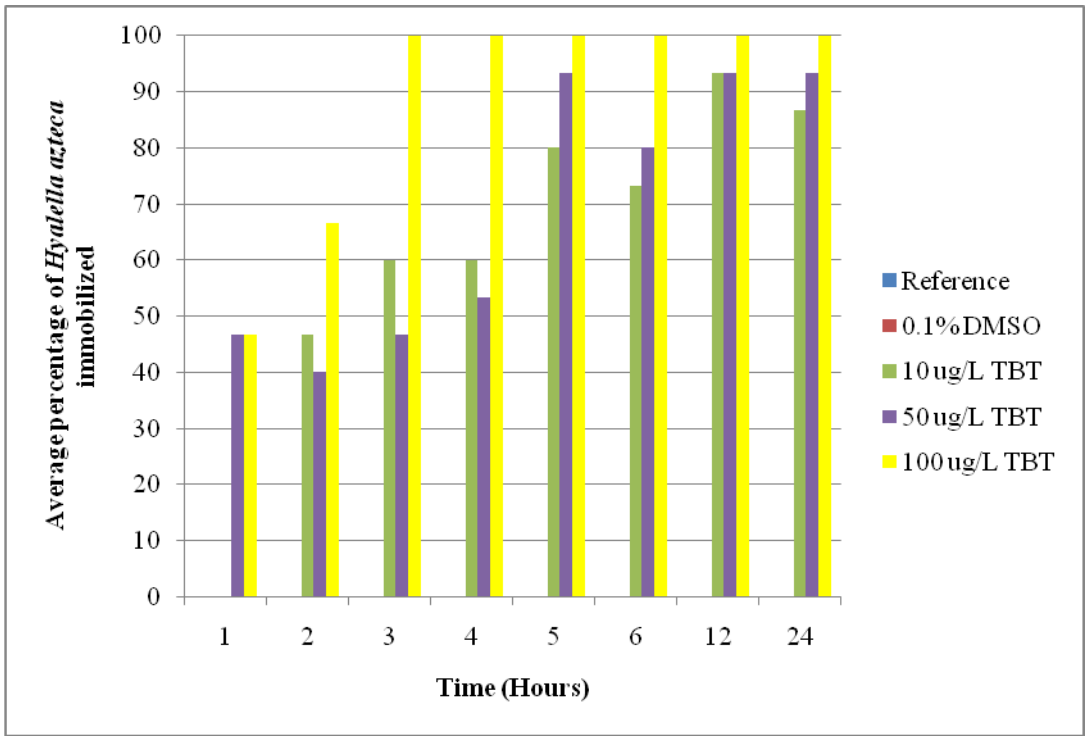

Figure 26: Average percentage of immobilized *Hyalella azteca* in three concentrations of TBT over a 24 hour period.

When comparing the reference and 0.1% DMSO treatments, there was no significant difference in the number of the organisms which were immobilized ($p = 1.000$) over the 24 hour test period. A significant increase in the number of the organisms which were immobilized was seen in the 10 µg/L TBT ($p = 0.000$), the 50 µg/L TBT ($p = 0.000$), and the 100 µg/L TBT ($p = 0.000$) treatments when compared to the reference. No immobilization of the organisms was seen in the reference or 0.1% DMSO treatments in the TBT bioassay. All organisms remained active throughout the bioassay. Increases in immobilization in the three atrazine treatments were seen to be dependent on concentration and time, with responses seen earlier and having more impact at higher concentrations (Figure 26). In the 100 µg/L treatments, no movement by any organisms was observed after 3 hours of exposure to the contaminant. Responses to the contaminant were seen in all three treatments after 2 hours of exposure.

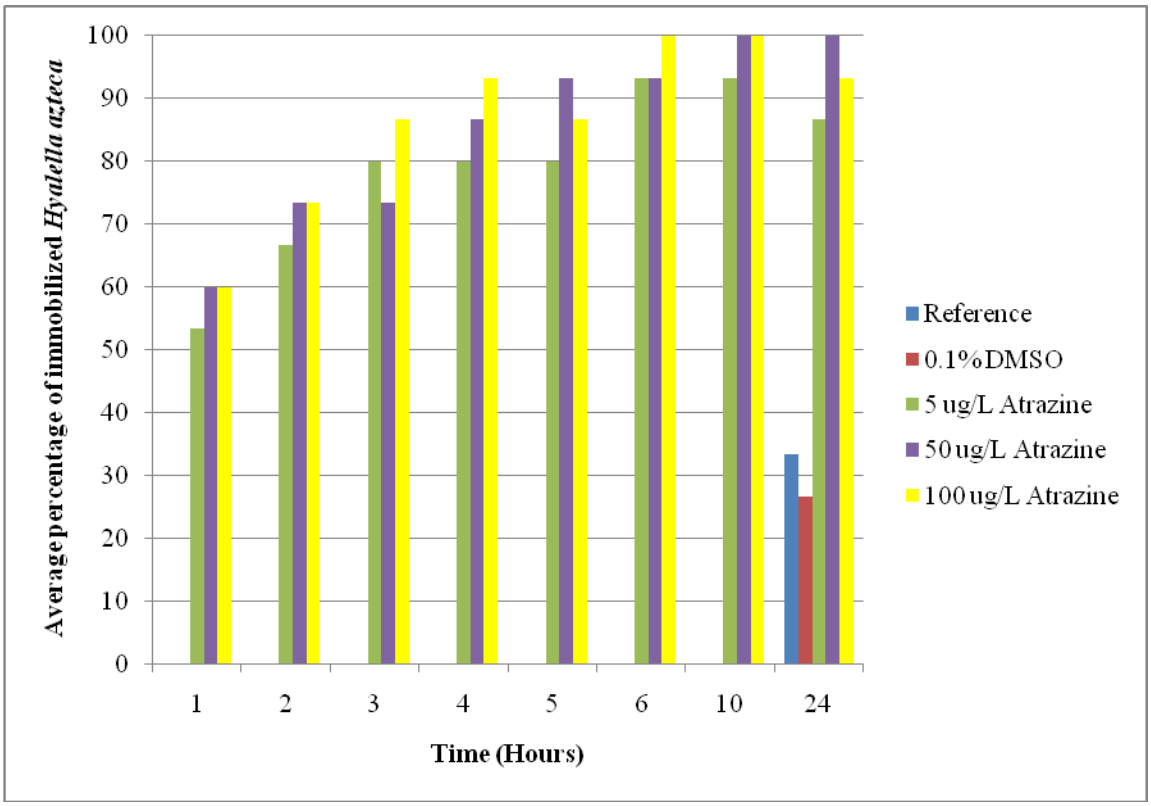

Figure 27: Average percentage of immobilized *Hyalella azteca* in three concentrations of atrazine over a 24 hour period.

When comparing the reference and 0.1% DMSO treatments, there was no significant difference in the number of organisms which were immobilized ($p = 0.976$) over the 24 hour test period. A significant increase in the number of organisms which were immobilized was seen in the 5 µg/L atrazine treatments ($p = 0.000$), the 50 µg/L atrazine treatments ($p = 0.000$), and the 100 µg/L atrazine treatments ($p = 0.000$) when compared to the reference. In general, a time and concentration dependent increase in *Hyalella* immobilization was seen in the three atrazine treatments (Figure 27), with responses seen in all three treatments after 1 hour of exposure to the contaminant.

All concentrations of TBT and atrazine produced a time and concentration dependent increase in *Hyalella* immobilization when compared to reference treatments. Changes in this type of behaviour were rapid in both contaminants, indicating that changes in overall mobility are highly sensitive to the two different classes of contaminant. The time and concentration dependent response patterns to both chemicals indicates that modelling based on changes in this parameter is viable for both contaminants, and it is likely that such a model could be used to

precisely predict individual contaminant concentrations based on changes in organism immobilization. Because of differences in timing of immobilization of organisms it is also likely possible that the model will be able to distinguish between the two classes of contaminants, in addition to providing information to water treatment facility managers about the overall concentration of the contaminant. Severe decreases in mobility are seen in all concentrations of atrazine and TBT with less than 2 hours of exposure, while no changes were seen in the reference and DMSO treatments. This indicates that immobilization is highly sensitive to changes in water quality caused by TBT and atrazine and that it should be included for evaluation in a multi-organism, early-warning biomonitoring technology to detect contaminants in drinking water.

Mobility is highly important for all biological functions in organisms including foraging, predator avoidance, mating and conspecific recognition. Immobilization in *Hyalella* likely occurs for similar reasons as in populations of daphnids discussed above, mainly the organisms suffer from a depletion of energy reserves trying to avoid or adapt to contaminants, or the organisms' internal functioning is directly altered by the contaminant leading to the inability to move.

iv) Burrowing

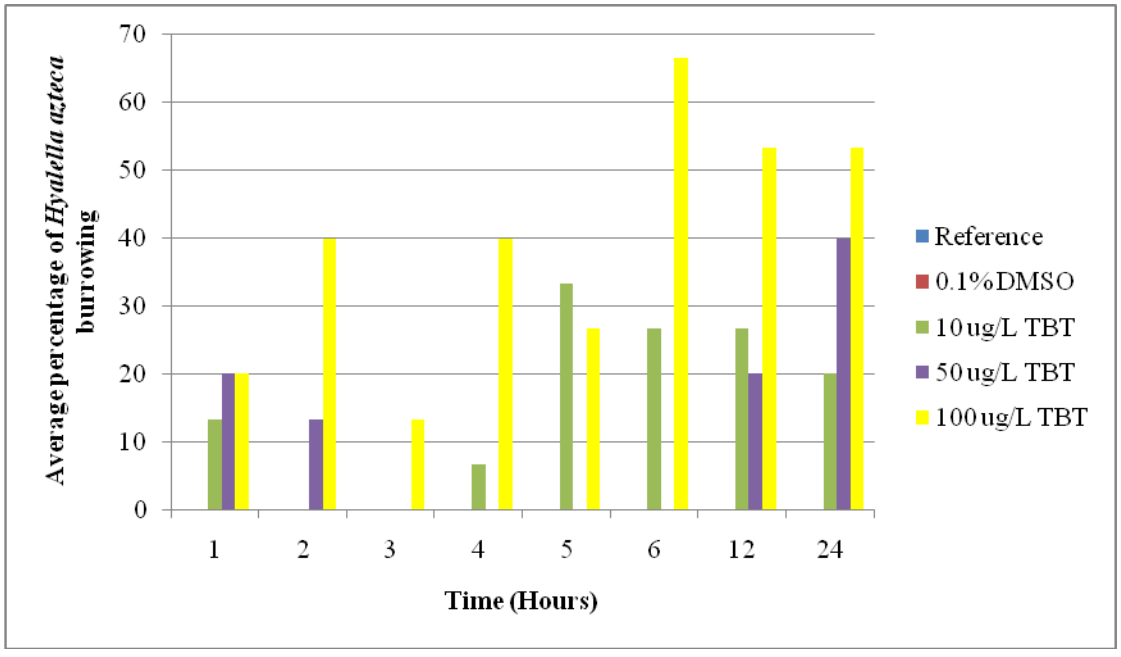

Figure 28: Average percentage of *Hyalella azteca* burrowed in three concentrations of TBT over a 24 hour exposure period.

There was no significant difference when comparing the number of the organisms burrowing in the reference and 0.1% DMSO treatments (p = 1.000). A significant difference was seen when comparing the 10 µg/L TBT treatments (p = 0.000), the 50 µg/L treatments (p = 0.002), and the 100 µg/L treatments (p = 0.000) to the reference treatments over 24 hours. No clear pattern was seen with respect to burrowing and TBT concentration as the organisms moved in and out of burrows throughout the experiment (Figure 28). No time or concentration dependence was seen in the bioassay.

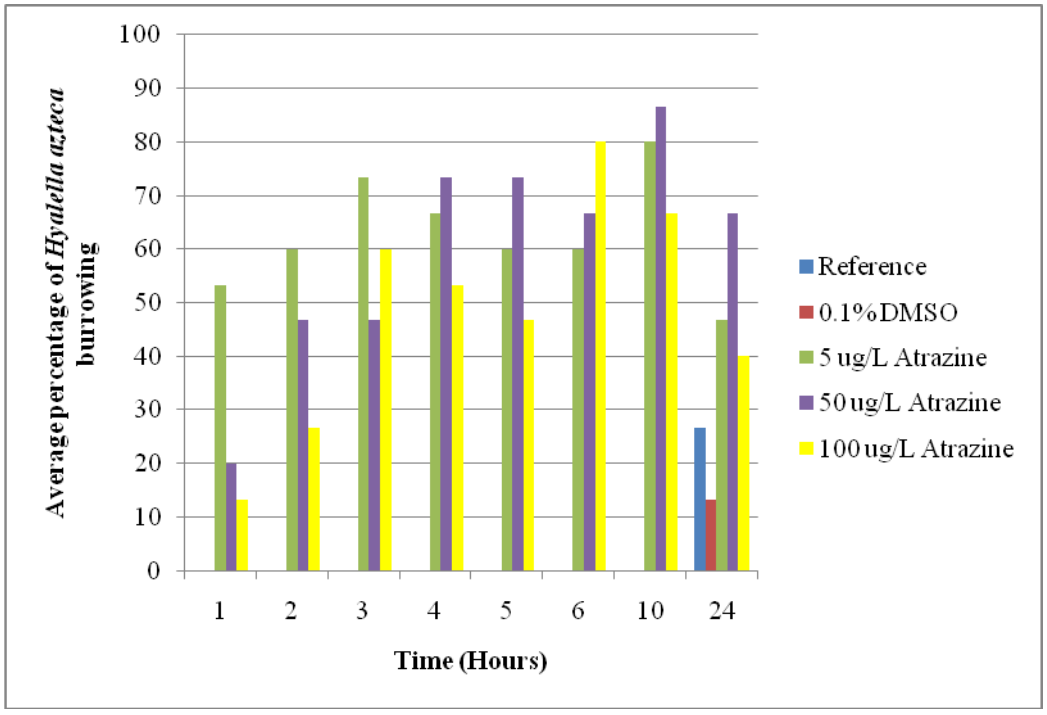

Figure 29: Average percentage of *Hyalella azteca* burrowed in three concentrations of atrazine over a 24 hour exposure period.

There was no significant difference when comparing the number of organisms burrowing in the reference and 0.1% DMSO treatments (p = 0.966). A significant difference was seen when comparing the 5 µg/L atrazine treatments (p = 0.000), the 50 µg/L atrazine treatments (p = 0.000), and the 100 µg/L atrazine treatments (p = 0.000) to the reference over the 24 hour experimental period. Burrowing was seen in all atrazine treatments with no concentration or time dependency (Figure 29). In all atrazine treatments, the number of the organisms burrowing fluctuated between the different observation periods.

Significant increases in organism burrowing were seen in all treatments of TBT and atrazine when compared to the reference treatments. Little or no burrowing was seen in the reference and DMSO treatments. Burrowing can therefore be considered to be a general indicator of stress caused by TBT and atrazine contamination, and changes in burrowing by the organisms should be evaluated in a multi-species, early-warning biomonitoring technology in order to detect the presence of these contaminants. However, as no clear pattern of response was seen when organisms were exposed to either contaminant, it is unlikely that this parameter would be useful for inclusion in a model designed to predict precise concentrations of the substances.

Burrowing in *Hyalella* is considered to be a type of avoidance behaviour (Hatch and Burton, 1999). Generally *Hyalella* burrow in response to predators, when frightened, or in response to other disturbances in their environments (Wang *et al.,* 2004). It follows that if the organisms are able to detect contaminants in the water column, they may burrow into uncontaminated sediment to avoid contact with chemicals. This may help to lessen the physiological effect of the contaminant on the organism (Oberlin and Blinn, 1997). Past long-term bioassays have shown that *Hyalella* exposed to increasing concentrations of fluoranthene will spend significantly more time burrowed than organisms in uncontaminated water (Hatch and Burton, 1999).

v) Grouping

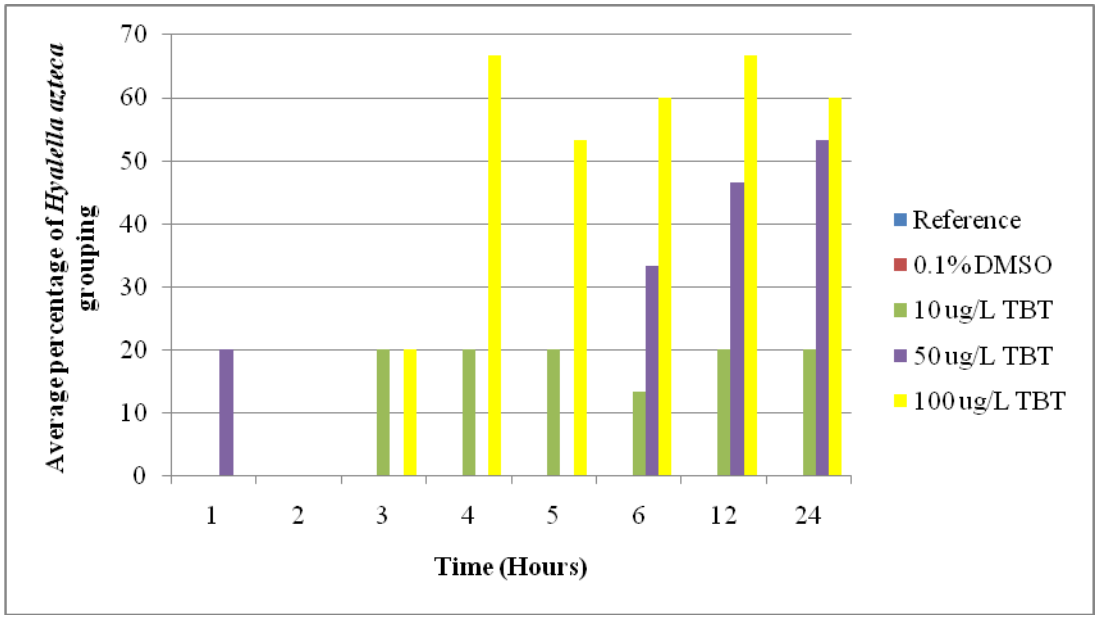

Figure 30: Average percentage of *Hyalella azteca* found in groups in three concentrations of TBT over a 24 hour period.

There was no significant difference in the grouping behaviour of the organisms in the reference and 0.1% DMSO ($p = 1.000$). When compared to the reference, a significant difference in the grouping behaviour of the *Hyalella* was seen after exposure to 10 µg/L TBT treatments ($p = 0.01$), 50 µg/L TBT treatments ($p = 0.005$), and 100 µg/L TBT treatments ($p = 0.000$) over the 24 hour test period. No grouping was observed in the reference and 0.1% DMSO treatments (Figure 30). Increased grouping was seen in all concentrations of TBT was seen; however, there was no clear concentration or time dependence until after 6 hours of exposure. At earlier timepoints, groups were either not formed or the organisms constantly moved into and out of groups.

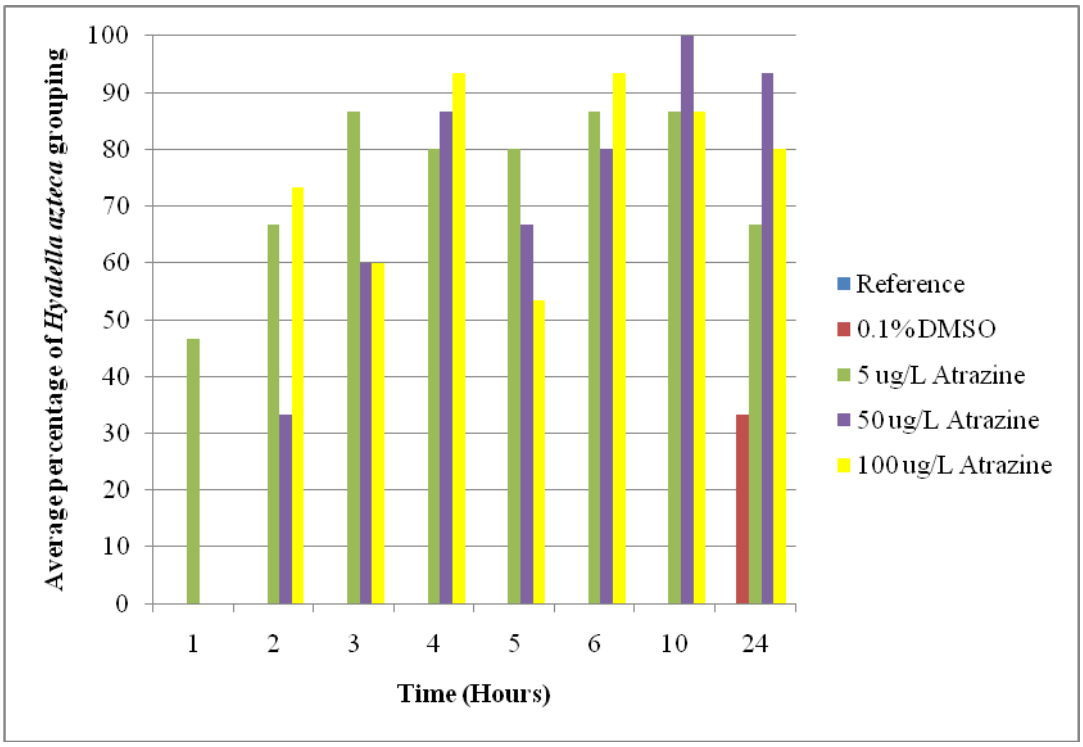

Figure 31: Average percentage of *Hyalella azteca* found in groups in three concentrations of atrazine over a 24 hour period.

There was no significant difference in the grouping behaviour of the organisms in the reference and 0.1% DMSO ($p = 0.317$). When compared to the reference, a significant difference in the burrowing behaviour of the *Hyalella* was seen after exposure to 5 µg/L atrazine treatments ($p = 0.000$), 50 µg/L atrazine treatments ($p = 0.000$), and 100 µg/L atrazine treatments ($p = 0.000$) over the 24 hour test period. Little or no grouping was seen in the reference and 0.1% DMSO treatments (Figure 31). Increased grouping was seen in all concentrations of atrazine, but no pattern of time or concentration dependency was seen, as lower concentrations of atrazine often affected a greater proportion of the organisms than higher concentrations at multiple timepoints.

Significant increases in *Hyalella* grouping was seen in all treatments of TBT and atrazine when compared to the reference treatments. Little or no grouping was seen in the reference and DMSO treatments. Grouping can therefore be considered to be a general indicator of stress caused by TBT and atrazine contamination, and changes in grouping by the organisms should be evaluated in a multi-species, early-warning biomonitoring technology in order to detect overall the presence of these contaminants. However, as no clear pattern of response was seen when

organisms were exposed to either contaminant, it is unlikely that this parameter would be useful for inclusion in a model designed to predict precise concentrations of the substances.

Increases in organism grouping in response to chemical contaminants have been seen in past long-term bioassays (Hatch and Burton, 1999). It is believed that grouping in *Hyalella* occurs in response to stress, but is not a behavioural adaptation to reduce impacts of contaminants (Hatch and Burton, 1999). It is not clear why increased group formation is seen when organisms are exposed to contaminants; however, it seems that using grouping as a parameter in a multi-species, early-warning biomonitoring would be appropriate based on responses seen in the TBT and atrazine bioassays.

vi) Body Length

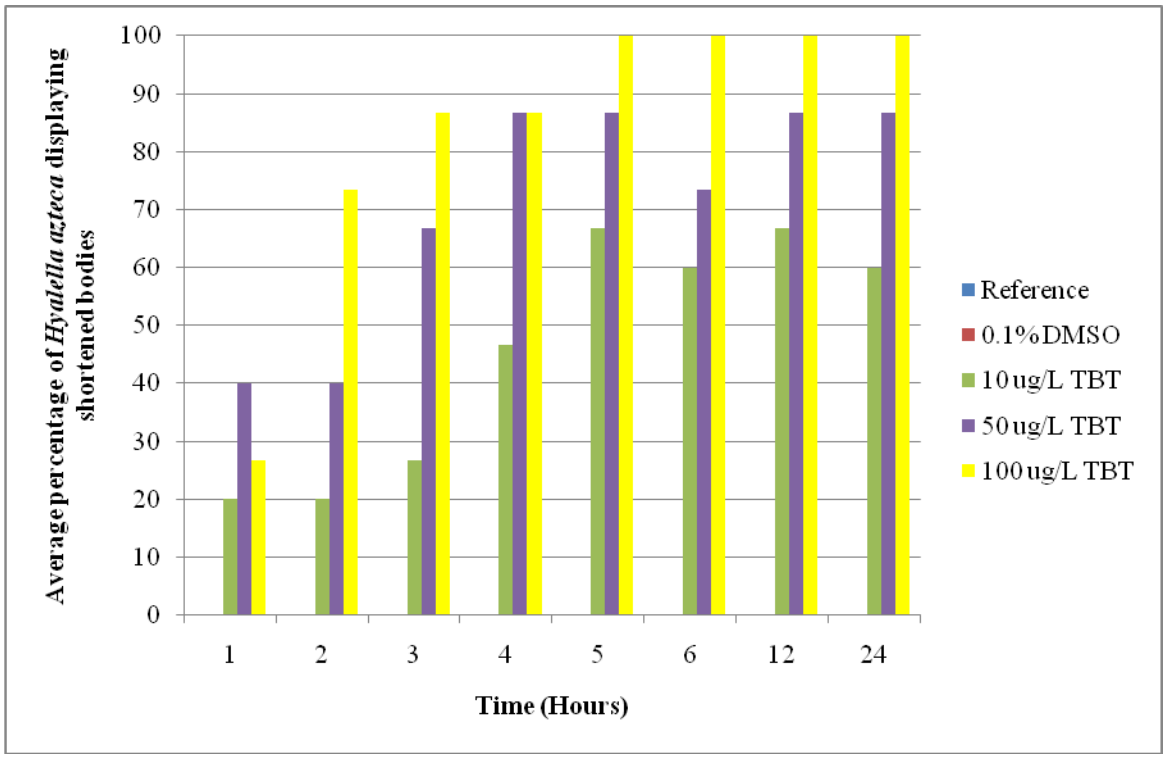

Figure 32: Average percentage of *Hyalella azteca* displaying shortened body lengths in three concentrations of TBT over a 24 hour exposure period.

There was no significant difference *Hyalella* body length when comparing the reference and 0.1% DMSO treatments ($p = 1.000$). A significant change in body length was seen in the 10 µg/L TBT treatments ($p = 0.000$), the 50 µg/L TBT treatments ($p = 0.000$), and the 100 µg/L

treatments (p = 0.000) compared to the reference over the 24 hour trial period. In the reference and 0.1% DMSO treatments, no organisms displayed shortened bodies (Figure 32). An increased number of the organisms displayed shortened bodies in all three concentrations of TBT. Increases in body shortening were time and concentration dependent, with higher concentrations of the contaminant causing body shortening in a greater proportion of the organisms, more rapidly than in lower concentrations.

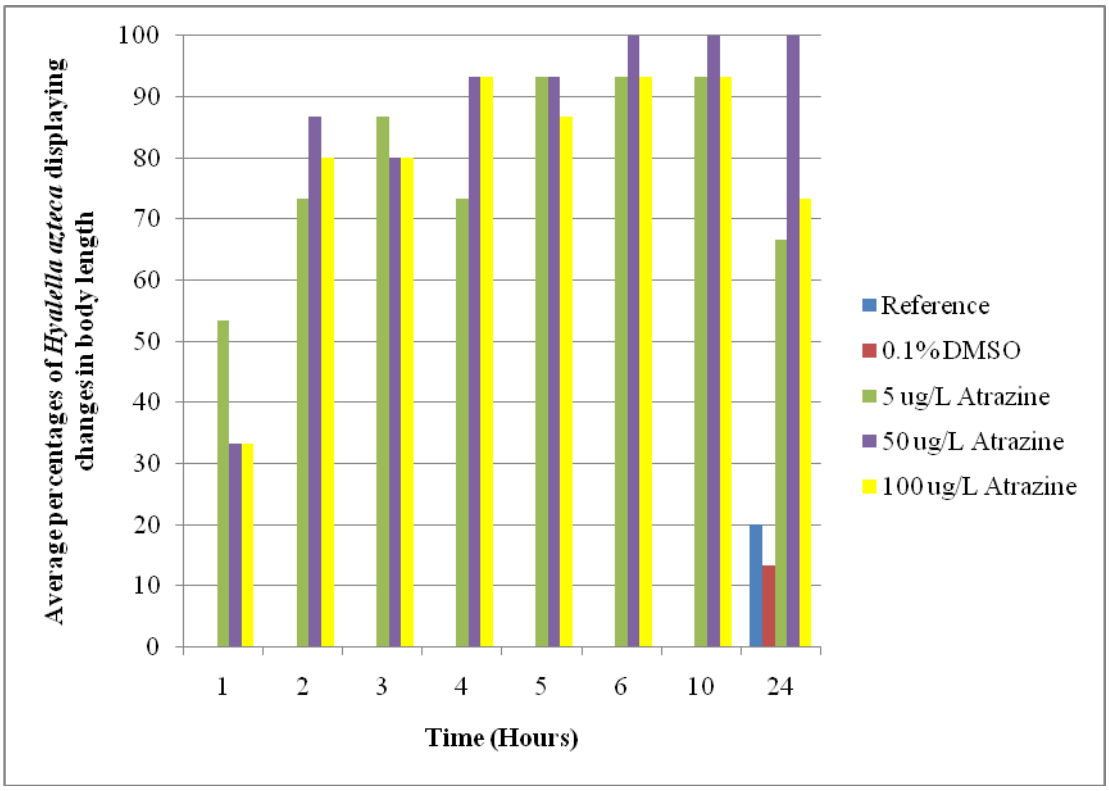

Figure 33: Average percentage of *Hyalella azteca* displaying shortened body lengths in three concentrations of atrazine over a 24 hour exposure period.

There was no significant difference in organism body length when comparing the reference and 0.1% DMSO treatments (p = 0.976). A significant change in body length was seen in the 5 µg/L atrazine treatments (p = 0.000), the 50 µg/L atrazine treatments (p = 0.000), and the 100 µg/L atrazine treatments (p = 0.000) compared to the reference over the 24 hour trial period. In the reference and 0.1% DMSO treatments, no organisms displayed shortened bodies (Figure 33). An increased number of the organisms displayed shortened bodies in all three concentrations of atrazine. No pattern of time or concentration dependence was seen in the three concentrations, as fluctuations in the average proportion of the organisms with shortened bodies

were seen at the various time points. Multiple observations of lower concentrations impacting greater proportions of the organisms than higher concentrations were also seen.

Significant changes in body length were seen in rapid response to all concentrations of TBT and atrazine, indicating that this is a sensitive parameter which can be changed in response to the overall presence of contaminants. Evaluations of changes in *Hyalella* body length should therefore be incorporated in a multi-organism, early-warning biomonitoring technology. A consistent time and concentration dependent response was seen in TBT treatments, indicating that changes in this parameter could potentially be included in a model which will help water treatment plant operators determine exact concentrations of the contaminant present. Atrazine, on the other hand, did not produce a consistent pattern of response. This suggests that elevated levels of atrazine could potentially be detected by examining changes in *Hyalella* body length but use of this parameter would not be helpful if incorporated into a model to help determinate precise concentrations of the contaminant.

vii) Body Orientation

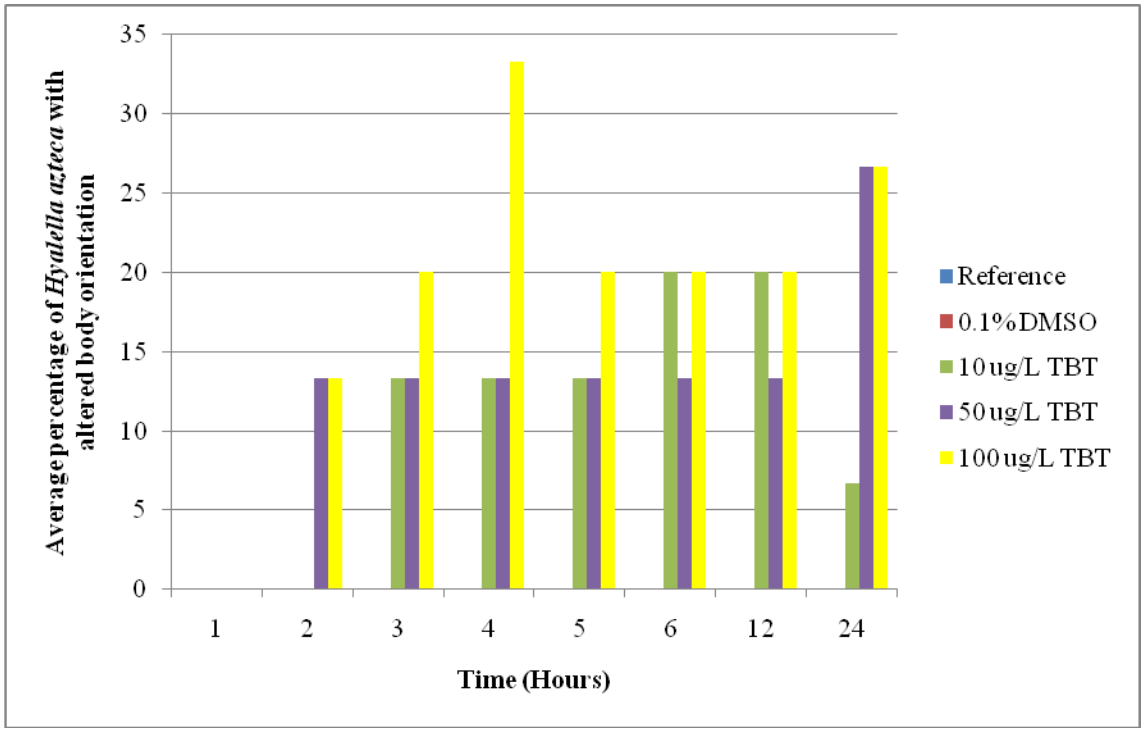

Figure 34: Average percentage of *Hyalella azteca* displaying changes in body orientation in three concentrations of TBT over a 24 hour exposure period.

There were no significant changes in body orientation when comparing the reference and 0.1% DMSO treatments (p = 1.000). Significant changes in body orientation were seen in the 10 µg/L TBT treatments (p = 0.000), the 50 µg/L TBT treatments (p = 0.001) and the 100 µg/L TBT treatments (p = 0.000) over the 24 hour experimental period. No changes in body orientation (organisms lying on sides instead of upright) were seen in the reference and 0.1% DMSO treatments (Figure 34). Compared to the reference treatments, an increase in abnormal body orientation was seen in all three TBT treatments. No concentration or time dependence was seen in the three TBT treatments, and a fluctuation of the organisms displaying altered body orientation was seen in all treatments over time. It appeared that organisms were able to recover to their normal upright body positions and that altered body positions were not maintained for the duration of the experiment.

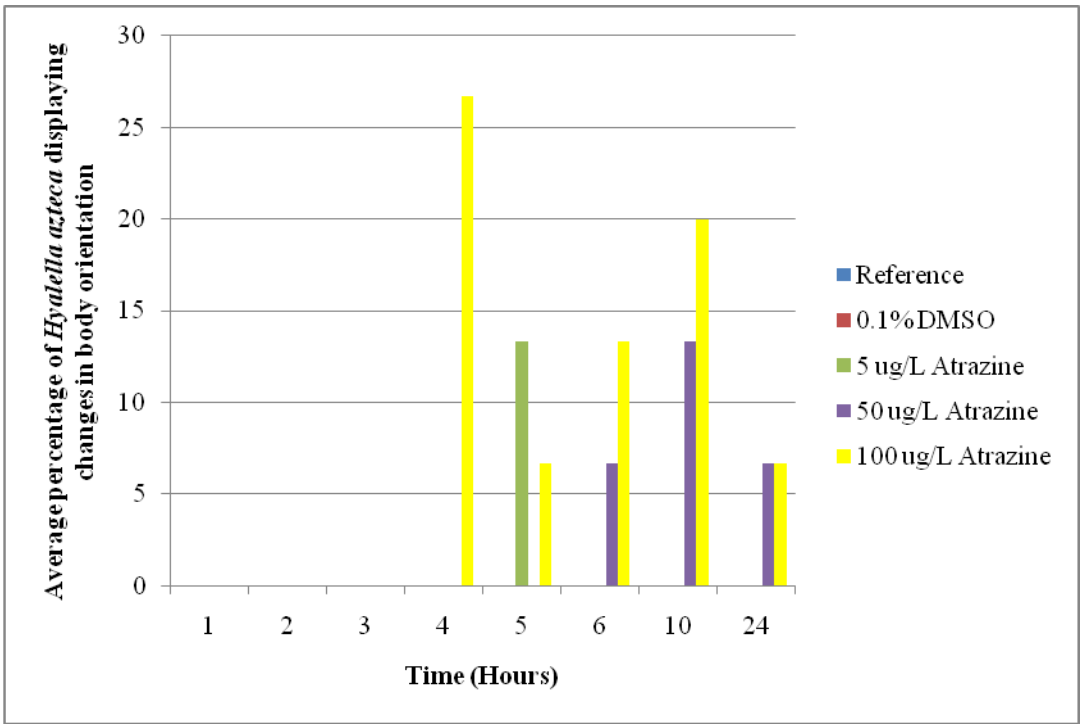

Figure 35: Average percentage of *Hyalella azteca* displaying changes in body orientation in three concentrations of atrazine over a 24 hour exposure period.

There was no significant change in body orientation of the organisms when comparing the reference treatments to the 0.1% DMSO treatments (p = 1.000), the 5 µg/L atrazine treatments (p = 0.317) and the 50 µg/L atrazine treatments (p = 0.077) over the 24 hour experimental period. A significant change in *Hyalella* body orientation was seen in the 100 µg/L

atrazine treatments compared to the reference ($p = 0.010$) during the 24 hour trial. A significant increase in abnormal body orientation was seen in the 100 µg/L treatments when compared to the reference treatments. No time dependence for changes in body orientation was noted at this concentration (Figure 35).

Significant changes in body orientation of the *Hyalella* were seen in all three concentrations of TBT. As responses were not concentration or time dependent, changes in this parameter may only be used to determine the presence of TBT and would not be useful in any type of model to inform treatment plant operators about the specific concentration of the contaminant present. Significant changes in body orientation were only seen in the highest concentration of atrazine, indicating that this may not be a sensitive parameter for detecting low concentrations of the contaminant. An extensive literature review found no previous bioassays which have been conducted examining changes in body orientation in *Hyalella azteca* in response to chemical contaminants in the environment. It seems that this parameter is not appropriate use for the detection of TBT and atrazine in a multi-species, early-warning biomonitoring technology, due to a lack of response by organisms and the ability of organisms to recover their upright positions.

Summary of Hyalella azteca *Motility Bioassay*

The results of the *Hyalella azteca* motility bioassay revealed that some movement behaviour parameters are more sensitive to chemical contaminants, specifically TBT and atrazine, than others and should therefore be evaluated in a multi-organism, early-warning biomonitoring technology. Since some parameters are more sensitive than others to the given contaminants, changes in these parameters should be considered more important stress indicators than other changes in behaviour and should be given higher priority when creating a model to detect types and concentrations of contaminants.

The most important parameter for evaluation in a multi-species, early-warning biomonitoring system appeared to be immobilization. This parameter displayed a time and concentration dependent response in both the TBT and atrazine, appeared rapidly in all concentrations of both contaminants and should allow for modelling based discrimination between concentrations and treatments. The second most important parameter to be evaluated

appeared to be substrate crawling. A concentration and time dependent response to TBT but not atrazine was seen, allowing for discrimination between the two compounds and the potential for modelling to determine exact TBT concentrations. A rapid onset with a high percentage of organisms showing altered behaviour was seen in all concentrations of both contaminants. The next most important parameter to be evaluated in our technology should be organism body length. A similar concentration and time dependent response to TBT but not atrazine was seen, allowing for discrimination between the two compounds and the potential for modelling to determine exact TBT concentrations. All concentrations of both contaminants affected body length and onset was again rapid. However, it appeared that overall a lesser number of organisms displayed changes in body length than in other parameters discussed above, indicating it might be a less sensitive characteristic of movement behaviour. The next most important parameter appeared to be burrowing, which had no time or concentration dependent response to either contaminant, a great deal of fluctuation in response, and could therefore be only used as a general indicator of the contaminants, not for discrimination between different concentrations. The parameter of grouping behaviour showed a similar response, but since it has less ecological relevance, it should be ranked as less important that burrowing behaviour. The final two parameters (swimming events and body orientation) should not be included for monitoring in a multi-species, early-warning biomonitoring technology. Swimming events declined in the reference and 0.1% DMSO treatments, indicating that acclimation may have taken place and that over a long period of time this parameter may not be viable for evaluation. Changes in body orientation occurred in all concentrations but with no pattern of time and concentration dependence. Organisms were able to recover their body positions and the parameter seemed to not be sensitive to low concentrations of atrazine.

Overall, the ranking of importance of the 7 parameters is: immobilization > substrate crawling > body length > burrowing > grouping > body orientation = swimming events. The first five parameters should be included for evaluation in a multi-species, early-warning biomonitoring technology, whereas body orientation and swimming events should not.

3.3 *Lumbriculus variegatus* Behaviour

3.3.1 *Lumbriculus variegatus* Burrowing

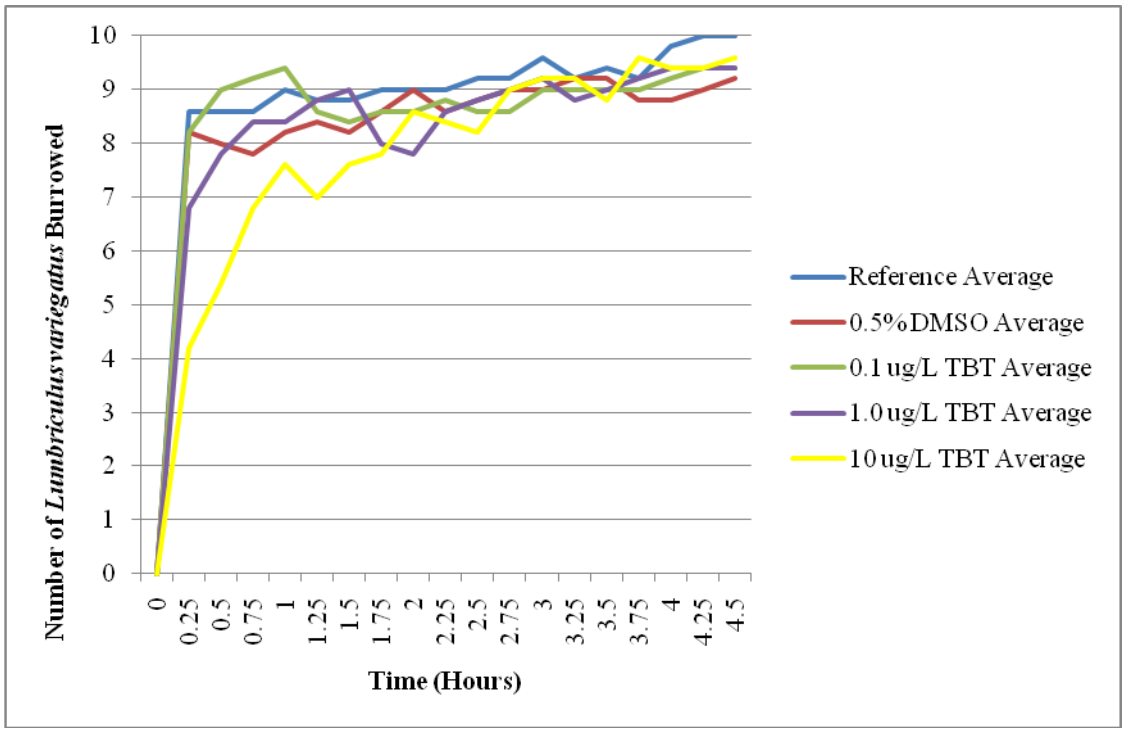

Figure 36: Average rate of *Lumbriculus* burrowing (N = 10 organisms total) over time for the five experimental treatments in the TBT bioassay.

Line graphs were utilized in the interpretation of *Lumbriculus variegatus* burrowing and grouping behaviours, rather than bar graphs, in order to aid in interpretation of rates at which the organisms burrowed and formed groups and to better present data overall. Qualitative observations of changes in the rate of *Lumbriculus variegatus* burrowing suggest that the organisms in the reference, DMSO and 0.1 µg/L treatments display a similar rate of burrowing (Figure 36). Similar numbers of the organisms were buried at the different time points and a similar endpoint was seen for the total average number of organisms in burrows. When compared to the reference treatment, the organisms exposed to 1.0 and 10 µg/L of TBT seem to have a lower rate of burrowing with fewer organisms burrowed overall, especially during the first 2 hours of the trial (Figure 36). The organisms appear to burrow more slowly during initial exposure, and have a lower endpoint for the total number of the organisms burrowed. This suggests that concentrations of TBT as low as 1.0 µg/L may affect the burrowing rate of

Lumbriculus variegatus and that increasing concentrations of TBT may have a greater impact as there is a greater difference in the change of rate as concentrations increase from 1.0 µg/L to 10.0 µg/L compared to the reference treatments.

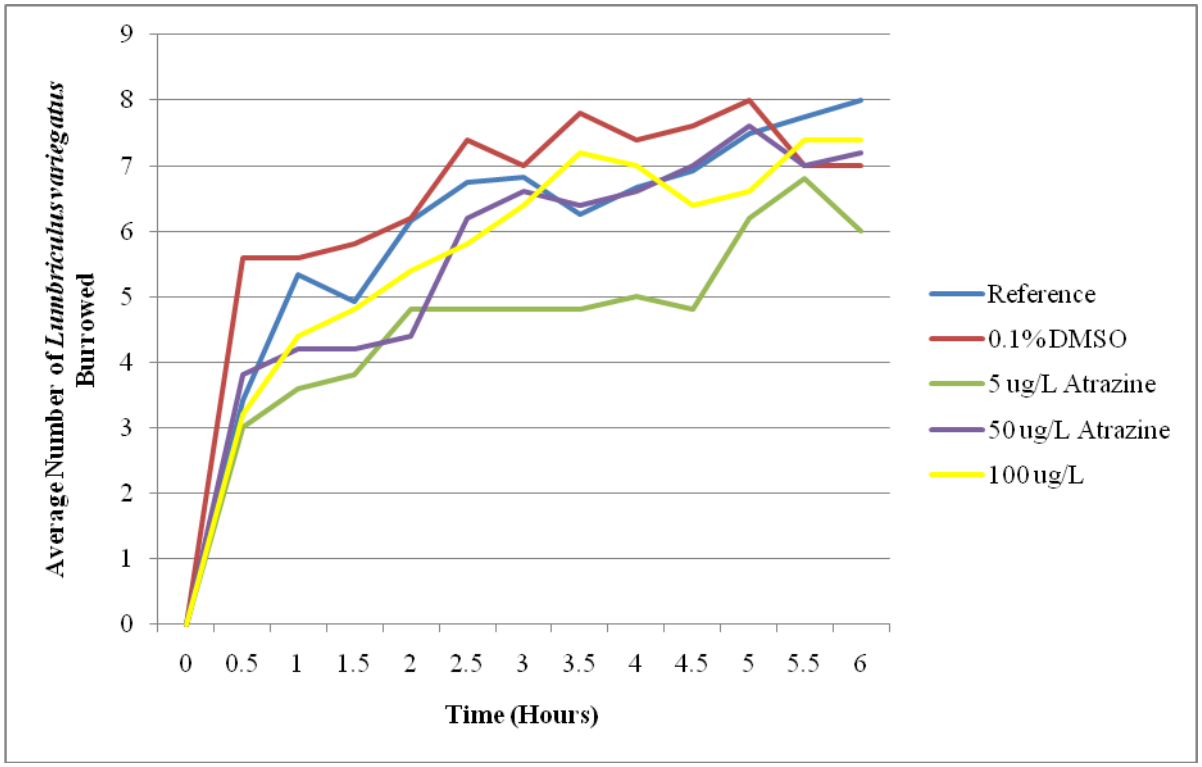

Figure 37: Average rate of burrowing of *Lumbriculus variegatus* (N = 10 organisms total) over a 6 hour experimental time period when exposed to five different treatments.

Qualitative observations of changes in the rate of *Lumbriculus variegatus* burrowing in atrazine suggests that the average rate of burrowing in the DMSO treatment is slightly higher than the reference, with a greater number of organisms buried in total at the end of the bioassay (Figure 37). The three atrazine treatments seem to have a lower average rate of burrowing than the reference. The impairment of burrowing behaviour in atrazine does not appear to be concentration dependent, as the organisms in the 5 µg/L displayed a lower rate of burrowing than the 50 and 100 µg/L, with less worms burrowed at all time points. The increase in burrowing rate in the DMSO and the decrease in burrowing in the atrazine treatments indicate that the two chemicals may affect the organisms in different manners. As the organisms in the DMSO treatment burrowed more quickly than in the reference and atrazine treatments, it seems that the

worms were able to detect and attempt to avoid the DMSO in the water and that movement behaviour was not impaired. This is unusual, as 0.1% DMSO did not affect the burrowing rate of the organisms in the TBT bioassay or any of the other behaviours examined in this project. Further modelling analyses beyond the scope of this thesis are necessary in order to determine if the presence of the solvent affects the rate of burrowing of the organisms. In the atrazine treatments, lower rates of burrowing were observed compared to the reference, which suggests that the ability of the organisms to move away from the atrazine contamination is impaired.

Burrowing in sediment is a normal behaviour in *Lumbriculus variegatus*, which will bury their head in the sediment and extend the tail for gas exchange (O'Gara *et al.,* 2004). However, organisms are also able to crawl on the surface of sediment in order to move from one area to another (Drewes, 1999). Burrowing can be seen as an avoidance response in organisms which are able to detect chemicals in their environment. Burrowing may allow organisms to avoid the contaminant by escaping to clean sediment, thus reducing the impacts of the contaminant (Wicklum *et al.,*1997). Varying results have been seen with regards to burrowing in the presence of contaminants. Gerhardt (2007) found that *Lumbriculus* showed increased burrowing activity in the presence of varying concentrations of lead. On the other hand, other studies have demonstrated that burrowing in the organisms will decrease in the presence of pollutants due to narcotic impacts of the contaminants (Landrum *et al.,* 2002). This indicates that different classes of contaminants may impact the rate of *Lumbriculus* burrowing in different ways.

An examination of *Lumbriculus* burrowing should be included in a multi-species, early-warning biomonitoring technology. It appears that both TBT and atrazine at concentrations of over 1.0 µg/L and 5 µg/L, respectively, will cause a decrease in the burrowing rate of the organisms, indicating that this parameter is highly sensitive to low concentrations of these two contaminants. As burrowing rates are decreased, it is likely that the contaminants are impacting the organism's ability to move and burrow, as discussed in greater detail below.

3.3.2 *Lumbriculus variegatus* Grouping

Initial grouping behaviour evaluations indicated that no response was seen to concentrations of TBT below 10 µg/L , necessitating modifications to experimental procedures including increased contaminant concentrations and longer observation periods.

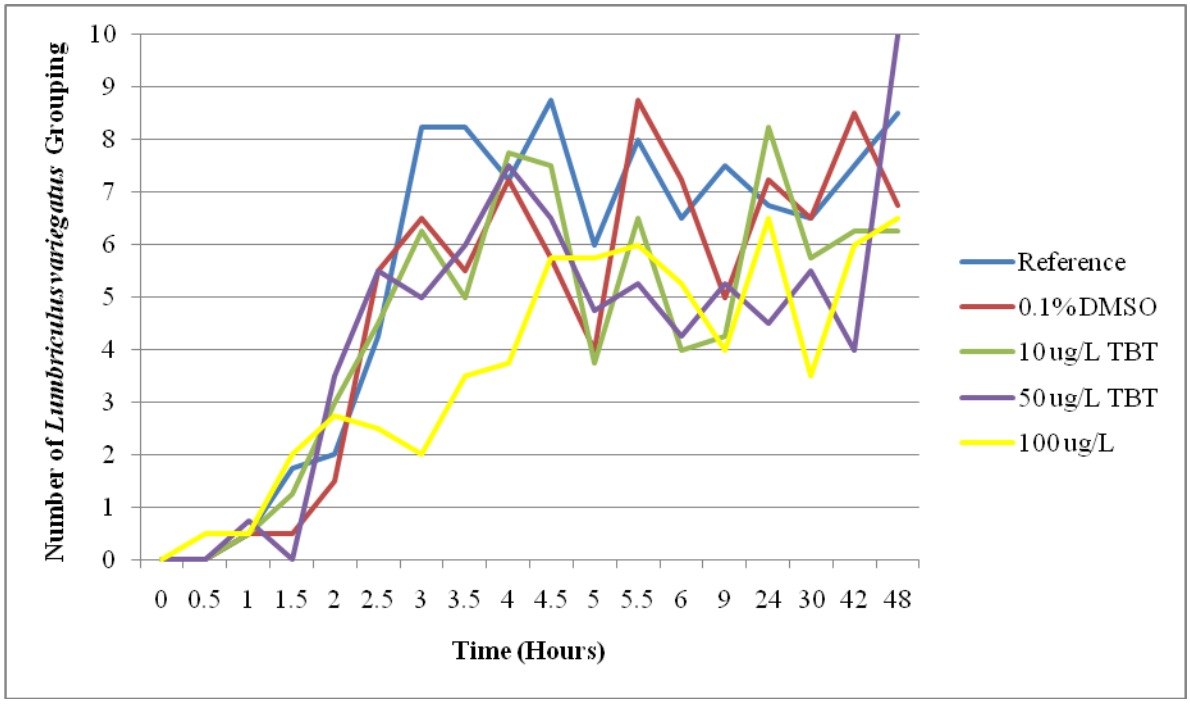

Figure 38: Average rate of the grouping of *Lumbriculus variegatus* (N = 10 organisms total) over time for the five experimental treatments during the TBT bioassay.

During the TBT colony formation bioassay, it appeared that the DMSO treatments and concentrations of TBT ≤ 50 µg/L had no impact on the grouping rate of the organisms when compared to the reference treatments (Figure 38). Worms in the 100 µg/L treatments appeared to have a lower rate of colony formation than the reference organisms. Large amounts of fluctuation in the number of the organisms involved in groups made qualitative observations difficult, necessitating further modelling beyond the scale of this thesis to determine if an impact was in fact seen.

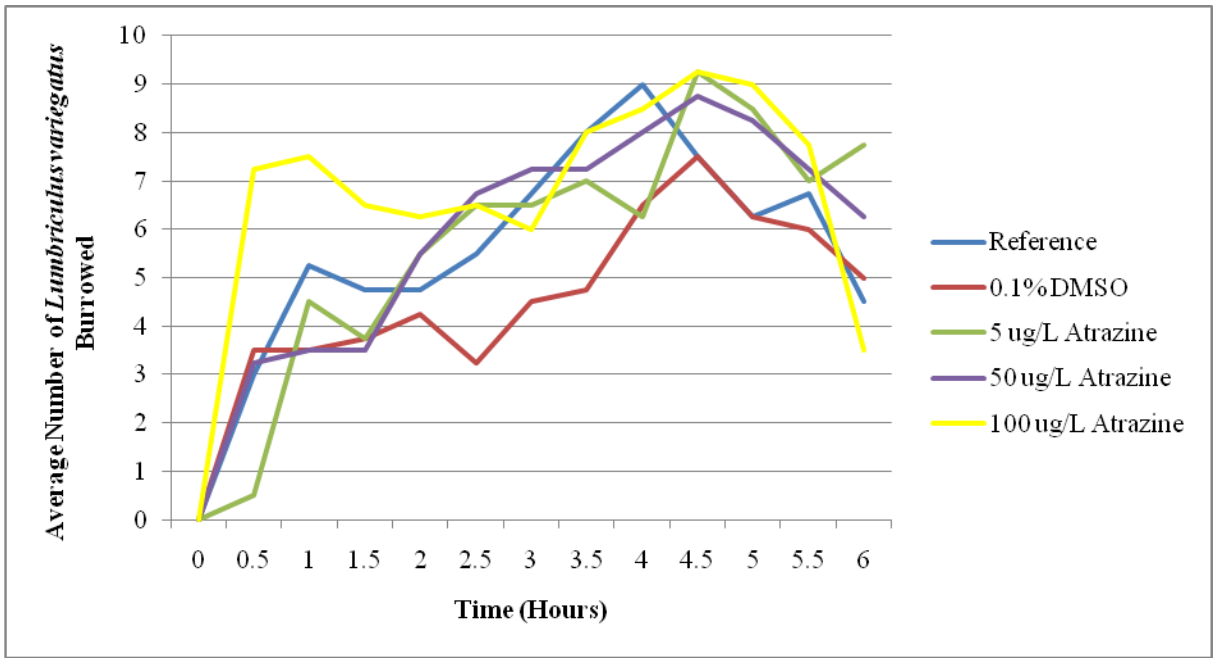

Figure 39: Average rate colony formation of *Lumbriculus variegatus* (N = 10 organisms total) over a 6 hour experimental time period when exposed to five different treatments in the atrazine colony formation bioassay.

During the atrazine colony formation bioassay, it appeared that all treatments had similar colony formation rates to the reference treatments due to fluctuation in the average number of the organisms involved in a colony in all treatments (Figure 39). Movement of the organisms in and out of colonies made qualitative observations of trends difficult, necessitating further modelling beyond the scale of this thesis to determine if an impact was in fact seen.

The rate of colony formation and changes in colony behaviour has not been widely studied in response to changes in water quality. It is known that in natural settings the organisms will group together to increase body heat by being near other organisms or will gather around a food source (Drewes, 1999). Although the organisms reproduced via asexual fragmentation when cultured in a laboratory, *Lumbriculus* have also been known to sexually mate in the wild (Drewes, 1997) and group formation may help to facilitate this process. The related species *Tubifex tubifex* has been used in past bioassays with the MFB examining the effects of nickel, copper, cadmium, and the pesticides imidacloprid and ivermectin on group movement and ventilation. Over time, decreased group locomotion and ventilation in the aggregation were found, with higher concentrations of the substances having a greater impact on the organisms

(Gerhardt, 2009). This indicates that monitoring of grouping behaviour is possible but that the determination of rate models may not be the best way to monitor the parameter.

None of the treatments of TBT or atrazine appeared to have had an effect on the intrinsic grouping rates of the organisms, indicating this parameter is not appropriate for detecting sub-lethal concentrations of the two contaminants. The organisms are constantly moving in and out of groups and group numbers can fluctuate greatly within short periods of time (Figures 38 and 39). The behaviour does not seem to have a measurable endpoint in any of the environmentally relevant contaminant treatments, including the reference. Because of these factors, and the potential difficulty of automating a system which could count worms in a colony, it is recommended that grouping behaviour not be considered as a parameter in a rapid automated biomonitoring system.

3.3.3 *Lumbriculus variegatus* Motility

Several aspects of *Lumbriculus* motility behaviour were evaluated during these bioassays and were found to change in the presence of varying concentrations of the contaminants TBT and atrazine. Changes in response to the contaminants are summarized below with a discussion of the ecological impacts of the change. Qualitative observations of general trends of behavioural changes were made to aid in the ranking of the importance of the various parameters. A discussion of the utility of the various parameters for evaluation in a multi-organism early-warning biomonitoring system is also included. The specific parameters evaluated included: i) position in bioassay vessel, ii) locomotion style, iii) immobilization, iv) body length, v) body orientation, and vi) group movement.

i) Position in Bioassay Vessel

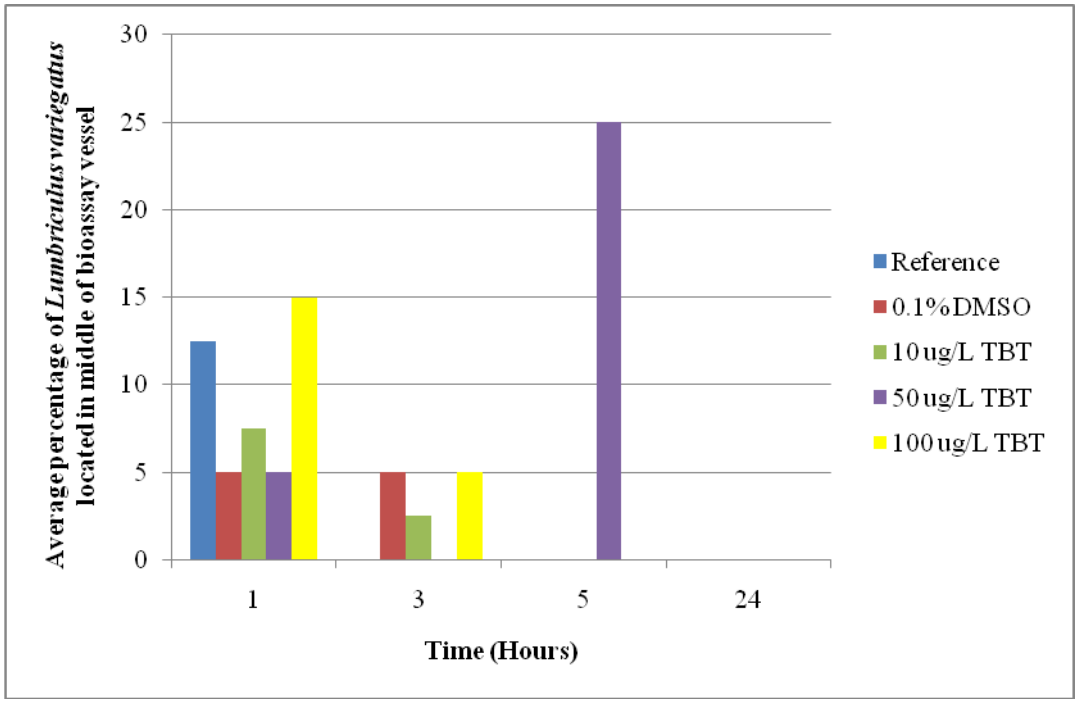

Figure 40: Average percentage of *Lumbriculus variegatus* positioned in the middle of the bioassay vessel when exposed to three concentrations of TBT over a period of 24 hours.

No significant differences in organism location within the bioassay vessel were seen when comparing the reference to the 0.1% DMSO treatments ($p = 0.676$), the 10 µg/L TBT treatments ($p = 0.978$), the 50 µg/L TBT treatments ($p = 0.602$), and the 100 µg/L TBT treatments ($p = 0.426$) over the 24 hour experiment period. Some positioning within the centre of the vessel was noted in all treatments after 1 hour of exposure; however, the organisms remained at the edges of the bioassay vessels for the majority of the bioassay (Figure 40) Overall, none of the TBT treatments had any effect on the location of the worms within the bioassay vessel.

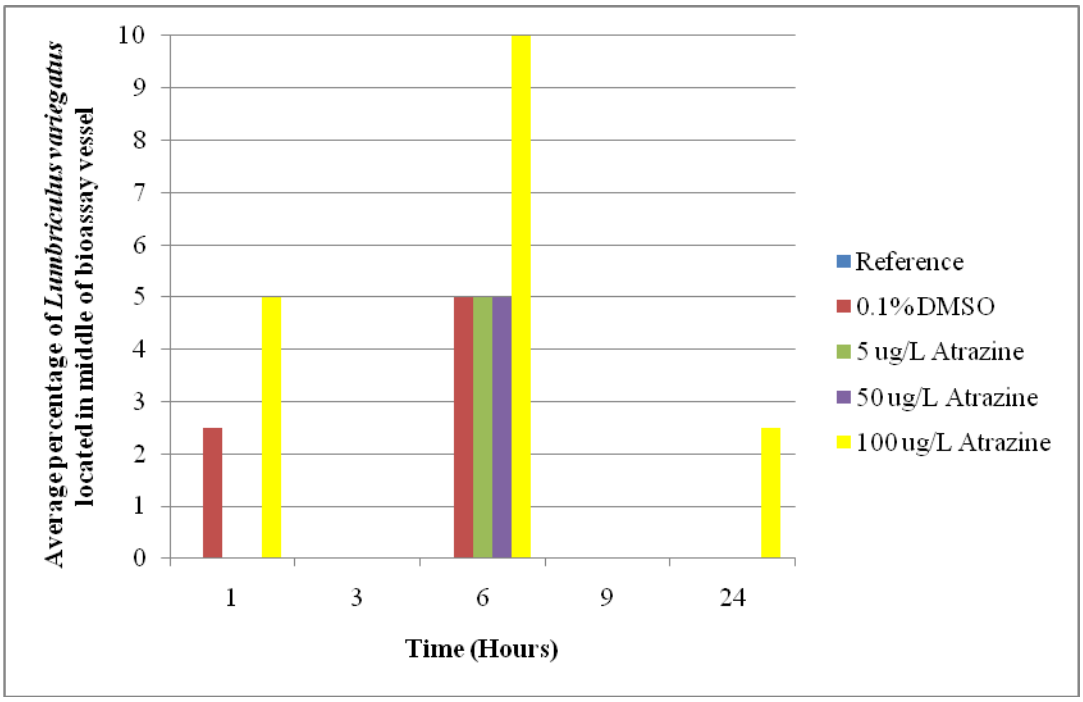

Figure 41: Average percentage of *Lumbriculus variegatus* positioned in the middle of the bioassay vessel when exposed to three concentrations of atrazine over a period of 24 hours.

No significant differences in organism positioning in the bioassay vessels was noted when comparing the reference to the 0.1% DMSO treatments ($p = 0.059$), the 5 µg/L atrazine treatments ($p = 0.077$), and the 50 µg/L atrazine treatments ($p = 0.077$). A significant change in body positioning in the bioassay vessel was noted in the 100 µg/L atrazine treatments ($p = 0.020$) when compared to the reference over the 24 hour experimental period. Overall, a greater number of the organisms were located in the middle of the bioassay vessel in the 100 µg/L atrazine treatments than in the reference where all the organisms remained at the edge of the vessel (Figure 41). A pattern of concentration dependence but not time dependence was seen in this bioassay, as the organisms in the 100 µg/L bioassays appeared to alternate in position between the edge and the middle of the vessel over the course of the experiment.

No changes in organism positioning within the bioassay vessel were seen in the three treatments of TBT and in the two lowest concentrations of atrazine. The organisms spent the majority of time moving along the edge of the glass beaker and were able to use the edge of the vessel to aid in locomotion. A significant increase in the number of the organisms found in the middle of the bioassay vessel was seen in the 100 µg/L atrazine treatments; however, the highest

average number of the organisms in the middle of the vessel was only 10%. This suggests that although there is a significant difference from the reference treatments, not that many worms are affected overall. Changes in position in the bioassay chamber have not been evaluated in response to contaminants in a multi-species, early-warning biomonitoring technology. The parameter has no real ecological significance as this type of restrictive space would not be found in the natural habitat of the *Lumbriculus*. Results of the TBT and atrazine bioassays indicate that this parameter should not be considered as part of a multi-species, early-warning biomonitoring technology due to low levels of response, inconsistencies in results, the ability of organisms to recover and move from one area to another, and potential problems monitoring with online biomonitoring chambers.

ii) Locomotion Style

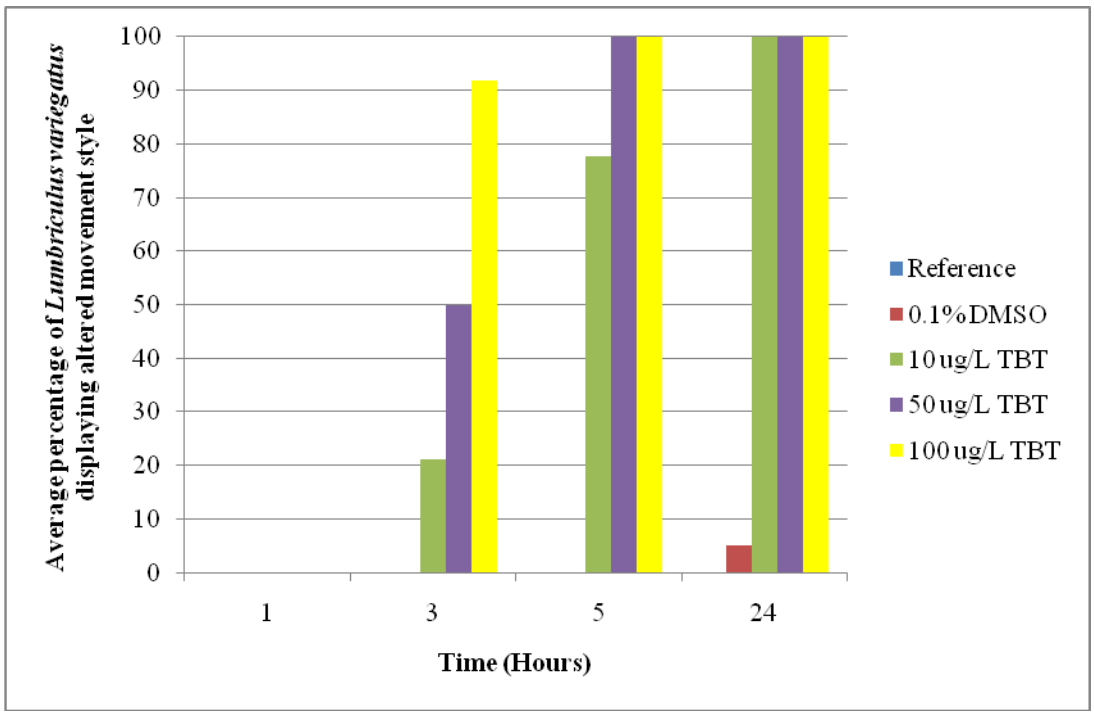

Figure 42: Average percentage of *Lumbriculus variegatus* displaying changes in locomotion style after exposure to three concentrations of TBT over a 24 hour period.

No significant differences in locomotion style were seen when comparing the reference and 0.1% DMSO treatments ($p = 0.283$) over the 24 hour experimental period. A significant difference in the number of the organisms displaying abnormal movement was seen in the 10 µg/L TBT treatments ($p = 0.000$), the 50 µg/L TBT treatments ($p = 0.000$), and the 100 µg/L

TBT treatments (p = 0.000) when compared to the reference treatments. All three concentrations of TBT affected the locomotion style of the worms in a time and concentration dependent manner, with responses seen after 3 hours of exposure (Figure 42). After 5 hours of exposure, all the organisms in the 50 µg/L and 100 µg/L TBT treatments displayed altered locomotion patterns (Figure 42). After 24 hours, all of the organisms in all concentrations displayed movement abnormalities.

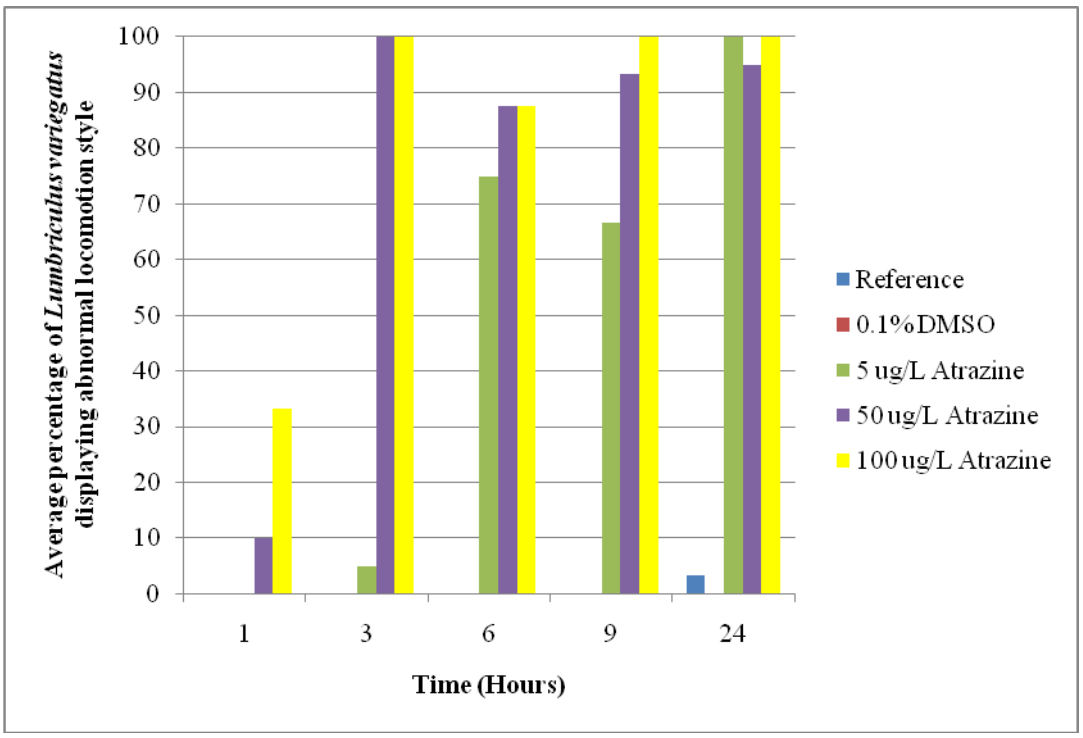

Figure 43: Average percentage of *Lumbriculus variegatus* displaying changes in locomotion style after exposure to three concentrations of atrazine over a 24 hour period.

No significant differences in locomotion style were seen when comparing the reference and 0.1% DMSO treatments (p = 0.317). A significant increase in abnormal movement behaviour was seen in the 5 µg/L atrazine treatments (p = 0.002), the 50 µg/L atrazine treatments (p = 0.000), and the 100 µg/L atrazine treatments (p = 0.000) when compared to the reference over the 24 hour experimental period. All three concentrations of atrazine affected the locomotion style of the *Lumbriculus* in a time and concentration dependent manner, with a greater number of the organisms displaying altered behaviour in the higher concentrations after shorter periods of exposure (Figure 43). After 24 hours of exposure, almost all of the organisms in the three treatments displayed movement style abnormalities.

A time and concentration dependent change in locomotion style of the worms was seen in response to all three concentrations of both TBT and atrazine. Nearly all worms in all contaminant treatments displayed altered movement patterns at the end of the bioassay period. This indicates that change in movement style is a highly sensitive parameter which should be investigated in a multi-species, early-warning biomonitoring technology. As a time and concentration dependent response was seen to both contaminants, it should be possible to incorporate these responses into a model to determine specific concentrations of each contaminant when present in in-coming water supplies. As response patterns differed between the two chemicals, it should also be possible to discriminate between the two classes of contaminants using a modelling based approach. Due to its high sensitivity and ecological importance, it is recommended that changes in movement style be measured when designing a multi-species, early-warning biomonitoring technology using *Lumbriculus variegatus.*

Changes in movement style have been noted in numerous past bioassays in response to a variety of contaminants. When exposed to varying concentrations of the pesticide ivermectin, *Lumbriculus* showed similar behavioural changes to those seen in the TBT and atrazine, bioassays including jerky movements and movements originating throughout the body rather than the head region (Ding *et al.,* 2001). Writhing and thrashing have also been observed in response to copper contamination (O'Gara *et al.,* 2004). Changes in movement behaviour could have many detrimental effects on the organisms. The ability to move rapidly is important for the organisms while trying to escape predators or other stimuli that organisms perceive to be dangerous (Drewes, 1999; Drewes and Cain, 1999). Changes in speed or movement efficiency may increase the vulnerability of organisms to predators. Thrashing behaviour may also attract the attention of predators, again increasing the likelihood of predation. Movement style changes may also affect the ability of organisms to find food sources and move away from areas of contamination.

iii) Immobilization

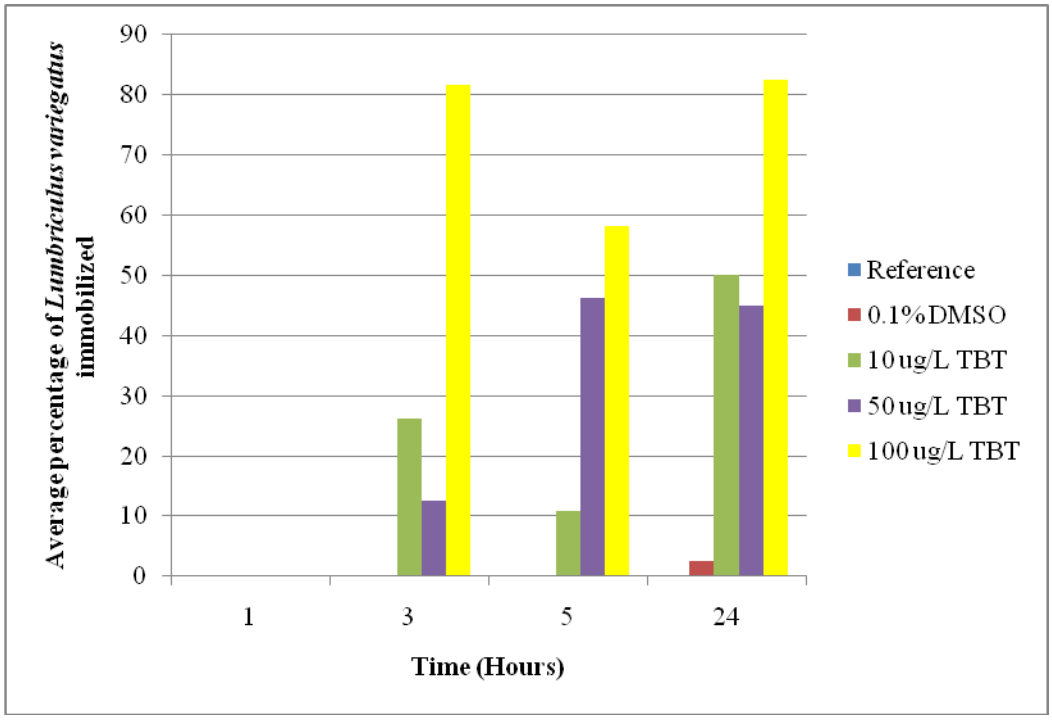

Figure 44: Average percentage of *Lumbriculus variegatus* immobilized in three concentrations of TBT over a 24 hour exposure period.

No significant differences in the number of individual organisms immobilized was seen when comparing the reference and 0.1% DMSO treatments ($p = 0.317$). A significant increase in the number of individual organisms immobilized was seen in the 10 µg/L TBT treatments ($p = 0.002$), the 50 µg/L treatments ($p = 0.002$), and the 100 µg/L TBT treatments ($p = 0.000$) when compared to the reference treatments over the 24 hour experimental period. An overall increase in the number of immobilized worms was seen in response to all three concentrations of TBT (Figure 44). However, it appeared that there was no pattern of time or concentration dependence, as at two of the observation points the average number of the organisms immobilized in the 5 µg/L treatment was higher than in the 50 µg/L treatments. A fluctuation in the number of the organisms immobilized was seen over time.

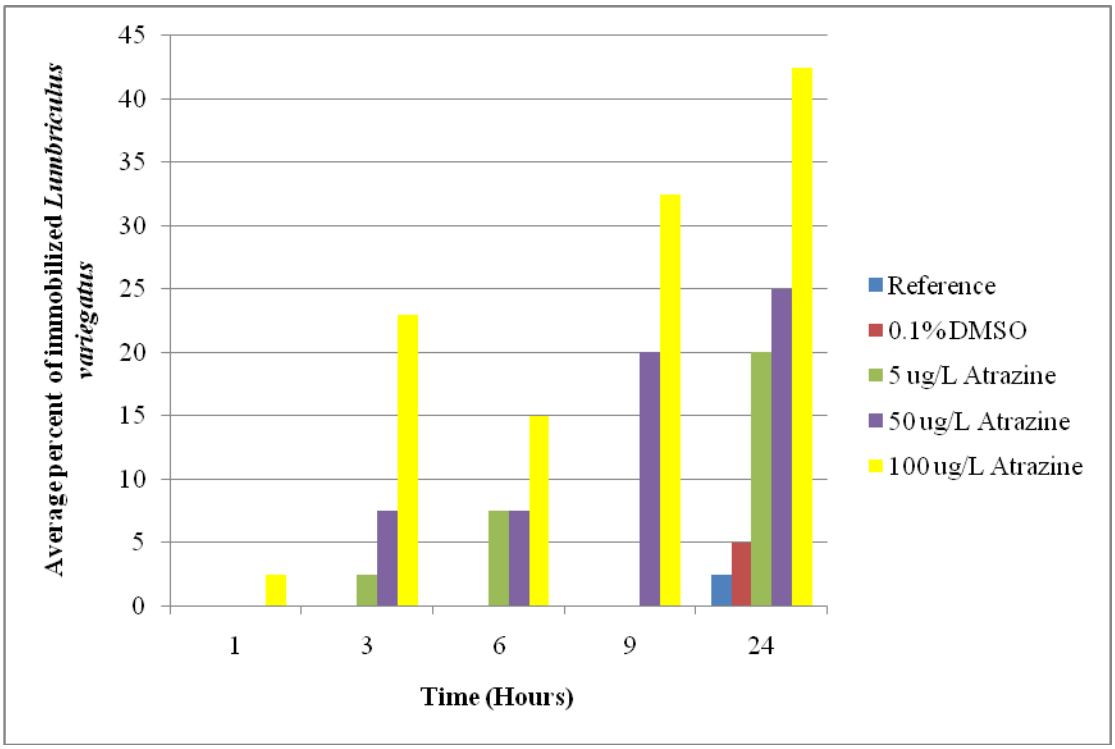

Figure 45: Average percentage of *Lumbriculus variegatus* immobilized in three concentrations of atrazine over a 24 hour exposure period.

No significant difference in the number of immobilized organisms was seen when comparing the reference and the 0.1% DMSO treatments ($p = 0.918$). A significant increase in organism immobilization was seen in the 5 µg/L atrazine treatments ($p = 0.011$), the 50 µg/L atrazine treatments ($p = 0.000$), and the 100 µg/L atrazine treatments ($p = 0.000$) when compared to the reference treatments over the 24 hour trial period. All three concentrations of atrazine produced a time and concentration dependent increase in immobilization (Figure 45), with a greater number of the organisms immobilized in a shorter period of time in higher concentrations than in lower concentrations.

A significant increase in worm immobilization was seen in all three concentrations of both TBT and atrazine. The concentration and time dependent response seen in the atrazine treatments indicates that immobilization could be incorporated into a model to help predict the exact concentration of the contaminant present in a sample. As no concentration or time dependence was noted for immobilization in response to TBT, it is likely that this parameter would be less likely to be useful in a model to determine the precise concentration of TBT in a water sample. However, increases immobilization could be used as a general indicator of the

presence of TBT. The two distinct responses to the different classes of contaminants indicates that discrimination between the two contaminants in a multi-species, early-warning biomonitoring technology should be possible. Decreases in movement were seen in response to all concentrations of the two contaminants, indicating that this parameter is highly sensitive to chemical stress and should be evaluated as part of a multi-species, early-warning technology.

Mobility is highly important for all biological functions in organisms including foraging, predator avoidance, mating and conspecific recognition. Immobilization in *Lumbriculus* likely occurs for similar reasons as in populations of daphnids and *Hyalella* discussed above, mainly organisms suffer from a depletion of energy reserves trying to avoid or adapt to contaminants or the organisms' internal functioning is directly altered by the contaminant leading to the inability to move. The benefits of lowering activity levels would have to outweigh the costs of immobility and are likely to allow for energy to be budgeted for other activities more necessary for the organism to survive (Wicklum *et al.*, 1997). Similar increases in immobility have been seen in *Lumbriculus* exposed to varying concentrations of copper (O'Gara *et al.*, 2004).

iv) Body Length

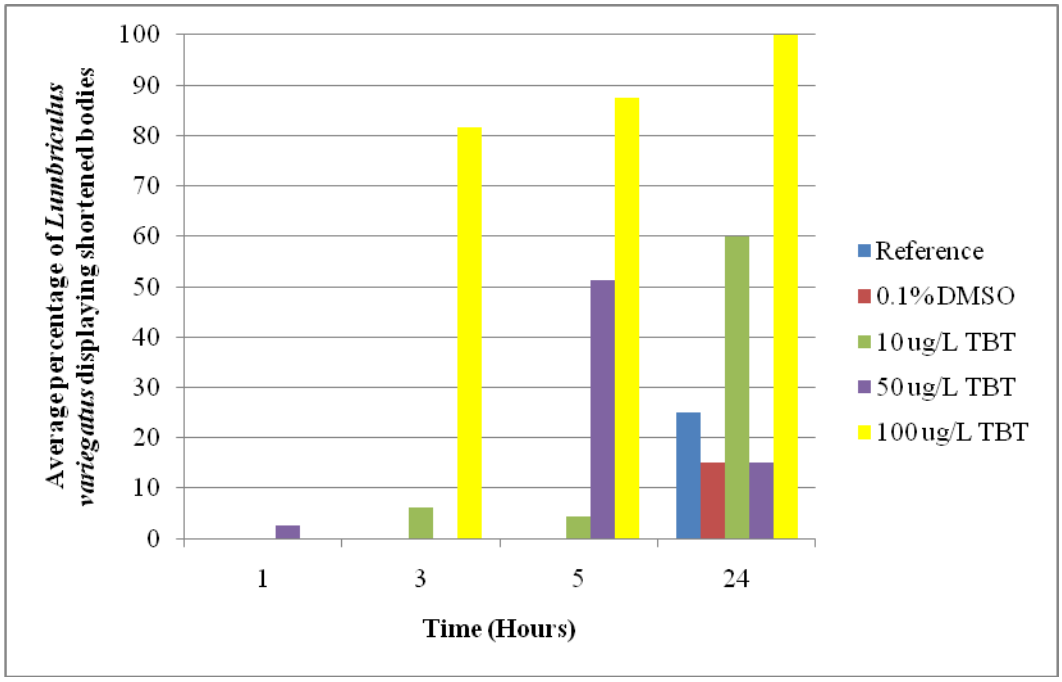

Figure 46: Average percentage of *Lumbriculus variegatus* displaying shortened bodies after exposure to three concentrations of TBT over a 24 hour test period.

No significant changes in body length were seen when comparing the reference and 0.1% DMSO treatments ($p = 0.563$) and the reference and 10 µg/L TBT treatments ($p = 0.108$). A significant difference was seen when comparing the 50 µg/L TBT treatments ($p = 0.039$) and the 100 µg/L TBT treatments ($p = 0.001$) to the reference over the 24 hour observation period. The DMSO treatments and concentrations of TBT $\leq$ 10 µg/L caused no significant changes in organism body length, with the majority of worms maintaining an elongated body length for the duration of the experiment (Figure 46). In the two highest concentrations a significant increase in the organisms showing shortened bodies was seen, but the pattern of response was independent of time. A concentration dependent response was seen when comparing the 50 µg/L and the 100 µg/L treatments, with a greater number of organisms in the 100 µg/L treatments displaying shortened bodies than in the 50 µg/L treatments.

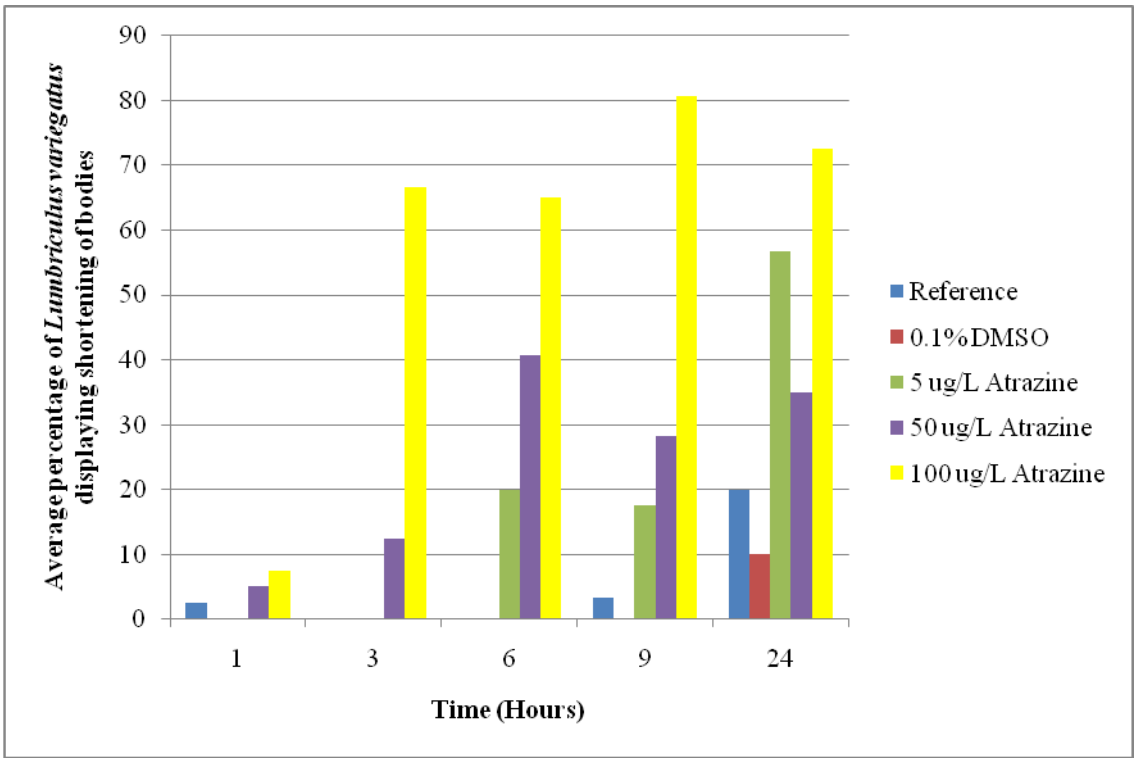

Figure 47: Average percentage of organisms displaying shortened bodies after exposure to three concentrations of atrazine over a 24 hour test period.

No significant changes in body length were seen when comparing the reference and 0.1% DMSO treatments ($p = 0.124$) and the 5 µg/L atrazine treatments ($p = 0.201$). A significant

increase in the number of the organisms with shortened bodies was seen in the 50 µg/L atrazine treatments ($p = 0.004$), and the 100 µg/L atrazine treatments ($p = 0.001$) when compared to the reference treatments over the 24 hour trial period. The DMSO treatments and concentrations of atrazine ≤ 5 µg/L caused no significant changes in organism body length, with the majority of worms maintaining an elongated body length for the duration of the experiment (Figure 47). A concentration dependent response, independent of time, was seen when comparing the 50 µg/L and 100 µg/L atrazine treatments.

A significant increase in the average number of the organisms displaying shortened bodies was seen in the 50 µg/L and 100 µg/L atrazine and TBT treatments. This suggests that this parameter may be useful for detecting high levels of both contaminants but not low levels. The lack of a consistent time and concentration dependent response to both contaminants indicates that this parameter may not be appropriate to incorporate into a model which can determine accurate concentrations of the contaminants. However, the evaluation of changes in body length should still be included in a multi-species, early-warning biomonitoring technology in order to help detect high contaminant concentrations, such as those which may be seen following a pulse introduction of chemical into the environment.

Body shortening has never been specifically examined for use in a multi-species, early-warning biomonitoring system. Body shortening has been generally observed when the organisms are exposed to sub-lethal concentrations of copper (O'Gara *et al.*, 2004), but the parameter has never been studied specifically. Body shortening in *Lumbriculus* is likely due to a loss of function in the muscles which contract and expand the body during locomotion (Drewes, 1999). The ability to extend the body is closely related to locomotion and organisms with permanently shortened bodies may not be able to move as efficiently as those with extended bodies. Shortening of the body may also help to reduce exposure to contaminated water, as less surface area is exposed for absorption of the contaminant.

v) Body Orientation

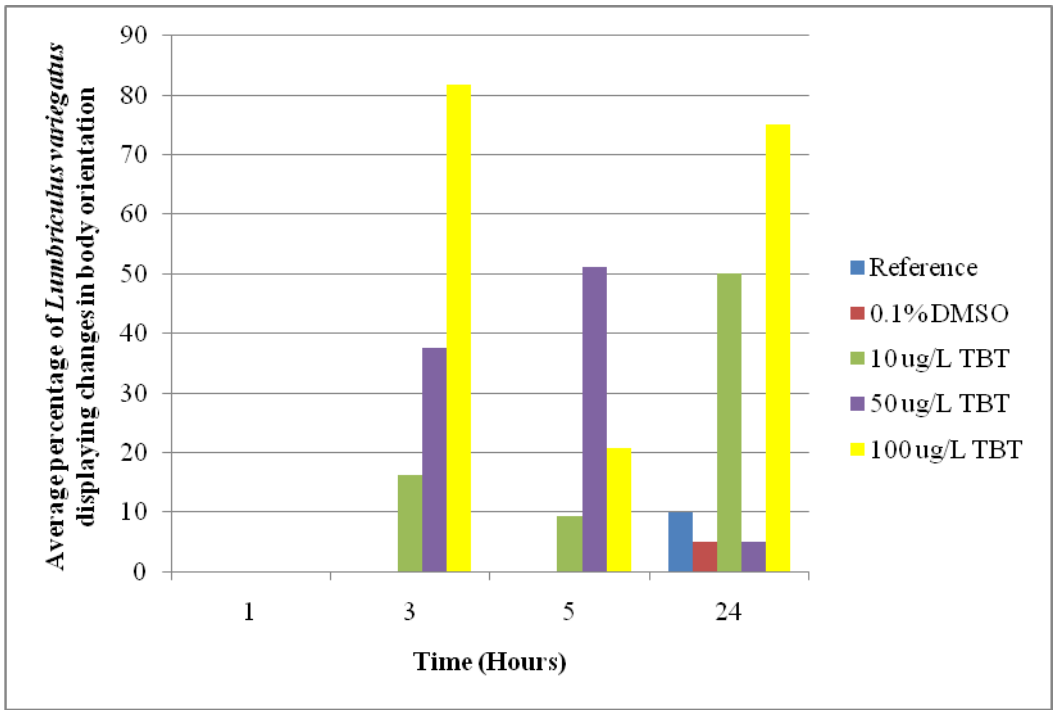

Figure 48: Average percentage of *Lumbriculus variegatus* showing changes in body orientation after exposure to three concentrations of TBT over a 24 hour period.

No significant changes in body orientation were noted in the organisms exposed to 0.1% DMSO treatments ($p = 0.536$) or 10 µg/L TBT treatments ($p = 0.057$) when compared to the reference. Significant changes in body orientation were seen in the 50 µg/L TBT treatments ($p = 0.021$) and the 100 µg/L TBT ($p = 0.002$) treatments when compared to the reference over the 24 hour trial. The 0.1% DMSO and 10 µg/L TBT treatments had no effect on the body orientation of the *Lumbriculus*. A significant increase in the number of the organisms displaying changes in body orientation (bends or kinks in the body, or body coiling) was seen in the 50 µg/L and 100 µg/L TBT treatments (Figure 48). The response pattern was dependent on concentration, as the organisms in the higher treatment displayed a higher average number of affected organisms. No pattern of time dependence was seen.

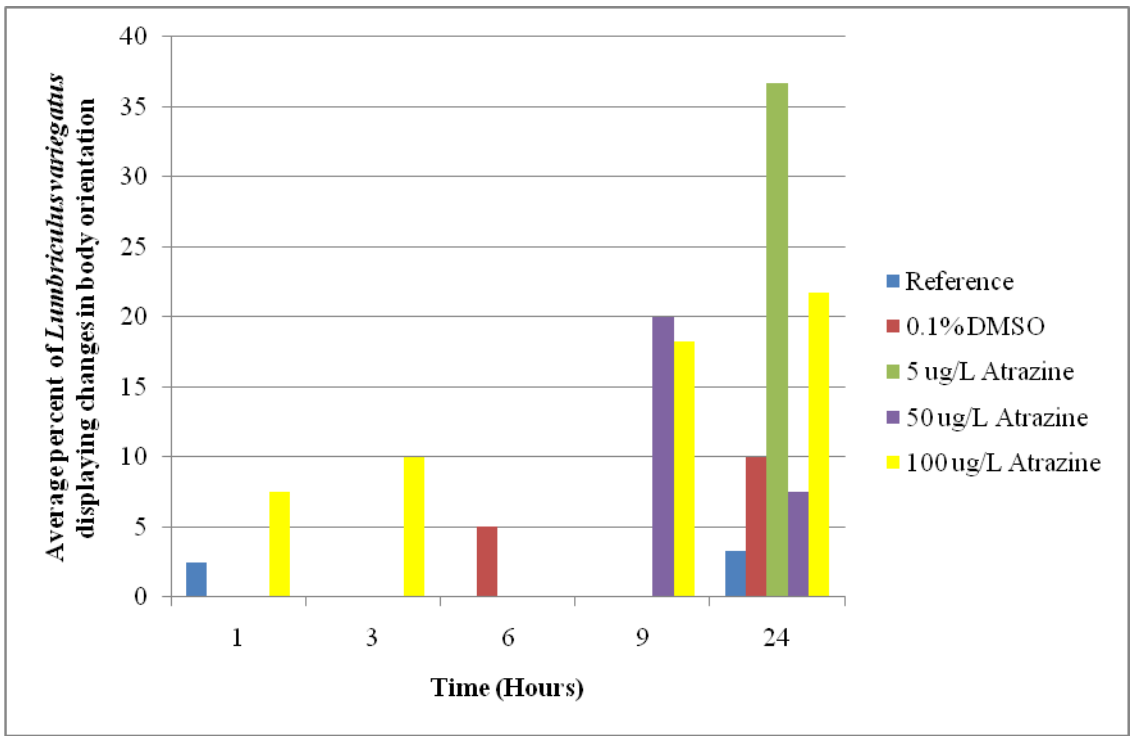

Figure 49: Average percentage of *Lumbriculus* showing changes in body orientation after exposure to three concentrations of atrazine over a 24 hour period.

No significant changes in body orientation were seen when comparing the reference treatments to the 0.1% DMSO treatments ($p = 0.944$), the 5 µg/L atrazine treatments ($p = 0.940$) and the 50 µg/L atrazine treatments ($p = 0.237$). A significant difference in body orientation was seen when comparing the reference and 100 µg/L atrazine treatments ($p = 0.048$) over the 24 hour experimental period. The DMSO treatments and concentrations of TBT ≤ 50 µg/L had no effect on the body orientation of the organisms. A significant increase in the number of the organisms displaying altered body orientation was seen when the organisms were exposed to the 100 µg/L treatments (Figure 49). No pattern of time or concentration dependence was seen.

In the TBT treatments only the two highest concentrations displayed alterations in body orientation, while the only the highest concentration of atrazine showed an effect. This indicates that this parameter might only be useful for detecting higher concentrations of the contaminant, and not lower levels. No pattern of time or concentration dependent change was seen for either contaminant, indicating that as this parameter may not be useful to incorporate into a model designed to determine precise concentrations of the toxins. The parameter may be useful for discriminating between the two chemicals and detecting if they are generally present, but not for

figuring out exact contaminant levels. Measurements of body orientation should therefore be incorporated into a multi-species, early-warning biomonitoring technology, but the importance of the parameter should be considered less than other more consistent responses.

Changes in body orientation have not been evaluated specifically for use in a multi-species, early-warning biomonitoring technology in past studies. Changes in body orientation and body coiling have been observed in response to increased copper concentrations (O'Gara *et al.*, 2004), but no studies have been performed to determine if this is an appropriate measure to examine in sub-acute studies. Changes in body orientation would likely have an impact on an organism's ability to perform locomotion and may result in increased likelihood of predation.

vi) Group Movement

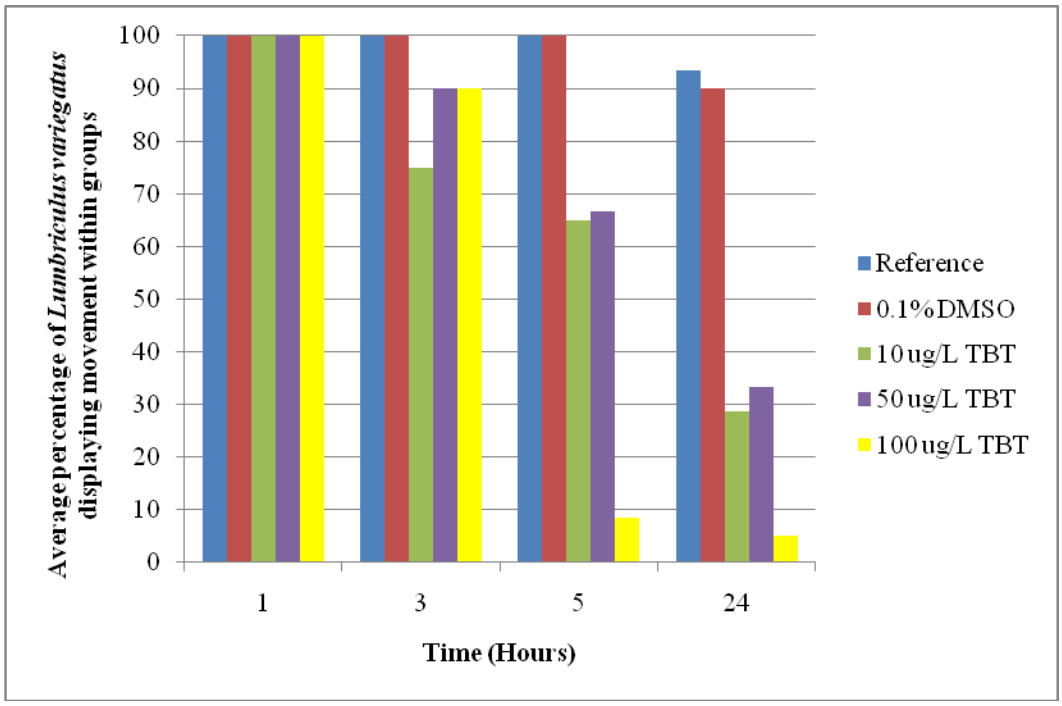

Figure 50: Average percentage of *Lumbriculus variegatus* displaying movement within groups after exposure to three concentrations of TBT over a 24 hour exposure period.

No significant differences in group movement were noted when comparing the reference and 0.1% DMSO treatments ($p = 0.884$), the reference and the 10 µg/L treatments ($p = 0.075$) and the reference, and the 50 µg/L treatments ($p = 0.058$). Significant differences in group movement were seen in when comparing the reference and 100 µg/L treatments ($p = 0.005$). No

significant changes in group movement were seen in concentrations of TBT ≤ 50 µg/L , while the organisms in the 100 µg/L TBT treatments showed a time dependent decrease in group movement (Figure 50).

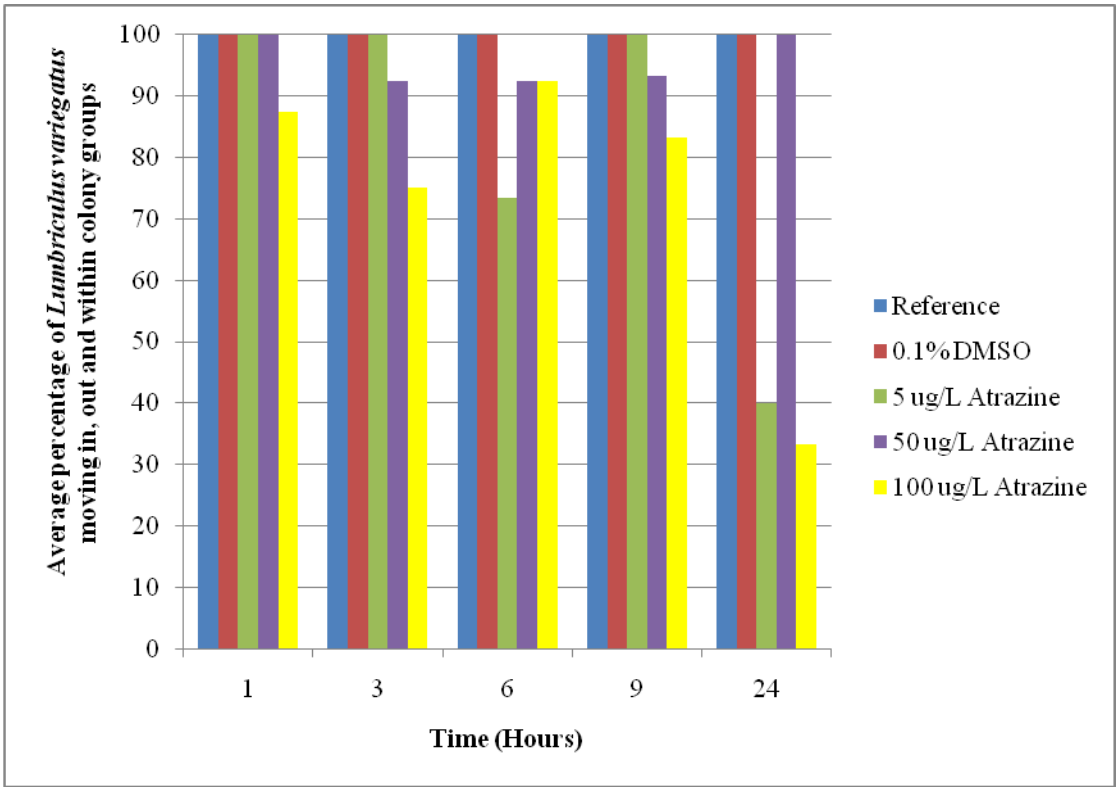

Figure 51: Average percentage of *Lumbriculus variegatus* displaying movement within groups after exposure to three concentrations of atrazine over a 24 hour exposure period.

No significant differences in group movement were noted when comparing the reference and the 0.1% DMSO treatments (p = 1.000) over the 24 hour trial period. Significant differences in group movement were seen in the 5 µg/L atrazine treatments (p = 0.025), the 50 µg/L treatments (p = 0.006), and the 100 µg/L treatments (p = 0.003) when compared to the reference treatments. All three treatments of atrazine affected the group movement of the *Lumbriculus* in a generally time and concentration dependent manner (Figure 51). Overall, a decrease in group movement was seen in all three treatments of the contaminant.

All three treatments of the atrazine affected group movement in the *Lumbriculus*; however, only the highest concentration of TBT had a significant effect on the parameter. This suggests that the parameter of group movement is more sensitive to atrazine contamination and

that it is a good parameter to use in a model to help discriminate between the two classes of contaminants. As a time and concentration dependent response was seen to the contaminants, this parameter should be useful for incorporation into a model to predict accurate concentrations of atrazine and TBT in a water sample. The parameter of group movement should therefore be evaluated in a multi-species, early-warning biomonitoring system.

Movement within colonies is of significance for a variety of reasons. Movement in, out and within the colony is indicative of the motility of the organisms overall and indicates that the organisms have not been immobilized and made unable to perform normal biological functions. Secondly, movement within the colony is related to respiration in the organisms. Worms extend their tail ends from the colony and allow them to sway back and forth and absorb oxygen from the water column (Gerhardt, 2009). Decreases in movement within the colony could therefore be indicative of decreased respiratory activity. Decreases in colony movement have been observed in the related worm *Tubifex tubifex* when exposed to various heavy metals and pesticides (Gerhardt, 2009).

Summary of Lumbriculus variegatus *Motility Bioassay*

The results of the *Lumbriculus variegatus* motility bioassay revealed that some movement behaviour parameters are more sensitive to chemical contaminants, specifically TBT and atrazine, than others and should therefore be evaluated in a multi-organism, early-warning biomonitoring technology. Since some parameters are more sensitive than others to the given contaminants, changes in these parameters should be considered more important stress indicators than other changes in behaviour and should be given higher priority when creating a model to detect types and concentrations of contaminants.

The most useful parameter for incorporation into a model to detect specific concentrations of contaminants appeared to be locomotion style. Changes in movement style were time and concentration dependent, had a rapid rate of onset, and affected a large proportion of the organisms in both contaminants. The second most important parameter appeared to be immobilization of organisms. All concentrations of the two contaminants increased organism immobilization. A time and concentration dependent increase in immobilization was seen in the TBT, but not the atrazine and discrimination between the two chemicals would likely be

possible. Increases in immobilization were less rapid than changes in locomotion style and affected fewer organisms in general. The third parameter which should be considered is group movement. All concentrations of atrazine and the highest concentration of TBT produced significant changes in group movement that were time and concentration dependent. The parameter appeared to be more sensitive to atrazine, allowing for discrimination between the two compounds but making the parameter less useful for detecting low concentrations of TBT. The next most important parameter appeared to be body length, which had no concentration or time dependent patterns of response and showed no changes to the lowest concentrations of the two contaminants. The parameter could only be used to judge if higher concentrations of TBT or atrazine were present, not an exact concentrations. The parameter appeared to be slightly more sensitive than body orientation. Body orientation would be the next most important parameter to evaluate, as again no concentration or time dependent patterns of response were seen. In the TBT treatments, the lowest concentration had no effect on body orientation and in the atrazine treatments, only the highest concentration had an effect, indicating that the parameter is not highly sensitive to either contaminant. Evaluation of this characteristic would only tell plant operators if a contaminant was present, not what concentration was in the water sample. The final characteristic studied, changes in vessel position, should not be included in a multi-species, early-warning biomonitoring technology. TBT had no effect on this parameter, and only the highest concentration of atrazine affected where organisms were located in the vessel, indicating the parameter is not sensitive. Overall, very few of the organisms were affected and the parameter has no real ecological significance, and therefore should not be evaluated.

Overall, the ranking of importance of the 6 parameters is: locomotion style > immobilization > group movement > body length > body orientation > position in vessel. The first 5 parameters should be included for evaluation in a multi-species, early-warning biomonitoring technology, whereas vessel position should not.

3.3.4 *Lumbriculus variegatus* 72 Hour Mortaliy TBT

There were no significant differences from the reference in the 0.1% DMSO ($p = 1.000$) or 10 µg/L TBT ($p = 0.371$) in terms of the number of *Lumbriculus* deaths which took place over 72 hours. Compared to the reference individual Kruskal-Wallis analysis showed that there

was a significant increase in the number of deaths which occurred in the 50 µg/L TBT treatments ($p = 0.011$) and the 100 µg/L TBT treatments ($p = 0.014$).

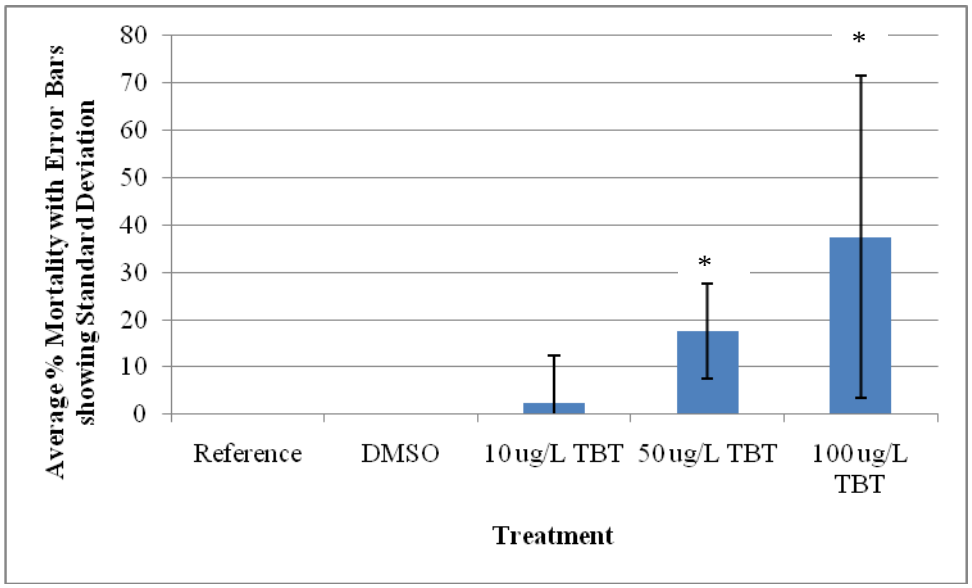

Figure 52: Average percent of *Lumbriculus variegatus* which died after 72 hours of exposure to varying concentrations of TBT. Error bars indicate standard deviation and any treatments which have a significantly different average mortality than the reference ($p < 0.05$) are indicated with a *.

3.4 Mode of Action for Toxicity of TBT

Research has been conducted on the long-term impacts of TBT on invertebrate movement in the past. Results of past bioassays with daphnids revealed that TBT exposure caused a slow shut-down of many biological functions in the organisms rather than disrupting a single organ system and that multiple cell types take up the contaminant, leading to a decline in biological function and making it difficult to determine a primary mode of toxicity (Schmidt *et al.*, 2005). Decreases in movement parameters in all three study organisms are likely related to the way in which TBT impairs muscle function. TBT impairs cellular metabolism by preventing the breakdown of ATP to ADP, thus causing muscles to be deficient in energy to perform locomotory activities (Alzieu, 1998). TBT has also been shown to increase intracellular calcium, decrease ATP synthesis and breakdown by ATPase and decrease phenoloxidase activity, leading to a lack of available energy for muscle activity and a decrease in muscle contraction ability (Schmidt *et al.*, 2005).

In daphnids, since muscle contraction is responsible for the movement of the secondary antennae, the use of these may be impaired by TBT. Impairment of secondary antennae is linked to changes in swimming style, swimming height, and immobilization. It is likely that spinning is an escape response early in exposure or to exposure to low concentrations of TBT, and that inhibition of secondary antennae use due to effects at the cellular level and the associated changes in swimming height, swimming style and immobilization will occur with extended exposure or with exposure to higher concentrations, as seen in the above results.

TBT likely affects *Hyalella azteca* by a similar impairment of muscle function, which likely explains why a decrease in swimming events and substrate crawling and an increase in organism immobilization was seen with exposure to TBT. The increased severity of reactions seen in higher concentrations of TBT is likely related to the fact that increasing concentrations of metal contaminants in the water column, such as cadmium and tin, results in higher internal accumulation within tissues, and thus a greater impact on organisms exposed to the higher environmental concentrations (Borgmann *et al.,* 1996). Burrowing was likely a behavioural response which helped to reduce exposure to TBT. As *Hyalella* accumulate most contaminants from the water column (Wang *et al.,* 2004) it is likely that escape to clean sediments was an attempt to reduce the impacts of the contaminant.

TBT likely also impairs *Lumbriculus* muscle function in a similar manner and leads to an overall decrease in movement parameters. *Lumbriculus* move through successive contractions of longitudinal and circular muscles which travel down the length of the body of the worm and propel it forward in the characteristic crawling motion (O'Gara *et al.,* 2004). If longitudinal and circular muscles were impaired by TBT, changes in movement would likely occur, as seen the TBT motility bioassay. Changes in movement style and the ability to perform locomotion are also closely related to colony movement and immobilization in organisms, and as TBT impairs muscle function it is likely these parameters would also be affected, as seen in the bioassay. Finally, if muscles are unable to extend and contract, this may explain why organisms exposed to TBT display shortened body lengths.

3.5 Mode of Action for Toxicity of Atrazine

No conclusive determination of the cause of sub-lethal behavioural changes in response to atrazine in non-target invertebrates has been reached (Wan *et al.,* 2006). However, several physiological changes which could potentially be related to changes in movement behaviour have been seen in a number of aquatic organisms.

Several studies have suggested a link between atrazine exposure and depression of the enzyme acetylcholinesterase (AChE) (Saglio and Trijasse, 1998; Key *et al.,* 2003; Forget *et al.,* 2003). AChE is an important enzyme found in a wide range of species which functions as a neurotransmitter, especially at neuromuscular junctions (Donkin *et al.,* 1997; Forget *et al.,* 2003). AChE is responsible for the breakdown of acetylcholine at neuromuscular junctions and the inhibition of the enzyme will result in a build-up of acetylcholine and a stimulation of nerve and muscle fibres (Forget *et al.,* 2003). If this stimulation is allowed to continue eventually muscle tetany and paralysis will occur, followed by the death of the organism affected (Forget *et al.,* 2003). The ACh/AChE regulatory system is essential for normal muscle function and impairment of this system may result in abnormal movement behaviour and immobility. Key and colleagues (2003) noted a decrease in AChE activity in grass shrimp found in Florida canals where the dominant contaminant was atrazine found in concentrations of between 15.4 and 29.4 ng/L. Another field study examining the relationship between atrazine contamination and AChE function found that AChE levels of the copepod *Tigriopus brevicornis* were depressed by 70-80% following the spring runoff in agricultural areas of France compared to uncontaminated reference areas (Forget *et al.,* 2003). The study concluded that there was a significant linear relationship between decreased presence of AChE and increased atrazine concentrations (7-148 ng/L) in water (Forget *et al.,* 2003). Both studies emphasized that it is not clear if atrazine alone affects AChE activity or if it acts synergistically with other contaminants which may be present in the water and that more research is needed in this area (Forget *et al.,* 2003; Key *et al.,* 2003). If AChE activity is indeed depressed by atrazine, this would explain the movement abnormalities and eventual immobilization of *Daphnia magna, Hyalella azteca,* and *Lumbriculus variegatus* in the atrazine bioassays.

Several other cellular level changes have also been seen in aquatic organisms in response to atrazine contamination. A major mode of toxicity at a cellular level appears to be oxidative

stress, wherein an increase in harmful reactive oxygen species (ROS) is seen after exposure to atrazine (Liu *et al.*, 2006; Sanchez *et al.*, 2008; Song *et al.*, 2009). The accumulation of ROS can cause damage proteins and lipids (Song *et al.*, 2009) making them unavailable for use as an energy source. Long-term accumulation of ROS in animal tissue can result in DNA damage including strand breaks, DNA lesions, removal of nucleotides and modification of nucleotide bases (Song *et al.*, 2009). It is likely that this type of damage to DNA is the mechanism by which atrazine increases the risk of tumours and cancer in a number of organisms (Song *et al.*, 2009). Atrazine has also been linked to mitochondrial dysfunction and may contribute to reduced availability of ATP for cellular function (Liu *et al.*, 2005; Sanchez *et al.*, 2009). The herbicide has also been shown to affect ion regulation in several species of fish (Waring and Moore, 2004). At concentrations of atrazine exceeding 6.5 µg/L increased plasma cortisol, increased plasma osmolalities, and elevated concentrations of sodium, potassium, and chloride were seen in Atlantic salmon (*Salma salar*) after 7 days of exposure (Waring and Moore, 2004). Stress brought on by increased internal ROS, mitochondrial dysfunction, and ion regulation abnormalities may have indirectly contributed to the overall depression of movement behaviour seen in all three test organisms during the atrazine bioassays.

3.6 *Daphnia magna* Respiration

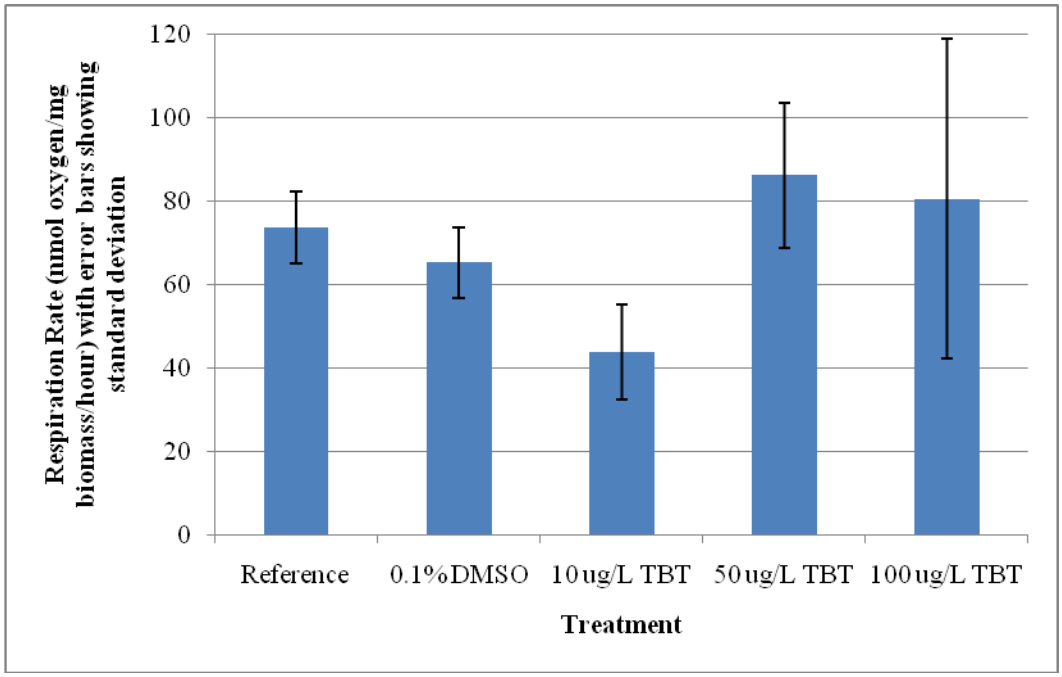

Figure 53: Respiration rates of 8 adult *Daphnia magna* exposed to varying concentrations of TBT over a 24-hour period, expressed in relation to total average body weight of the organisms. Significant differences ($p \leq 0.05$) in treatments from the reference are denoted by *.

No significant differences were observed between the reference and the 0.1% DMSO ($t = 1.22$, $p = 0.05$), the 10 µg/L ($t = 3.64$, $p = 0.05$), the 50 µg/L ($t = 1.11$, $p = 0.05$) or the 100 µg/L ($t = .695$, $p = 0.05$) TBT treatments (Figure 53). This indicates that (1) concentrations of TBT ≤ 100 µg/L do not elicit a change in respiration rate in *Daphnia* or that (2) 8 organisms do not consume enough oxygen to produce a noticeable change using Winkler titrations. For future experiments, modifications of increasing the concentration of TBT or increasing the number of organisms used should be made.

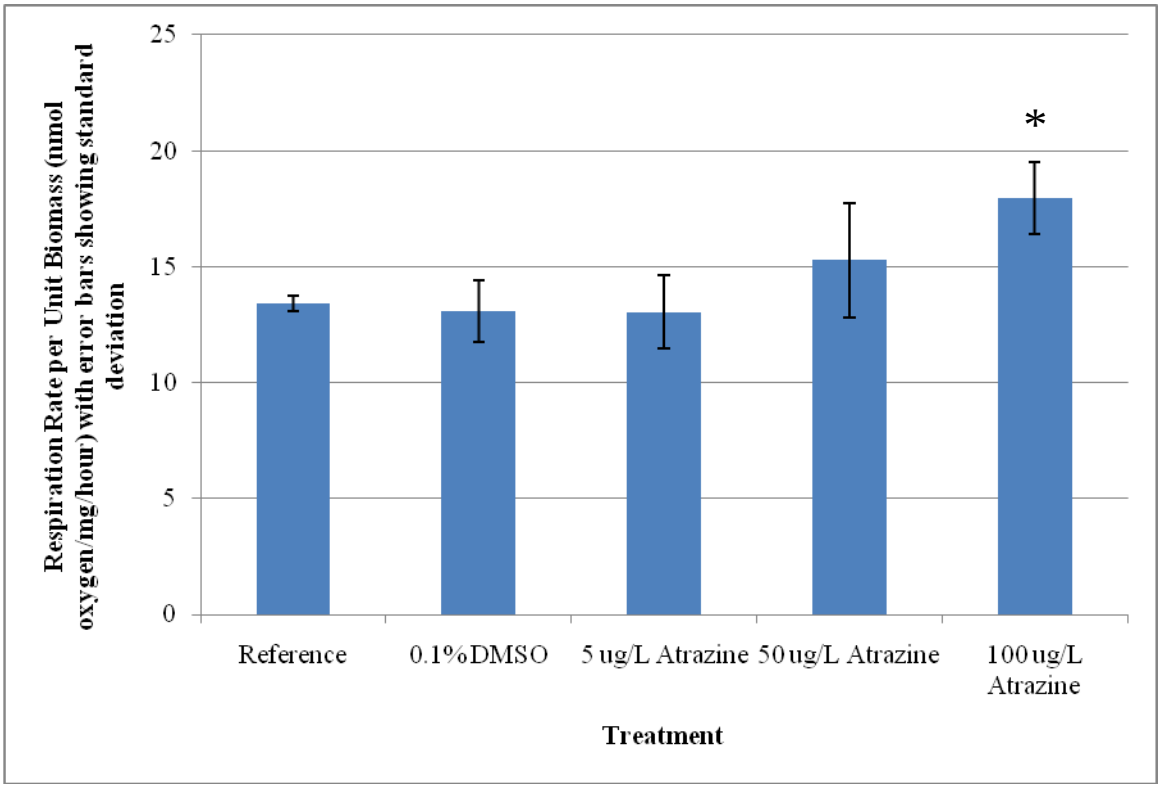

Figure 54: Respiration rates of 15 adult *Daphnia magna* exposed to varying concentrations of atrazine over a 2-hour period, expressed in relation to total average body weight of the organisms. Significant differences ($p \leq 0.05$) in treatments from the reference are denoted by *.

No significant changes in respiration rates were seen between the reference and the 0.1% DMSO treatment ($p = 0.999$), the reference and the 5 µg/L atrazine treatment ($p = 0.998$), and the 50 µg/L atrazine treatment ($p = 0.630$) (Figure 54). A significant increase in respiration was seen in the 100 µg/L treatment when compared to the reference ($p = 0.039$). This indicates that concentrations of atrazine ≤ 50 µg/L will not affect the respiration rates of a population of daphnids, but that at concentrations over 100 µg/L respiration rates will increase.

In theory, under low to moderate toxic stress the metabolic rate and demand for oxygen should be increased. Under stress, energy demanding cellular repair mechanisms will be activated causing an increase in oxygen consumption needed to perform these functions (Knops *et al.*, 2001). This response was seen in the *Daphnia magna* when exposed to atrazine at concentrations ≥ 100 µg/L , where respiration rates significantly increased following 2 hours of exposure to the contaminant. This suggests that changes in respiration of *Daphnia magna* are a

sensitive parameter for detecting higher concentrations of atrazine, with responses which are rapidly seen at environmentally relevant concentrations. Similar increases in respiration rates of daphnids have been seen in response to other chemical contaminants commonly found in aquatic systems. Sigmon (1979) noted a significant increase in oxygen consumption in daphnids exposed to 1 and 3 ppm of the pesticides 2,4-D and 2,4,5-T over a period of 9 hours exposure. A significant increase in respiration in *Daphnia magna* has also been seen in response to concentrations of cadmium greater than 5 ppb and concentrations of 3,4-dichloroaniline (DCA) greater than 50 ppb after 48 hours of exposure (Barber *et al.,* 1990). Other species of aquatic organisms have also displayed an increase in overall respiration in response to contamination by atrazine. Fathead minnow (*Pimephales promelas*) larvae exposed to 150 µg/L atrazine for 24 hours experienced a significant increase in oxygen consumption compared to control conditions (Sanchez *et al.,* 2008). This further supports the idea that changes in respiration of aquatic organisms may be a sensitive parameter to use to detect atrazine contamination in drinking water supplies.

No change in the respiration rates of *Daphnia magna* were seen in response to the addition of varying concentrations of TBT. Changes in behavioural parameters of the *Daphnia* were noted, including an increase in spinning behaviour and a decrease in movement up and down through the water column. It is likely that decreases in certain behaviours compensate for increases in other behaviours, therefore causing overall energy usage to remain the same. Similar responses to various contaminants have been seen in past studies using various species of *Daphnia.* No change in oxygen consumption was seen in *Daphnia pulex* exposed to naphthalene, phenanthrene, fuel oil extract, and coal-tar creosote for 24 hours at concentrations representing 48 hour LC_{20} and LC_{30} values for the substances (Geiger and Buikema, 1981). Another study found that concentrations of 17-52 µg/L CTAB, 4.1-12 µg/L copper and 0.74-8.4 µg/L cadmium have no effect on the respiration of *Daphnia magna* over a 3 day period of exposure (Knops *et al.,* 2001).

Several possible reasons have been proposed for the lack of change seen in respiration rates when organisms are exposed to contaminants. Firstly, the metabolic costs of increased repair processes are masked by other effects of the contaminant. For example, decreased energy spent on movement, food acquisition and growth in response to toxicants allows for more energy

to be spent on repair and adaptation processes (Knops *et al.,* 2001). Overall, there is no change in energy use, so no change in oxygen consumption would be seen. A second reason for respiration rates to remain the same could be that cellular repair and adaptation energy demands are very low compared to total metabolic demands of the organisms, and that they would not require an increase in oxygen consumption in order to occur (Knops *et al.,* 2001). A final reason that respiration rates did not change could be that there was no additional metabolic costs associated with chemical stress, and therefore no need for oxygen consumption to be increased (Knops *et al.,* 2001).

It is difficult to determine a single cause for a lack of change in oxygen consumption. In the TBT bioassay, it seems most likely that changes in respiration rates may be masked by decreases in other activities which require energy. TBT caused a severe decrease in a number of parameters associated with locomotion, as seen when examining the results of the *Daphnia* swimming behaviour bioassay. As less locomotory activities are performed, less energy will be allocated to these and more will be allocated to repair processes. The TBT treatments will therefore appear to have a similar rate of oxygen consumption as the reference and DMSO treatments where organisms are performing normal swimming.

Both the TBT and atrazine bioassays demonstrated that it is possible to monitor the oxygen consumption of daphnids over time, indicating that this could potentially be monitored in a multi-species, early-warning biomonitoring technology. Results of the respiration bioassays suggest that changes in oxygen consumption occur in *Daphnia magna* in response to elevated concentrations of atrazine, but not TBT. Therefore, the use of this parameter would be more suitable for detecting atrazine and similar halogenated pesticides than TBT and related organotin compounds.

3.7 *Hyalella azteca* Respiration

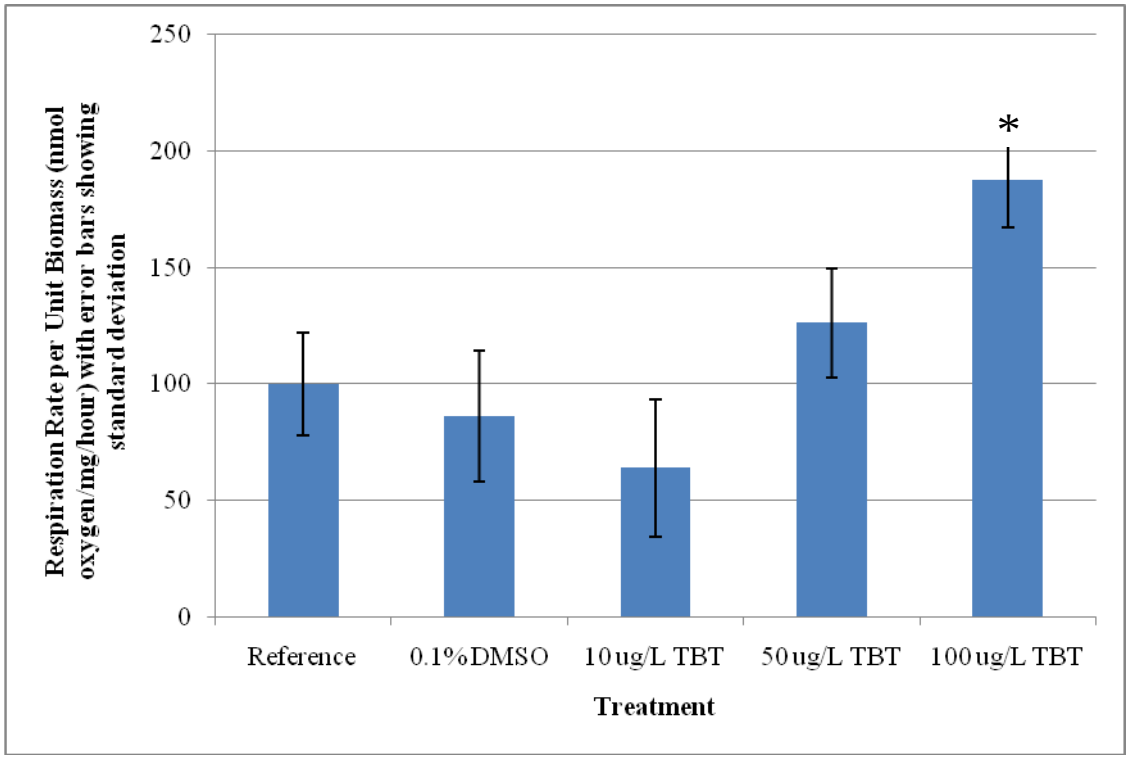

Figure 55: Respiration rates of 3 adult *Hyalella azteca* exposed to varying concentrations of TBT over a 10-hour period, expressed in relation to total average body weight of the organisms. Significant differences ($p \leq 0.05$) in treatments from the reference are denoted by *.

No significant differences were observed between the reference and the 0.1% DMSO ($t = 0.68$, $p = 0.05$), the 10 µg/L ($t = 1.71$, $p = 0.05$), or the 50 µg/L ($t = 1.40$, $p = 0.05$) TBT treatments (Figure 55). A significant difference in the respiration rate of the reference and 100 µg/L organisms was found ($t = 5.006$, $p = 0.05$). A significant increase in respiration is seen in organisms exposed to 100 µg/L TBT. Concentrations ≤ 50 µg/L TBT do not produce a significant increase or decrease in the respiration rate of *Hyalella azteca.*

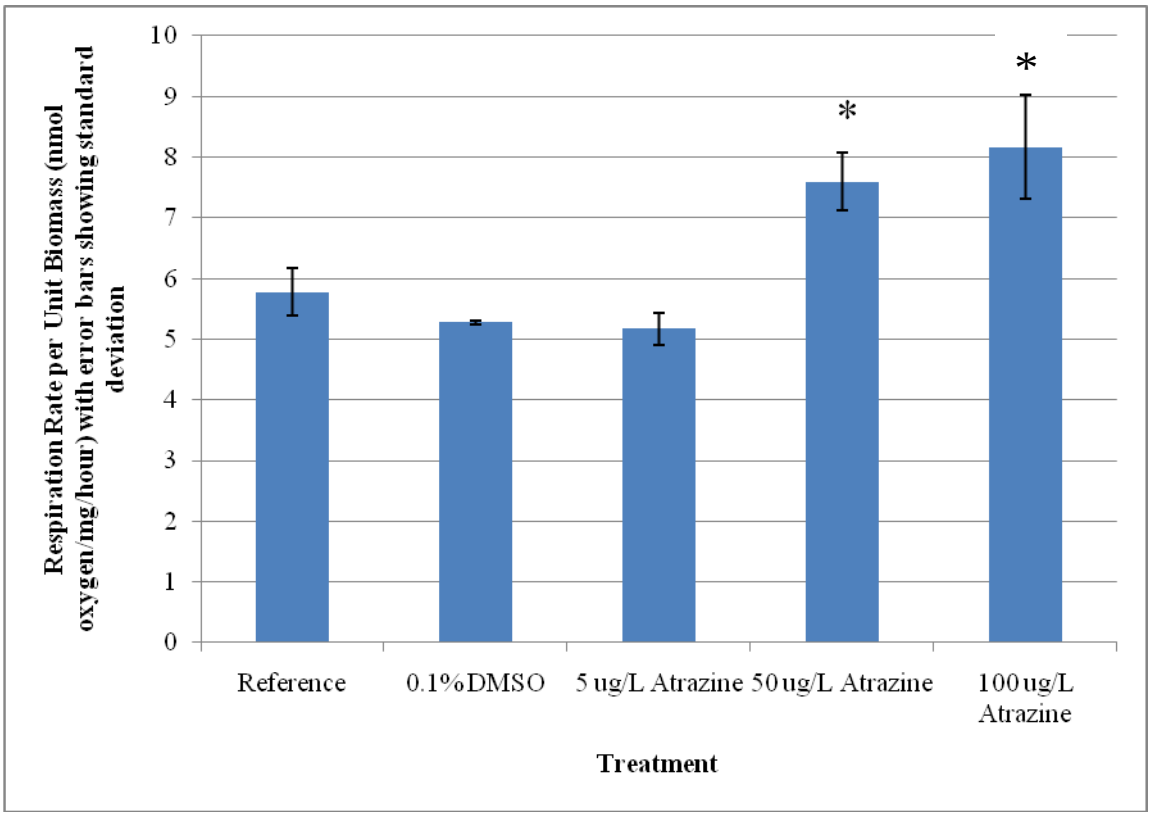

Figure 56: Respiration rates of 8 adult *Hyalella azteca* exposed to varying concentrations of atrazine over a 4-hour period, expressed in relation to total average body weight of the organisms. Significant differences ($p \leq 0.05$) in treatments from the reference are denoted by *.

No significant changes in respiration rates were seen between the reference and the 0.1% DMSO treatment ($p = 0.723$) and the reference and the 5 µg/L atrazine treatment ($p = 0.578$) (Figure 56). A significant increase in respiration rate was seen in both the 50 µg/L atrazine treatment ($p = 0.007$) and the 100 µg/L atrazine treatment ($p = 0.001$) when compared to the reference treatment. This indicates that concentrations of atrazine ≥ 50 µg/L will cause a significant increase in the respiration rate of a population of *Hyalella azteca* following 4 hours of exposure to the contaminant.

Results for both the TBT and atrazine respiration bioassays were consistent with results seen in experiments conducted by Oberlin and Bunn (1997), who found that *Hyalella montezuma* increase their respiration and metabolic rates in response to stress from increased habitat temperatures. This indicates that concentrations of TBT ≥100 µg/L (up to a point) and concentrations of atrazine ≥ 50 µg/L (to an upper limit) may increase cellular repair processes within the organism, and thus increase the oxygen demand and consumption by the *Hyalella*. In

the 10 and 50 µg/L treatments of TBT and the 5 µg/L treatment of atrazine the effects of the contaminants on respiration may again be hidden by decreases in energy demands for locomotion behaviour, causing the TBT treatments to appear to have similar respiration rates as the reference treatments. In the 100 µg/L TBT treatment and the 50 and 100 µg/L atrazine treatments it is likely that metabolic demands for cellular repair exceeded the savings in energy from reduced locomotion activity, thus requiring increased oxygen uptake.

Evaluating changes in respiration rates may be a useful parameter for detecting TBT concentrations higher than 100 µg/L and atrazine concentrations greater than 50 µg/L, which could potentially be seen after a pulse introduction of the contaminant into the environment. Responses were seen to lower concentrations of atrazine in a shorter period of time, indicating that changes in oxygen consumption in *Hyalella* may be more sensitive to atrazine and herbicides of similar structure than to TBT and related compounds. Further bioassays with other classes of contaminants are required, but results found in this set of experiments indicate that *Hyalella* respiration is a good characteristic to monitor in a multi-species, early-warning biomonitoring technology due to the rapid response seen to high concentrations of TBT and atrazine.

3.8 *Lumbriculus variegatus* Respiration

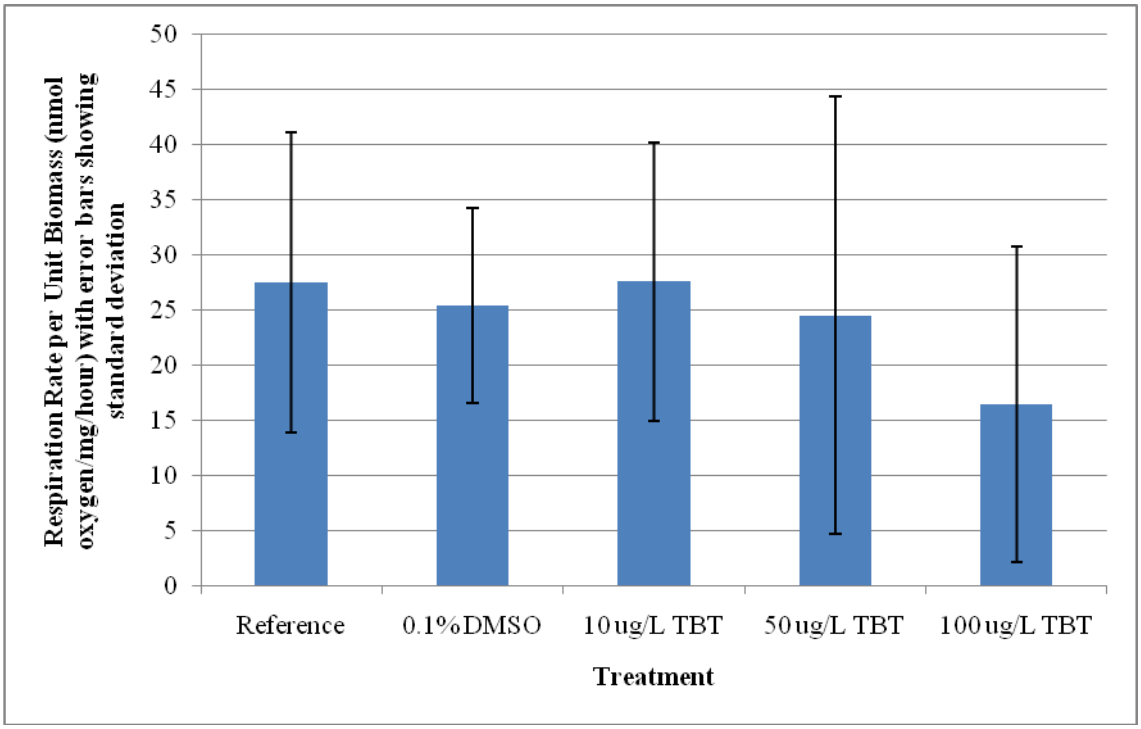

Figure 57: Respiration rates of 10 adult *Lumbriculus variegatus* exposed to varying concentrations of TBT over a 24-hour period, expressed in relation to total average body weight of the organisms. Significant differences ($p \leq 0.05$) in treatments from the reference are denoted by *.

No significant differences were observed between the reference and the 0.1% DMSO (t value = 0.22, p = 0.05), the 10 µg/L (t value = 0.009, p = 0.05), the 50 µg/L (t value = 0.21, p = 0.05) or the 100 µg/L (t value = 0.96, p = 0.05) treatments (Figure 57). This indicates either (1) concentrations ≤ 100 µg/L TBT do not produce a significant increase or decrease in the respiration rate of *Lumbriculus variegatus* or (2) more organisms need to be used in bioassays in order to detect greater change in DO content over time using Winkler titrations. For future bioassays higher concentrations and more organisms should be used in order to determine if TBT has an effect on the respiration of *Lumbriculus variegatus*.

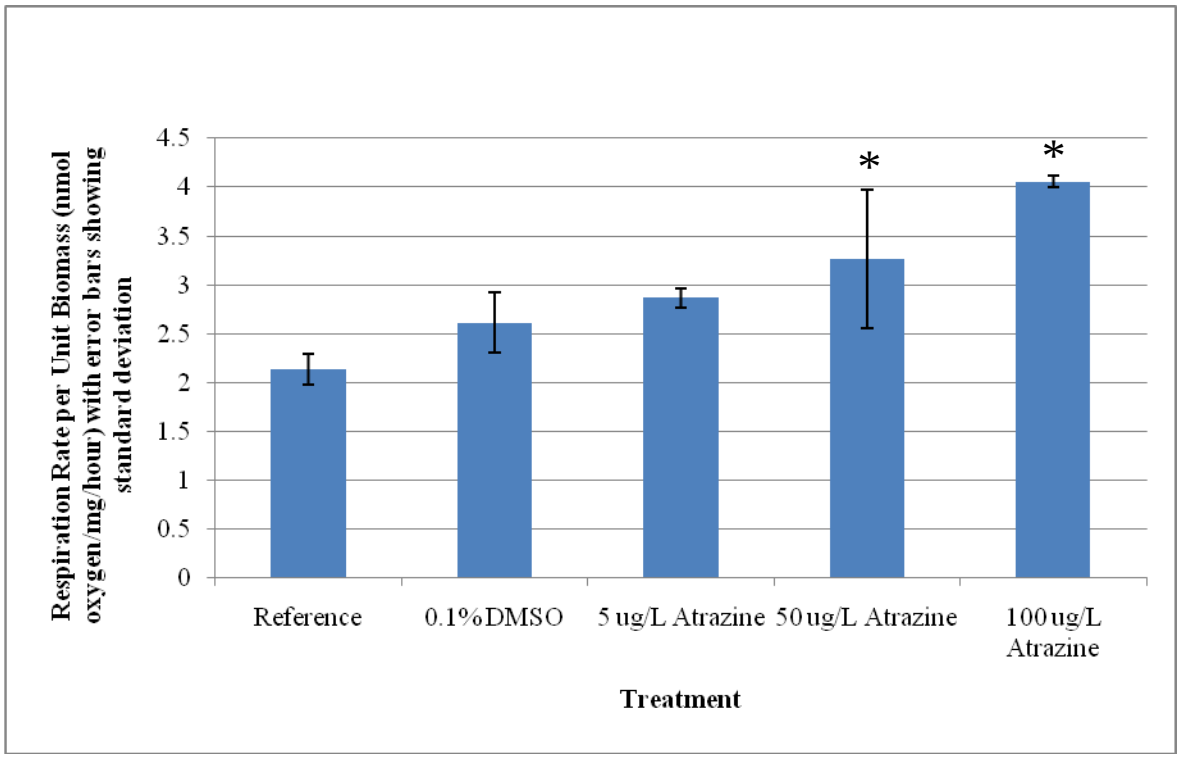

Figure 58: Respiration rates of 20 adult *Lumbriculus variegatus* exposed to varying concentrations of atrazine over a 2-hour period, expressed in relation to total average body weight of the organisms. Significant differences ($p \leq 0.05$) in treatments from the reference are denoted by *.

No significant changes in respiration rates were seen between the reference and the 0.1% DMSO treatment ($p = 0.509$) and the reference and the 5 µg/L atrazine treatment ($p = 0.167$) (Figure 58). A significant increase in respiration rate was seen in both the 50 µg/L atrazine treatment ($p = 0.020$) and the 100 µg/L atrazine treatment ($p = 0.000$) when compared to the reference treatment. This indicates that concentrations of atrazine ≥ 50 µg/L will cause a significant increase in the respiration rate of a population of *Lumbriculus variegatus* following 2 hours of exposure to the contaminant.

No changes in oxygen consumption were seen in the three TBT treatments. Similar to the daphnids discussed above, it is likely that (1) effects of the contaminant on respiration were masked by a decrease in energy demands for other processes, (2) repair mechanisms represent a negligible demand of the total metabolic expenses of the organism or (3) there are no metabolic costs associated with the contaminants (Knops *et al.*, 2001). As a significant reduction in several parameters associated with motility were seen when *Lumbriculus* were exposed to the

experimental concentrations, it seems likely that a reduction in energy demand by these parameters may have masked any increases in respiration in the atrazine treatments which may have occurred.

A significant increase in oxygen consumption was seen in the two highest concentrations of atrazine. Results obtained in the atrazine bioassay are consistent with observations made by Pentinnen and Kukkonen (2000) who found that exposure to sub-lethal concentrations of PCP increased the respiration rates of *Lumbriculus variegatus.* Results indicate that concentrations of atrazine $\geq$ 50 µg/L (up to a point) will increase cellular repair processes within the organism and thus increase the oxygen demand and consumption by the *Lumbriculus*. In the 10 µg/L treatments the effects of TBT on respiration may again be hidden by decreases in energy demands for locomotion behaviour, causing the TBT and atrazine treatments to appear to have similar respiration rates as the reference treatments. In the 50 µg/L and 100 µg/L treatments it is likely that metabolic demands for cellular repair exceeded the savings in energy from reduced locomotion activity, thus requiring increased oxygen uptake. Pentinnen and Kukkoken (2000) also examined internal tissue concentrations of *Lumbriculus* exposed to PCP simultaneously to examining respiration rates. They found that a threshold tissue concentration of PCP was required before a change in respiration occurred (Pentinnen and Kukkoken, 2000). It is likely that a similar threshold exists for TBT and atrazine and that exposure to low concentrations of the two contaminants for 24 hours does not result in an internal concentration high enough to effect respiration.

Measurement of changes in oxygen consumption appears to be a rapid and sensitive parameter to use to evaluate stress in *Lumbriculus variegatus* induced by the presence of atrazine. However, examining changes in respiration does not appear to be a valid way to determine if TBT is present in the aquatic environment as no change in the respiration rate of *Lumbriculus* was seen over 24 hours of exposure to environmentally relevant concentrations. Changes in respiration rates should therefore be incorporated into a rapid automated biomonitoring system in order to help detect atrazine and similar compounds, but should not be relied upon in order to detect TBT and related chemicals.

4.0 Summary and Future Directions

4.1 Summary

Various parameters of movement behaviour in *Daphnia magna* were observed to rapidly and consistently change in response to all concentrations of TBT and atrazine, supporting past research which indicates that these organisms are highly sensitive to contamination and are very useful for biomonitoring using a real-time detection system. Results of this study showed that swimming height, swimming style, immobilization, secondary antennae use, and spinning should be evaluated in a multi-organism, early-warning biomonitoring technology. When considering parameters to incorporate in a model to predict the exact concentrations of a given substance, the parameters should be ranked as listed above, with swimming height being the best indicator of potential stressors and spinning being the least reliable. Body orientation should not be monitored. Results of the respiration bioassay showed that direct oxygen measurements of respiration rates using daphnids is possible; however, not all contaminants may affect oxygen consumption rates as TBT had no significant effect on the oxygen used by the organisms. Respiration rate measurements of daphnids should be included in a multi-organism, early-warning biomonitoring technology, but should be used in conjunction with behavioural monitoring to ensure that all potential contaminants are detected.

Changes in several movement-based parameters were also noted in *Hyalella azteca* exposed to TBT and atrazine, suggesting that these organisms should be included in a multi-species, early-warning biomonitoring system. Changes in immobilization, substrate crawling, body length, burrowing, and grouping should be monitored in a multi-species, early-warning biomonitoring technology, and immobilization considered to be the most important parameter and grouping the least important parameter for incorporation into future modelling. Body orientation and swimming events should not be evaluated in a multi-species, early-warning biomonitoring system. Changes in respiration rates were seen in response to both contaminants, indicating that evaluation of oxygen consumption by *Hyalella* should be evaluated in a multi-species, early-warning biomonitoring technology. However, changes in respiration should again be evaluated at the same time as behaviour, as the behavioural changes seem to have been seen more rapidly and at lower concentrations than the respiration rate changes.

Finally, changes in several locomotory parameters of *Lumbriculus variegatus* were also seen in rapid response to both TBT and atrazine, suggesting that these organisms should be included in a multi-species, early-warning biomonitoring system evaluating behaviour. Locomotion style, immobilization, group movement, body length and body orientation should be monitored for changes in response to chemical contaminants, with importance placed in on parameters in this order when performing modelling to predict classes and concentrations of contaminants. Position in the bioassay vessel or monitoring chamber should not be evaluated as part of a multi-species, early-warning biomonitoring technology. Changes in burrowing behaviour of the *Lumbriculus* should also be evaluated, whereas changes in grouping behaviour should not be included for monitoring in a multi-species, early-warning biomonitoring technology. Results of the respiration bioassay showed that direct oxygen measurements of respiration rates using *Lumbriculus variegatus* is possible; however, not all contaminants may affect oxygen consumption rates, as TBT had no significant effect on the oxygen used by the worms. Changes in respiration must therefore be evaluated simultaneously with behavioural monitoring, to make sure that all concentrations and types of contaminants are detected.

Results of the aforementioned bioassays showed that all three study organisms should be utilized in a multi-species, early-warning biomonitoring technology. Both behaviour and respiration rates should be evaluated in all three organisms; however, it appears that overall behavioural changes are more sensitive to contaminants than respiration changes. All concentrations of TBT and atrazine cause changes in behaviour in the three organisms, whereas mixed results were seen for respiratory responses. Changes in behaviour were also seen more rapidly in general than changes in respiration, indicating that behavioural parameters may be more sensitive and thus more useful for rapid detection of chemicals.

4.2 Future Directions of Research

This thesis research is a small part of a large-scale NSERC-funded project aimed at developing a holistic multi-species, early-warning biomonitoring technology over the next five years. An outline of the project as a whole is provided below (Figure 59), with the work of this thesis highlighted in yellow and presented in relation to the entire project.

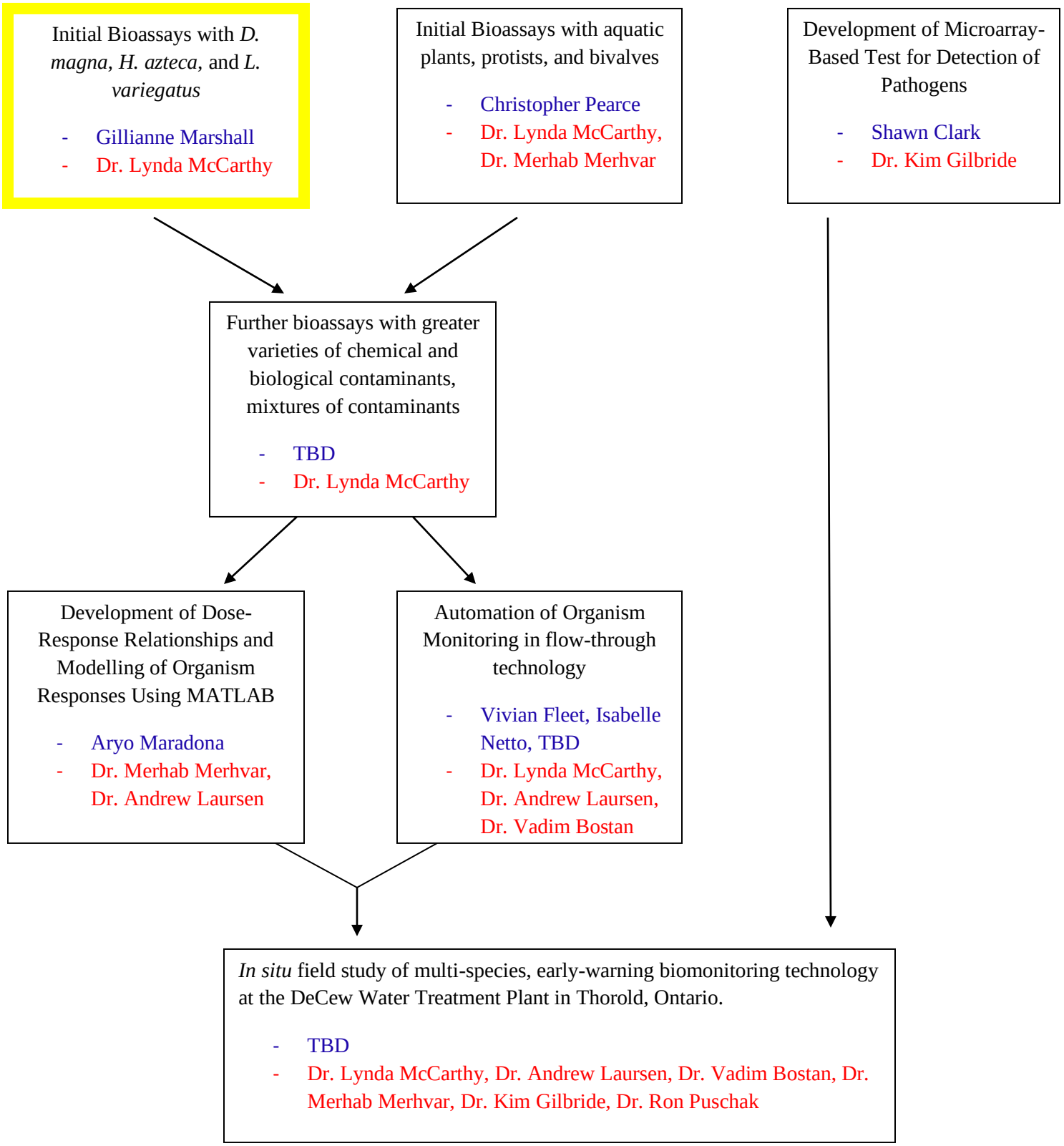

Figure 59: Outline of the steps involved in developing our multi-species, early-warning biomonitoring system. Graduate students involved in the project are highlighted in blue, while primary investigators are highlighted in red. Work performed as part of this thesis is highlighted in yellow.

The behavioural data collected in the bioassays discussed above are currently being processed and used to create a MATLAB-based model which will predict the type and concentration of contaminant present in a water sample based on a combined assessment of the behavioural changes of the three organisms. This model will be part of a "library" of response patterns to various types of contaminants and will be used as part of a multi-organism, early-warning biomonitoring technology.

However, bioassays conducted for this thesis only examined two potential contaminants in the aquatic environment. Further laboratory-based bioassays are required for heavy metals, poly-aromatic hydrocarbons (PAHs), pathogens, and a wide array of other classes of pollutants in order to add to the "library" of responses and to strengthen existing modelling. Laboratory bioassays also must be performed examining the effects of biological pathogens on the behaviour and physiology of the test organisms, in order to determine if the potential exists for this system to detect agents introduced to water supplies through acts of bioterrorism. The effects of mixtures of various contaminants, as would be seen in a natural freshwater setting, must also be evaluated. This thesis will help to greatly streamline future laboratory bioassays, as it has developed procedures for bioassays and determined the relevant characteristics which should be examined by future graduate students when performing laboratory bioassays. The next step will be to automate the recording and evaluation of the various behavioural and physiological parameters discussed above by modifying previously developed softwares and biomonitoring systems or by creating new technologies capable of automation of such monitoring. Integration of the MATLAB models based on laboratory bioassays will also need to occur in order to make the technology complete and unique from past BEWS.

The field application of our multi-species, early-warning biomonitoring technology will then take place at the DeCew Water Treatment Plant in Thorold, Ontario. At this facility, water is taken from the Welland Canal and is treated and distributed to citizens in the Niagara Region. Water enters from the canal through a channel and sits in a holding reservoir for 3-4 days before treatment and distribution. Our multi-species, early-warning biomonitoring technology will be established at the inflow to this reservoir. In the event of an adverse response to a chemical or biological contaminant, intake into the water treatment facility can be stopped and water can be

held in the reservoir until the nature of the problem can be established and treated appropriately by plant operators. The multi-species, early-warning biomonitoring technology will be established in a small shed constructed on the site. The shed will house a number of pieces of equipment capable of automatically and remotely monitoring the behaviours of the organisms discussed in this study, as well as the behaviours of photosynthetic organisms, protists, and bivalves which have been studied in a thesis project conducted simultaneously to this research (Figure ?). The large number of organisms being used will allow for greater sensitivity of the technology and will help in predicting the type and concentration of a given contaminant. Water inflow into the shed will be temperature controlled, as changes in this abiotic condition could potentially affect the behaviour of the organisms used in the technology. Overall, the technology will not replace more traditional chemical and physical means of detecting water contaminant; rather, it will be used in conjunction with these methods to help detect potentially dangerous contaminants in our water, especially pulses of contaminants which may be not be detected with periodic chemical analysis. The continuous nature of our biomonitoring technology, as well as it's time and cost efficiency make it an appealing technology for use in areas where access to chemical analysis facilities may be limited.

The field testing of our technology at the DeCew Water Treatment Plant will help to determine if the use of multiple species in a biomonitoring system is possible in a field setting, if the behaviour of bioassay organisms is consistent in the field, and if changes in these parameters can be used with the incorporated models to detect various types and concentrations of environmental contaminants in an incoming drinking water supply. If *in situ* use of our multi-species, early-warning biomonitoring technology is successful at the DeCew Water Treatment Plant, the next logical step would be for increased use of the technology throughout the country, particularly in marginalized areas such as First Nations reserves, and for policy-based changes to occur which would mandate that all drinking water facilities in Ontario and Canada utilize biomonitoring technology, in addition to traditional chemical and physical testing. Future employment of our system at water treatment facilities throughout the country would help to greatly increase the safety of Canada's freshwater drinking supply and help to prevent detrimental impacts on human health caused by polluted drinking water. Finally, the use of such a sensitive, reliable, and relatively inexpensive early-warning biomonitoring technology could be used in developing countries where clean drinking water is at a premium.

REFERENCES

Alfa Aesar (2007) MSDS for Sodium chloride.

Alzieu, C., Sanjuan, J., Deltreil, J.P. and Borel, M. (1986) Tin contamination in Archaron Bay: effects on oyster shell anomalies. Marine Pollution Bulletin 17: 494-498.

Alzieu, C. and Heral, M. (1984) Ecotoxicological effects of tributyltin compounds on oyster culture. Ecotoxicological Testing for the Marine Environment 2: 187-197.

Alzieu, C. (1998) Tributyltin: case study of a chronic contaminant in the coastal environment. Ocean and Coastal Management 40: 23-36.

American Society of Limnology and Oceanography (ASLO) (2004) Image of *Hyalella azteca*. Retrieved from http://www.aslo.org/photopost/showphoto.php/photo/398

Anderson, T.D. and Lydy, M.J. (2002) Increased toxicity to invertebrates associated with a mixture of atrazine and organophosphate insecticides. Environmental Toxicology and Chemistry 21: 1507-1514.

Anderson, T.D. and Zhu, K.Y. (2004) Synergistic and antagonistic effects of atrazine on the toxicity of organophosporodithioate and organophosphorotioate insecticides to *Chironomus tentans* (Diptera: Chironomidae). Pesticide Biochemistry and Physiology 80: 54-64.

Arner, M. and Koivisto, S. (1993) Effects of salinity on metabolism and life history characteristics of *Daphnia magna*. Hydrobiologia 259: 69-77.

Baillieul, M. and Blust, R. (1999) Analysis of the swimming velocity of cadmium-stressed *Daphnia magna*. Aquatic Toxicology 44: 245-254.

Baillieul, M. and Scheunders, P. (1998) Online determination of the velocity of simultaneously moving organisms by image analysis for the detection of sublethal toxicity. Water Research 32: 1027-1034.

Barber, I., Baird, D.J. and Calow, P. (1990) Clonal variation in general responses of Daphnia magna Straus to Toxic stress. II. Physiological Effects. Functional Ecology 4: 409-414.

Beitinger, T. (1990) Behavioural reactions for the assessment of stress in fish. Journal of Great Lakes Research 16: 495-528.

Blockwell, S.J., Maund, S.J. and Pascoe, D. (1998) The Acute Toxicity of Lindane to Hyalella Azteca and the Development of a Sublethal Bioassay Based on Precopulatory Guarding Behaviour. Archives of Environmental Contamination and Toxicology 35: 432-440.

Borgmann, U., Chau, Y.K., Wong, P.T.S., Brown, M. and Yaromich, J. (1996) The relationship between tributyltin (TBT) accumulation and toxicity to *Hyalella azteca* for use in identifying TBT toxicity in the field. Journal of Aquatic Ecosystem Health 5: 199-206.

Bunn, S.E. (1995) Biological monitoring of water quality in Australia: Workshop summary and future directions. Australian Journal of Ecology 20: 220-227.

Butterworth, F. M., Gunatilaka, A. and Diehl, P. (2002) The Rhine/Danube Monitoring Paradigm: Broader Applications of Sensor Technologies. Proceedings: International IWA Conference on Automation of Water Quality Monitoring. pp. 299-305. Retrieved from http://www.poyry.at/linked/en/general/rhine_danube_paradigm.pdf

Canadian Drinking Water Quality Guidelines (2008) Retrieved from http://www.hc-sc.gc.ca/ewh-semt/water-eau/drink-potab/index-eng.php

Chau, Y.K., Maguire, R.J., Brown, M., Yang, F. and Batchelor, S.P. (1997) Occurrence of organotin compounds in the Canadian aquatic environment five years after the regulation of antifouling uses of Tributyltin. Water Quality Research Journal of Canada 32: 453-521.

Christensen, B.T., Lauridsen, T.L., Ravn, H.W. and Bayley, M. (2005) A comparison of feeding efficiency and swimming ability of *Daphnia magna* exposed to cypermethrin. Aquatic Toxicology 73: 210-220.

Clotfelter, E.D., Bell, A.M. and Leverin, K.R. (2004) The role of animal behaviour in the study of endocrine-disrupting chemicals. Animal Behaviour 68: 665-676.

Collyard, S.A., Ankley, G.T., Hoke, R.A. and Goldenstein, T. (1994) Influence of age on the relative sensitivity of *Hyalella azteca* to diazinon, alkylphenol ethoxylates, copper, cadmium and zinc. Archives of Environmental Contamination and Toxicology 26: 110-113.

Cunha, S.R., Goncalves, R., Silva, S.R., and Correira, A.D. (2008) An automated marine biomonitoring system for assessing water quality in real-time. Ecotoxicology 17: 558-564.

Cushing, D.H. (1951) The vertical migration of planktonic crustacean. Biological Reviews 26: 158-192.

Denoyelles, F., Kettle, W.D. and Sinn, D.E. (1982) The responses of plankton communities in experimental ponds to atrazine, the most heavily used pesticide in the United States. Ecology 63: 1285-1293.

Detenbeck, N.E., Hermanutz, R., Allen, K. and Swift, M. (1996) Fate and effects of the herbicide atrazine in flow-through wetland mesocosms. Environmental Toxicology and Chemistry 14: 937-946.

Dewey, S. (1986) Effects of the herbicide atrazine on aquatic insect community structure and emergence. Ecology 67: 148-162.

Ding, J., Drewes, C.D. and Hsu, W.H. (2001) Behavioural effects of ivermectin in a freshwater Oligichaete, *Lumbriculus variegates*. Environmental Toxicology and Chemistry 20: 1584-1590.

Dodson, S.I. and Hanazato, T. (1995) Commentary on Effects of Anthropogenic and Natural Organic Chemicals on Development, Swimming Behavior, and Reproduction of Daphnia, a Key Member of Aquatic Ecosystems. Environmental Health Perspectives 103: 7-11.

Dodson, S.I., Hanazato, T. and Gorski, P.R. (1995) Behavioural responses of *Daphnia pulex* exposed to Carbaryl and *Chaoborus* Kairomone. Environmental Toxicology and Chemistry 14: 43-50.

Donkin, P., Widdows, J., Evans, S.V., Staff, F.J. and Yan, T. (1997) Effect of neurotoxic pesticides on the feeding rate of marine mussels (*Mytilus edulis*). Pesticide Science 49: 196-209.

Donna, A., Betta, P., Robutti, F., Crosignani, P., Berrino, F. and Bellingeri, D. (1984) Ovarian mesothelial tumors and herbicides: a case-control study. Carcinogenesis 5: 941-942.

Doving, K. (1991) Assessment of animal behaviourr as a method to indicate environmental toxicity. Comparative Biochemistry and Physiology 1/2: 247-252.

Drewes, C.D. (1999) Helical swimming and body reversal behaviors in *Lumbriculus variegates* (Annelida: Clitellata: Lumbriculidae). Hydrobiologia 406: 263-269.

Drewes, C.D. and Cain, K. (1999) As the Worm Turns: Locomotion in a Freshwater Oligochaete Worm. The American Biology Teacher 61: 438-442.

Drewes, C.D. and Fourtner, C.R. (1989) Hindsight and Rapid Escape in a Freshwater Oligochaete. Biology Bulletin 177: 363-371.

Environment Canada (1996) Biological Test Method : Acute Lethality Test Using *Daphnia* spp.

Environment Canada (1997) Biological Test Method: Test for Survival and Growth in Sediment Using the Freshwater Amphipod *Hyalella azteca*.

Fent, K. and Looser, P.W. (1995) Bioaccumulation and Bioavailability of Tributyltin Chloride: Influence of pH and Humic Acids. Water Research 29: 1631-1637.

Fent, K. (1996) Ecotoxicology of Organotin Compounds. Critical Reviews in Toxicology 26: 3-117.

Fischer, J.M., Fields, P.A., Pryzbylkowski, P.G., Nicolai, J.L. and Neale, P.J. (2006) Sublethal exposure to UV radiation affects respiration rates of the freshwater cladoceran *Daphnia catawba.* Photochemistry and Photobiology 82: 547-550.

Flaherty, C.M. and Dodson, S.I. (2005) Effects of pharmaceuticals on *Daphnia* survival, growth and reproduction. Chemosphere 61: 200-207.

Foran, J.A. and Brosnan, T.M. (2000) Early Warning Systems for Hazardous Biological Agents in Potable Water. Environmental Health Perspectives 108: 993-996.

Forget, J., Beliaff, B. and Bocquene, G. (2003) Acetylcholinesterase activity in copepods (*Tigriopus brevicornis*) from the Vilaine River Estuary, France, as a biomarker of neurotoxic contaminants. Aquatic Toxicology 62: 195-204.

Gerhardt, A. (2009) Screening the Toxicity of Ni, Cd, Cu, Ivermectin, and Imidacloprid in a Short-Term Automated Behavioural Toxicity Test with *Tubifex tubifex* (Muller 1774)(Oligochaeta). Human and Ecological Risk Assessment 15: 27-40.

Gerhardt, A., Kienle, C., Allan, I.J., Greenwood, R., Guigues, N., Fouillac, A., Mills, G. and Gonzalez, C. (2007) Biomonitoring with *Gammarus pulex* at the Meuse (NL), Aller, (GER) and Rhine (F) rivers with the online Multispecies Freshwater Biomonitor. Journal of Environmental Monitoring 9: 979-985.

Gerhardt, A. (2007a) Importance of Exposure Route for Behavioural Responses in *Lumbriculus variegates* Muller (Oligichaeta: Lumbriculida) in Short-Term Exposures to Pb. Environmental Science and Pollution Research 14: 430-434.

Gerhardt, A. (2007b) Aquatic Behavioral Ecotoxicology – Prospects and Limitations. Human and Ecological Risk Assessment 13: 481-491.

Gerhardt, A., Bisthoven, L.J. and Schmidt, S. (2006a) Automated recording of vertical negative phototactic behaviour in *Daphnia magna* Straus (Crustacea). Hydrobiologia 559: 433-441.

Gerhardt, A., Ingram, M.K., Kang, I.J. and Ulitzur, S. (2006b) In situ on-line toxicity biomonitoring in water: recent developments. Environmental Toxicology and Chemistry 25: 2263-2271.

Gerhardt, A., Bisthoven, L.J. and Penders, E. (2003) Quality control of drinking water from the River Rhine with the Multispecies Freshwater Biomonitor. Aquatic Ecosystem Health and Management 6: 159-166.

Gerhardt, A. and Bistoven, L.J. (1995) Behavioural, developmental and morphological responses of Chironomus gr. Thumni larvae (Diptera, Nematocera) to aquatic pollution. Journal of Aquatic Ecosystem Health 4: 205-214.

Geiger, J.G. and Buikema, A.L. (1981) Oxygen consumption and filtering rate of *Daphnia pulex* after exposure to water-soluble fractions of naphthalene, phenathrene, No. 2 Fuel Oil and coal-tar creosote. Bulletin of Environmental Contamination and Toxicology 27: 783-789.

Goodrich, M.S. and Lech, J.J. (1990) A Behavioral Screening Assay for *Daphnia magna*: A Method to Assess the Effects of Xenobiotics on Spacial Orientation. Environmental Toxicology and Chemistry 9: 21-30.

Graymore, M., Stagnitti, F. and Allinson, G. (2001) Impacts of atrazine in aquatic ecosystems. Environment International 26: 483-495.

Green, U., Kremer, J.H., Zillmer, M. and Moldaenke, C. (2003) Detection of Chemical Threat Agents in Drinking Water by an Early Warning Real-Time Biomonitor. Environmental Toxicology 18: 368-374.

Hamilton, P.B., Lean, D.R.S., Jackson, G.S., Kaushik, N.K. and Solomon, K.R. (1989) The effect of two applications of atrazine on the water quality of freshwater enclosures. Environmental Pollution 60: 291-304.

Hatch, A.C. and Burton, G.A. (1999) Photo-induced toxicity of PAHs to *Hyalella azteca* and *Chironomus tentans*: effects of mixtures and behavior. Environmental Pollution 106: 157-167.

Health Canada (1993) Atrazine. Retrieved from: http://www.hc-sc.gc.ca/ewh-semt/pubs/water-eau/atrazine/index-eng.php.

Hoang, T.C., Gallagher, J.S. and Klaine, S.J. (2006) Responses of *Daphnia magna* to pulsed exposures of arsenic. Environmental Toxicology 22: 308-317.

Hoar, Z., Blair, A., Holmes, F.F., Boysen, C., Robel, R.J., Hoover, R. and Fraumeni, J.F. (1988) A case-control study of non-Hodgkin's lymphoma and agricultural factors in eastern Nebraska. American Journal of Epidemiology 128: 901-910.

Horry, H., Durand, M.J., Picart, P., Bendriaa, L., Daniel, P. and Thouand, G. (2004) Development of a biosensor for the detection of Tributyltin. Environmental Toxicology 19: 342-345.

IARC (1999) Atrazine. Retrieved from http://monographs.iarc.fr/ENG/Monographs/vol73/mono73-8.pdf

Jiang, H., Adams, C., Graziano, N., Robertson, A., McGuire, M., and Khiari, D. (2006) Occurrence and Removal of chloro-s-triazines in water treatment plants. Environmental Science and Technology 40: 3609-3616.

Key, P.B., Fulton, M.H., Harman-Fetcho, J.A. and McConnell, L.L. (2003) Acetylcholinesterase activity in grass shrimp and aqueous pesticide levels from south Florida drainage canals. Archives of Environmental Contamination and Toxicology 45: 371-377.

Kieu, N.D., Michels, E. and De Meester, L. (2001) Phototactic Behavior of *Daphnia* and the continuous monitoring of water quality: interference of fish kairomones and food quality. Environmental Toxicology and Chemistry 20: 1098-1103.

Kiss, I., Kovats, N. and Szalay, T. (2003) Evaluation of some alternative guidelines for risk assessment of various habitats. Toxicology Letters 140: 411-417.

Konstantinou, I.K. and Albanis, T.A. (2004) Worldwide occurrence and effects of antifouling paint booster biocides in the aquatic environment: a review. Environment International 30: 235-248.

Knops, M., Altenburger, R. and Segner, H. (2001) Alterations of physiological energetic, growth and reproduction of *Daphnia magna* under toxicant stress. Aquatic Toxicology 53: 79-90.

Landrum, P.F., Gedeon, M.L., Burton, G.A., Greenberg, M.S. and Rowland, C.D. (2002) Biological responses of *Lumbriculus variegates* exposed to fluoranthene-spiked sediment. Environmental Contamination and Toxicology 42: 292-302.

Lechelt, M., Blohm, W., Kirschneit, B., Pfeiffer, M., Gresens, E., Liley, J., Holz, R., Luring, C. and Moldaenke, C. (2000) Monitoring of Surface Water by Ultrasensitive *Daphnia* toximeter. Environmental Toxicology 15: 390-400.

Leppanen, M.T. and Kukkoken, J.V.K. (1998) Relationship between reproduction, sediment type and feeding activity of *Lumbriculus variegates* (Muller): Implications for sediment toxicity testing. Environmental Toxicology and Chemistry 17: 2196-2202.

Liu, X., Shao, J., Xiang, L. and Chen, X. (2006) Cytotoxic effects and apoptosis induction of atrazine in a grass carp (*Ctenopharyngodon idellus*) cell line. Environmental Toxicology 10: 80-89.

Maguire, R.J. (1987) Environmental aspects of tributyltin. Applied Organometallic Chemistry 1: 475-498.

Martins, J., Soares, M.L., Saker, M.L., Oliva-Teles, L. and Vasconelos, V.M. (2007a) Phototactic behavior in *Daphnia magna* Straus as an indicator of toxicants in the aquatic environment. Ecotoxicology and Environmental Safety 67: 417-422.

Martins, J.C., Saker, M.L., Teles, L.F.O. and Vasconcelos, V.M. (2007b) Oxygen consumption by *Daphnia magna* Straus as a marker of chemical stress in the aquatic environment. Environmental Toxicology and Chemistry 26: 1987-1991.

McCarthy, L.H. (2007) NSERC Strategic Grant Proposal.

Michels, E., Semsari, S., Bin, C. and De Meester, L. (2000) Effect of Sublethal Doses of Cadmium on the Phototactic Behavior of *Daphnia magna*. Ecotoxicology and Environmental Safety 47: 261-265.

Mikol, Y.B., Richardson, W.R., van der Schalie, W.H., Shedd, T.R. and Widder, M.W. (2007) An online real-time biomonitor for contaminant surveillance in water supplies. Journal of the American Water Works Association 99: 107-116.

Mills, C.L., Shukla, D.H. and Compton, G.J. (2006) Development of a new low cost high sensitivity system for behavioural ecotoxicity testing. Aquatic Toxicology 77: 197-201.

MIT (2006) Environmental Chemistry of Boston Harbor. Retrieved from http://ocw.mit.edu/NR/rdonlyres/Earth--Atmospheric--and-Planetary-Sciences/12-097January--IAP--2006/35FAFFC3-135B-4266-93B4-2144F7487004/0/dissolved_oxygen.pdf

Moore, M.T., Lizotte, R.T., Knight, S.S., Smith, S. and Cooper, C.M. (2006) Assessment of pesticide contamination in three Mississippi Delta oxbow lakes using *Hyalella azteca*. Chemosphere 67: 2184-2191.

National Center for Biotechnology Information PubChem (2009a) Tributyltin Hydroxide. Retrieved from http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=6327813&loc=ec_rcs

National Center for Biotechnology Information PubChem (2009b) Atrazine. Retrieved from http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=2256&loc=ec_rcs

National Center for Biotechnology Information PubChem (2009c) Dimethyl Sulfoxide. Retrieved from http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=679&loc=ec_rcs

Oberlin, G.E. and Blinn, D.W. (1997) The affect of temperature on the metabolism and behaviour of an endemic amphipod, *Hyalella montezuma*, from Montezuma Well, Arizona, U.S.A. Freshwater Biology 37: 55-59.

OECD (2004) OECD Guideline for testing of chemicals: *Daphnia* sp., Acute Immobilization Test.

O'Gara, B.A., Bohannon, V.K., Teagure, M.W. and Smeaton, M.B. (2004) Copper-induced changes in locomotor behaviours and neuronal physiology of the freshwater oligocaete, *Lumbriculus variegates*. Aquatic Toxicology 69: 51-66.

Paul, R.J., Colmorgen, M., Huller, S., Tyroller, F. and Zinkler, D. (1997) Circulation and respiratory control in millimeter-sized animals (*Daphnia magna, Folsomia candida*) studied by optical methods. Journal of Comparative Physiology 167:399-408.

Penttinen, O.P. and Kukkoken, J.V.K. (2000) Metabolic Response of *Lumbriculus variegatus* to Respiratory Uncoupler in Cold and Anoxic Water. Environmental Toxicology and Chemistry 19: 2073-2075.

Putzer, V.M., de Zwaan, A. and Wieser, W. (1990) Anaerobic energy metabolism in the oligochaete *Lumbriculus variegates* Muller. Journal of Comparative Physiology 159: 707-715.

Ren, Z., Li, Z., Ma, M., Wang, Z. and Fu, R. (2009) Behavioural responses of *Daphnia magna* to Stresses of Chemicals with Different Toxic Characteristics. Bulletin of Environmental Contamination and Toxicology 82: 310-316.

Ren, Z.M., Li, Z.L., Zha, J.M., Rao, K.F., Ma, M., Wang, Z. and Fu, R.S. (2008) The Avoidance Responses of *Daphnia magna* to the Exposure of Organophosphorus Pesticides in an On-Line Biomonitoring System. Environmental Modelling and Assessment 14: 405-410.

Ren, Z., Zha, J., Ma, M., Wang, Z. and Gerhardt, A. (2007) The early warning of aquatic organophosphorus pesticide contamination by on-line monitoring behavioural changes of *Daphnia magna*. Environmental Monitoring and Assessment 134: 373-383.

Revsbech, N.P. (1989) An Oxygen Microsensor with a guard cathode. Limnology and Oceanography 34: 474-478.

Ryan, S.M. and Dodson, S.I. (1998) Seasonal analysis of *Daphnia pulicaria* swimming behavior. Hydrobiologia 384: 11-118.

Saglio, P. and Trijasse, S. (1998) Behavioural responses to atrazine and diuron in goldfish. Archives of Environmental Contamination and Toxicology 35: 484-491.

Sanchez, B.C., Ochoa-Acuna, H., Porterfield, D.M. and Sepulveda, M.S. (2008) Oxygen flux as an indicator of physiological stress in fathead minnow embryos: a real-time biomonitoring system of water quality. Environmental Science and Technology 42: 7010-7017.

Scherer, E. (1992) Behavioural responses as indicators of environmental alterations: approaches, results and developments. Journal of Applied Ichthyology 8: 122-131.

Schmidt, K., Steinberg, C.E.W., Staaks, G.B.O. and Pflugmacher, S. (2005) Influence of Xenobiotic Mixture (PCB and TBT) Compared to Single Substances on Swimming Behaviour or Reproduction of *Daphnia magna*. Acta Hydrochimistria and Hydrobiologia 33: 287-300.

Scott, G.R. and Sloman, K.A. (2004) The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquatic Toxicology 68: 369-392.

Sigmon, C. (1979) Oxygen consumption in *Daphnia pulex* Exposed to 2,4-D or 2,4,5-T. Bulletin of Environmental Contamination and Toxicology 21: 822-825.

Song, Y., Zhu, L.S., Wang, J., Wang, J.H., Liu, W. and Xie, H. (2009) DNA damage and effects on antioxidative enzymes in earthworm (*Eisenia foetida*) induced by atrazine. Soil Biology and Chemistry 41: 905-909.

Steinbeck, C.E.W., Lorenz, R. and Spieser, O.H. (1995) Effects of atrazine on swimming behaviour of zebrafish, *Brachydanio rerio*. Water Research 29: 981-985.

Suedel, B.C. and Rodgers, J.H. (1996) Toxicity of Fluoranthene to *Daphnia magna*, *Hyalella azteca, Chironomus tentans,* and *Stylaria lacustris* in water-only and whole sediment exposures. Bulletin of Environmental Contamination and Toxicology 57: 132-138.

Szulkin, M., Dawidowicz, P. and Dodson, S.I. (2006) Behavioural uniformity as a response to cues of predation risk. Animal Behaviour 71: 1013-1019.

Underwood, A.J. and Peterson, C.H. (1988) Towards an ecological framework for investigating pollution. Marine Ecology Progress Series 46: 227-234.

Unisense (2009) Oxygen sensor. Retrieved from http://www.unisense.com/Default.aspx?ID=443.

University of Iowa (2009) Image of *Lumbriculus variegatus.* Retrieved from http://www.eeob.iastate.edu/faculty/DrewesC/htdocs/Lv-KSNr.jpg

Untersteiner, H., Kahapka, J. and Kaiser, H. (2003) Behavioural response of the cladoceran *Daphnia magna* STRAUS to sublethal Copper stress – validation by image analysis. Aquatic Toxicology 65: 435-442.

USEPA (2000) Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants in freshwater invertebrates.

USEPA (2002) Methods for measuring acute toxicity of effluents and receiving waters to freshwater and marine organisms.

USEPA (2003) Interim Reregistration Eligibility Decision for Atrazine. Retrieved from http://www.epa.gov/oppsrrd1/reregistration/REDs/atrazine_ired.pdf

Van der Schalie, W.H., Shedd, T.R., Knechtges, P.L. and Widder, M.W. (2001) Using higher organisms in biological early warning systems for real-time toxicity detection. Biosensors and Bioelectronics 16: 457-465.

Van Hoof, F., Sluyts, H., Paulussen, J., Berckmans, D. and Bloemen, H. (1994) Evaluation of a biomonitor based on the phototactic behaviour of *Daphnia magna* using infrared detection and digital image processing. Water Science and Technology 10; 79-86.

Villegas-Navarro, A., Esperanze, R.L. and Reyes, J.L. (2003) The heart of *Daphnia magna*: effects of four cardiovascular drugs. Comparative Biochemistry and Physiology. 136: 127-134.

Wan, M.T., Buday, C., Schroeder, G., Kuo, J. and Pasternak, J. (2006) Toxicity to *Daphnia magna, Oncorhynchus mykiss, Onchorynchus tshawytscha* and *Rana catesbeiana* of Atrazine, Metolachlor, Simazine, and their formulated products. Bulletin of Environmental Contamination and Toxicology 76: 52-58.

Wang, F., Goulet, R.R. and Chapman, P.M. (2004) Testing sediment biological effects with the freshwater amphipod *Hyalella azteca*: the gap between laboratory and nature. Chemosphere 57: 1713-1724.

Waring, C.P. and Moore, A. (2004) The effect of atrazine on Atlantic salmon (*Salmo salar*) smolts in fresh water and after sea water transfer. Aquatic Toxicology 66: 93-104.

Watson, S.B., Juttner, F. and Koster, O. (2007) *Daphnia* behavioural responses to taste and odour compounds: ecological significance and application as an inline treatment plant monitoring tool. Water Science and Technology 55: 23-31.

Wicklum, D., Smith, D.E.C. and Davies, R.W. (1997) Motality, Preference, Avoidance and Activity of a Predatory Leech Exposed to Cadmium. Archives of Environmental Contamination and Toxicology 32: 178-183.

Winkelmann, D.A. and Klaine, S.J. (1991) Degradation and bound residue formation of four atrazine metabolites, deethylatrazine, deisopropylatrazine, dealkylatrazine and hydroxyatrazine, in a western Tennessee soil. Environmental Toxicology and Chemistry 10: 347-354.

Wollenberger, L., Halling-Sorensen, B. and Kusk, K.O. (2000) Acute and chronic toxicity of veterinary antibiotics to *Daphnia magna*. Chemosphere 40: 723-730.

Zala, S.M. and Penn, D.J. (2004) Abnormal behaviours induced by chemical pollution: a review of the evidence and new challenges. Animal Behaviour 68: 649-664.

APPENDICES

Appendix A Dilution Calculations

Preparation of 100 mg/L (TBT in DMSO) Substock

A 100 mL volume of 100 mg/L TBT in DMSO stock solution was made for utilization in bioassays.

$$D = \frac{m}{v}$$

$$V = \frac{100\ mg\ TBT}{1.103\ \frac{g}{cm^3}}$$

$$V = 0.09066\ cm^3$$

$$V = 0.09066\ mL\ TBT\ in\ 1\ L\ DMSO$$

$$V = 90.66\ uL\ TBT\ in\ 1\ L\ DMSO$$

$$V = 9.066\ uL\ TBT\ in\ 100\ mL\ DMSO$$

Preparation of 50 mg/L (TBT in DMSO) Substock

A 100 mL substock of 50 mg/L TBT in DMSO was also made for use in bioassays.

$C_1V_1 = C_2V_2$

100 mg/L (V_1) = 50 mg/L (0.1 L)

V_1 = 0.05 L = 50 mL

The substock was made by adding 50 mL of the 100 mg/L substock to 50 mL of DMSO.

Preparation of 10 mg/L (TBT in DMSO) Substock

A 100 mL substock of 10 mg/L TBT in DMSO was also made for use in bioassays.

$C_1V_1 = C_2V_2$

100 mg/L (V_1) = 10 mg/L (0.1 L)

V_1 = 0.01 L = 10 mL

The substock was made by adding 10 mL of the 100 mg/L substock to 90 mL of DMSO.

Preparation of 100 mg/L (Atrazine in DMSO) Substock

A 100 mL volume of 100 mg/L TBT in DMSO stock solution was made for utilization in bioassays.

$$D = \frac{m}{v}$$

$$V = \frac{100\ mg\ TBT}{1.187\ \frac{g}{cm^3}}$$

$$V = 0.084\ cm^3$$

$$V = 0.084\ mL\ TBT\ in\ 1\ L\ DMSO$$

$$V = 84.00\ uL\ TBT\ in\ 1\ L\ DMSO$$

$$V = 8.4\ uL\ TBT\ in\ 100\ mL\ DMSO$$

Preparation of 50 mg/L (Atrazine in DMSO) Substock

A 100 mL substock of 50 mg/L Atrazine in DMSO was also made for use in bioassays.

$C_1V_1 = C_2V_2$

100 mg/L (V_1) = 50 mg/L (0.1 L)

V_1 = 0.05 L = 50 mL

The substock was then made by adding 50 mL of the 100 mg/L substock to 50 mL of DMSO.

Preparation of 10 mg/L (Atrazine in DMSO) Substock

A 100 mL substock of 5 mg/L atrazine in DMSO was also made for use in bioassays.

$C_1V_1 = C_2V_2$

100 mg/L (V_1) = 5 mg/L (0.1 L)

V_1 = 0.005 L = 5 mL

The substock was made then by adding 5 mL of the 100 mg/L substock to 95 mL of DMSO.

Dilution calculations for *Daphnia magna, Hyalella azteca* and *Lumbriculus variegatus* motility bioassays and *Lumbriculus variegatus* grouping behaviour bioassays in TBT.

Test Concentration	Total Volume	TBT Substock Used	Volume Substock Added
0.1% DMSO	150 mL	DMSO	150 uL
10 µg/L	150 mL	10 mg/L	150 uL
50 µg/L	150 mL	50 mg/L	150 uL
100 µg/L	150 mL	100 mg/L	150 uL

Dilution calculations for *Daphnia magna, Hyalella azteca* and *Lumbriculus variegatus* motility bioassays and *Lumbriculus variegatus* burrowing and grouping behaviour bioassays in atrazine.

Test Concentration	Total Volume	Atrazine Substock Used	Volume Substock Added
0.1% DMSO	150 mL	DMSO	150 uL
5 µg/L	150 mL	5 mg/L	150 uL
50 µg/L	150 mL	50 mg/L	150 uL
100 µg/L	150 mL	100 mg/L	150 uL

Dilution calculations for *Daphnia magna, Hyalella azteca* and *Lumbriculus variegatus* respiration bioassays in TBT.

Test Concentration	Total Volume	Atrazine Substock Used	Volume Substock Added
0.1% DMSO	25 mL	DMSO	25 uL
10 µg/L	25 mL	10 mg/L	25 uL
50 µg/L	25 mL	50 mg/L	25 uL
100 µg/L	25 mL	100 mg/L	25 uL

Dilution calculations for *Daphnia magna, Hyalella azteca* and *Lumbriculus variegatus* respiration bioassays in TBT.

Test Concentration	Total Volume	Atrazine Substock Used	Volume Substock Added

0.1% DMSO	25 mL	DMSO	25 uL
5 µg/L	25 mL	5 mg/L	25 uL
50 µg/L	25 mL	50 mg/L	25 uL
100 µg/L	25 mL	100 mg/L	25 uL

Appendix B Winkler Titration Procedure

The Winkler titration procedure used in the TBT respiration bioassays was based on (MIT, 2006). At each time period in the bioassays, the following steps of the titration were performed:

1. 50 µL of MnCl solution was added to each scintillation vial, followed immediately by the addition of 50 µL of NaI solution.
2. Each vial was then closed, shaken vigorously for 30 seconds and left to sit for 10 minutes in order to allow for the formation and settling of floc.
3. Each vial was then shaken vigorously again for 30 seconds and allowed to sit for an additional 30 minutes.
4. Sulfuric acid (50 µL) was then added to each vial, which was then shaken again. Samples were then stored in the dark until titration took place (maximum of 24 hours later).
5. Samples were titrated in a 300-mL beaker using an Eppendorf multi-pipetter. Samples were poured into the beaker, where a stirring rod was in place to ensure mixing of the titrant and the sample.
6. One millilitre of starch indicator solution was added to each sample in the beaker, turning the colour of the solution to a dark blue.
7. The sample was titrated with sodium thiosulfate solution using the multi-pipetter. Titrant was added in 10 µL increments until the straw-colour of the sample returned. The amount of titrant used was then recorded and used to determine the concentration of oxygen in the sample.

Appendix C Unisense Oxygen Microelectrode Function

A Clark-type oxygen microelectrode (Unisense, Denmark) was used in the atrazine bioassays for all three test organisms. The probe functions as follows. Oxygen from the sample diffuses across a silicone membrane to an oxygen reducing cathode, which is polarized against an Ag/AgCl anode (Figure 1) (Unisense, 2009). An internal guard cathode is also present to remove all oxygen which diffuses toward the tip from the internal electrolyte reservoir, allowing for greater stability and sensitivity of the probe (Revsbech, 1989). The flow of electrons from anode to cathode reflects the partial pressure of oxygen at the tip of the probe and is measured in picoamperes by a highly sensitive picoammeter attached to the probe (Unisense, 2009). Exact concentrations of oxygen in a given sample can then be determined by creating a calibration curve based on samples saturated with oxygen and saturated with nitrogen (no oxygen present).

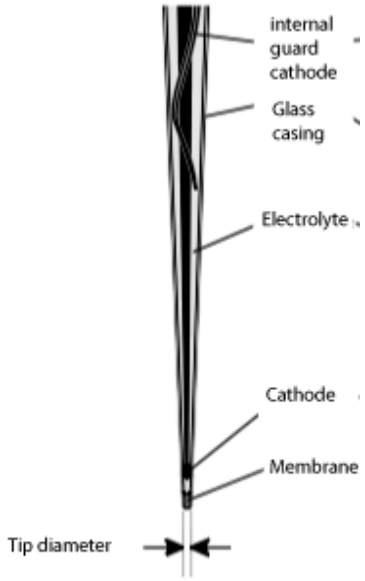

Figure 1: Schematic diagram of Unisense Clark-type oxygen microelectrode (Unisense, 2009)

Appendix D Daphnia magna Swimming Bioassay TBT Data

Swimming Height

Percentage of *Daphnia magna* Showing Changes in Swimming Height in the Water Column

	Time (Hours)	**Reference**	**0.1% DMSO**	**10 µg/L TBT**	**50 µg/L TBT**	**100 µg/L TBT**
Replicate 1	0	0	0	0	0	0
Replicate 2	0	0	0	0	0	0
Replicate 3	0	0	0	0	0	0
Replicate 1	1	0	0	60	80	80
Replicate 2	1	0	0	60	80	80
Replicate 3	1	0	0	100	100	100
Replicate 1	2	0	40	40	80	100
Replicate 2	2	0	0	60	100	80
Replicate 3	2	0	0	80	100	100
Replicate 1	3	0	0	100	100	100
Replicate 2	3	0	0	80	80	80
Replicate 3	3	0	0	80	80	100
Replicate 1	4	0	0	100	100	100
Replicate 2	4	0	0	80	80	80
Replicate 3	4	0	0	80	100	100
Replicate 1	5	0	0	60	100	100
Replicate 2	5	0	0	60	100	80
Replicate 3	5	0	0	100	100	100
Replicate 1	6	0	0	80	100	100
Replicate 2	6	0	0	80	100	100
Replicate 3	6	0	0	100	100	100
Replicate 1	12	0	0	100	100	80
Replicate 2	12	0	0	40	100	100
Replicate 3	12	0	0	100	100	100
Replicate 1	24	0	0	100	100	100
Replicate 2	24	0	0	80	100	80
Replicate 3	24	0	0	100	80	100

Spinning

Percentage of *Daphnia magna* displaying spinning behaviour

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	0	0	0	0	0	0
Replicate 2	0	0	0	0	0	0
Replicate 3	0	0	0	0	0	0
Replicate 1	1	0	0	40	20	20
Replicate 2	1	0	0	60	20	20
Replicate 3	1	0	0	60	40	0
Replicate 1	2	0	0	40	40	40
Replicate 2	2	0	0	0	0	80
Replicate 3	2	0	0	60	20	0
Replicate 1	3	0	0	40	20	40
Replicate 2	3	0	0	80	60	60
Replicate 3	3	0	0	20	40	0
Replicate 1	4	0	0	40	20	40
Replicate 2	4	0	0	40	40	40
Replicate 3	4	0	0	20	40	0
Replicate 1	5	0	0	0	20	20
Replicate 2	5	0	0	60	40	0
Replicate 3	5	0	0	20	40	20
Replicate 1	6	0	20	60	0	100
Replicate 2	6	0	20	40	40	40
Replicate 3	6	0	0	20	0	0
Replicate 1	12	0	0	20	0	20
Replicate 2	12	0	0	40	40	20
Replicate 3	12	0	0	0	0	20
Replicate 1	24	40	40	40	0	0
Replicate 2	24	20	20	40	0	0
Replicate 3	24	0	0	80	20	0

Body Orientation

Percentage of *Daphnia magna* displaying altered body orientation

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	0	0	0	0	0	0
Replicate 2	0	0	0	0	0	0
Replicate 3	0	0	0	0	0	0
Replicate 1	1	0	0	0	0	0
Replicate 2	1	0	0	0	20	0
Replicate 3	1	0	0	20	20	40
Replicate 1	2	0	0	0	0	0
Replicate 2	2	0	0	40	0	20
Replicate 3	2	0	0	40	20	0
Replicate 1	3	0	0	0	0	0
Replicate 2	3	0	0	20	0	60
Replicate 3	3	0	0	20	40	20
Replicate 1	4	0	0	0	0	0
Replicate 2	4	0	0	20	0	60
Replicate 3	4	0	0	0	0	0
Replicate 1	5	0	0	0	0	0
Replicate 2	5	0	0	40	20	20
Replicate 3	5	0	0	40	0	20
Replicate 1	6	0	0	0	0	40
Replicate 2	6	0	0	60	40	20
Replicate 3	6	0	0	20	20	60
Replicate 1	12	0	0	0	0	40
Replicate 2	12	0	0	20	20	20
Replicate 3	12	0	0	0	20	60
Replicate 1	24	0	0	20	0	40
Replicate 2	24	0	0	0	60	0
Replicate 3	24	0	0	0	20	0

Immobilization

Percentage of *Daphnia magna* immobilized

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	0	0	0	0	0	0
Replicate 2	0	0	0	0	0	0
Replicate 3	0	0	0	0	0	0
Replicate 1	1	0	0	0	0	0
Replicate 2	1	0	0	0	40	20
Replicate 3	1	0	0	20	60	40
Replicate 1	2	0	0	20	20	40
Replicate 2	2	0	0	40	0	40
Replicate 3	2	0	0	0	40	40
Replicate 1	3	0	0	20	40	40
Replicate 2	3	0	0	40	0	40
Replicate 3	3	0	0	40	40	60
Replicate 1	4	0	0	20	40	0
Replicate 2	4	0	0	0	20	0
Replicate 3	4	0	0	40	40	40
Replicate 1	5	0	0	0	40	60
Replicate 2	5	0	0	40	0	60
Replicate 3	5	0	0	40	60	60
Replicate 1	6	0	0	40	40	60
Replicate 2	6	0	0	60	60	60
Replicate 3	6	0	0	40	60	60
Replicate 1	12	0	0	60	40	60
Replicate 2	12	0	0	20	100	80
Replicate 3	12	0	0	40	80	60
Replicate 1	24	0	0	60	40	100
Replicate 2	24	0	0	20	40	80
Replicate 3	24	0	0	40	60	100

Secondary Antennae Use

Percentage of *Daphnia magna* displaying abnormal secondary antennae use

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	0	0	0	0	0	0
Replicate 2	0	0	0	0	0	0
Replicate 3	0	0	0	0	0	0
Replicate 1	1	0	0	0	0	0
Replicate 2	1	0	0	0	20	20
Replicate 3	1	0	0	40	60	40
Replicate 1	2	0	0	0	0	40
Replicate 2	2	0	0	40	20	60
Replicate 3	2	0	0	40	40	40
Replicate 1	3	0	0	20	40	40
Replicate 2	3	0	0	20	40	40
Replicate 3	3	0	0	40	80	60
Replicate 1	4	0	0	20	80	0
Replicate 2	4	0	0	40	40	40
Replicate 3	4	0	0	40	80	60
Replicate 1	5	0	0	0	60	80
Replicate 2	5	0	0	40	60	60
Replicate 3	5	0	0	40	80	60
Replicate 1	6	0	0	40	80	80
Replicate 2	6	0	0	60	60	80
Replicate 3	6	0	0	40	80	60
Replicate 1	12	0	0	60	60	60
Replicate 2	12	0	0	20	80	80
Replicate 3	12	0	0	60	80	80
Replicate 1	24	0	0	60	40	60
Replicate 2	24	0	0	20	40	80
Replicate 3	24	0	0	60	60	40

Swimming Style

	Percentage of *Daphnia magna* Showing Altered Swimming Style					
	Time (Hours)	**Reference**	**0.1% DMSO**	**10 µg/L TBT**	**50 µg/L TBT**	**100 µg/L TBT**
Replicate 1	0	0	0	0	0	0
Replicate 2	0	0	0	0	0	0
Replicate 3	0	0	0	0	0	0
Replicate 1	1	0	0	0	60	0
Replicate 2	1	0	0	20	80	60
Replicate 3	1	0	0	20	60	60
Replicate 1	2	0	0	0	80	80
Replicate 2	2	0	0	60	60	80
Replicate 3	2	0	0	60	60	60
Replicate 1	3	0	0	20	80	80
Replicate 2	3	0	0	40	60	80
Replicate 3	3	0	0	60	100	60
Replicate 1	4	0	0	20	100	60
Replicate 2	4	0	0	40	60	80
Replicate 3	4	0	0	60	100	60
Replicate 1	5	0	0	0	60	80
Replicate 2	5	0	0	60	60	80
Replicate 3	5	0	0	80	100	80
Replicate 1	6	0	0	40	60	60
Replicate 2	6	0	0	80	100	100
Replicate 3	6	0	0	60	80	80
Replicate 1	12	0	0	60	60	80
Replicate 2	12	0	0	80	80	80
Replicate 3	12	0	0	80	100	80
Replicate 1	24	0	0	60	40	80
Replicate 2	24	0	0	60	80	100
Replicate 3	24	0	0	60	80	100

Appendix E Daphnia magna Swimming Bioassay Atrazine Data

Swimming Height

Percentage of *Daphnia magna* displaying changes in swimming height

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	20	20	0	100	60
Replicate 2	1	0	0	0	100	80
Replicate 3	1	0	0	0	0	80
Replicate 1	2	0	0	80	60	100
Replicate 2	2	0	0	80	80	80
Replicate 3	2	0	0	0	40	100
Replicate 1	3	0	0	60	100	60
Replicate 2	3	0	0	60	80	80
Replicate 3	3	0	0	40	80	100
Replicate 1	4	0	0	100	100	100
Replicate 2	4	0	0	80	80	100
Replicate 3	4	0	0	60	80	80
Replicate 1	5	0	0	80	100	80
Replicate 2	5	0	0	80	100	100
Replicate 3	5	0	0	60	100	100
Replicate 1	6	0	0	100	80	100
Replicate 2	6	0	0	100	100	100
Replicate 3	6	0	0	60	80	100
Replicate 1	10	0	0	100	100	100
Replicate 2	10	0	0	100	100	100
Replicate 3	10	0	0	60	60	100
Replicate 1	24	0	0	100	100	100
Replicate 2	24	0	0	100	100	100
Replicate 3	24	0	0	100	100	100

Spinning

Percentage of *Daphnia magna* displaying spinning behaviour

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	0	0	0	20	20
Replicate 2	1	0	0	40	0	0
Replicate 3	1	0	0	40	100	40
Replicate 1	2	0	0	0	40	60
Replicate 2	2	0	0	20	20	0
Replicate 3	2	0	0	40	60	40
Replicate 1	3	0	0	0	40	60
Replicate 2	3	0	0	40	20	0
Replicate 3	3	0	0	60	40	20
Replicate 1	4	0	0	40	40	60
Replicate 2	4	0	0	40	40	0
Replicate 3	4	0	0	60	60	40
Replicate 1	5	0	0	40	20	0
Replicate 2	5	0	0	20	20	60
Replicate 3	5	0	0	0	0	60
Replicate 1	6	0	0	40	60	0
Replicate 2	6	0	0	40	20	0
Replicate 3	6	0	0	0	20	40
Replicate 1	10	0	0	0	20	20
Replicate 2	10	0	0	80	0	40
Replicate 3	10	0	0	40	20	20
Replicate 1	24	0	0	40	0	0
Replicate 2	24	0	0	60	20	0
Replicate 3	24	0	0	0	40	0

Body Orientation

Percentage of *Daphnia magna* displaying abnormal body orientation

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	0	0	0	40	0
Replicate 2	1	0	0	0	20	40
Replicate 3	1	0	0	0	0	0
Replicate 1	2	0	0	0	20	40
Replicate 2	2	0	0	0	20	40
Replicate 3	2	0	0	0	20	0
Replicate 1	3	0	0	20	60	40
Replicate 2	3	0	0	20	60	60
Replicate 3	3	0	0	0	40	20
Replicate 1	4	0	0	20	40	40
Replicate 2	4	0	0	0	20	60
Replicate 3	4	0	0	0	20	0
Replicate 1	5	0	0	0	60	40
Replicate 2	5	0	0	0	40	40
Replicate 3	5	0	0	40	40	40
Replicate 1	6	0	0	0	40	80
Replicate 2	6	0	0	40	60	80
Replicate 3	6	0	0	60	60	60
Replicate 1	10	0	0	0	0	80
Replicate 2	10	0	0	40	0	100
Replicate 3	10	0	0	0	20	40
Replicate 1	24	0	0	0	0	40
Replicate 2	24	0	0	0	40	40
Replicate 3	24	0	0	20	40	60

Immobilization

Percentage of *Daphnia magna* immobilized

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	0	0	0	0	0
Replicate 2	1	0	0	0	0	40
Replicate 3	1	0	0	0	0	60
Replicate 1	2	0	0	0	40	0
Replicate 2	2	0	0	20	60	40
Replicate 3	2	0	0	0	40	60
Replicate 1	3	0	0	0	60	40
Replicate 2	3	0	0	20	80	60
Replicate 3	3	0	0	40	40	80
Replicate 1	4	0	0	0	80	40
Replicate 2	4	0	0	0	20	40
Replicate 3	4	0	0	40	40	40
Replicate 1	5	0	0	0	60	0
Replicate 2	5	0	0	40	80	40
Replicate 3	5	0	0	60	80	40
Replicate 1	6	0	0	0	80	100
Replicate 2	6	0	0	0	60	80
Replicate 3	6	0	0	60	80	60
Replicate 1	10	0	0	80	60	80
Replicate 2	10	0	0	0	100	60
Replicate 3	10	0	0	40	40	60
Replicate 1	24	0	0	0	80	60
Replicate 2	24	0	0	0	60	100
Replicate 3	24	0	0	60	60	100

Secondary Antennae Use

Percentage of *Daphnia magna* displaying abnormal use of secondary antennae

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	0	0	0	0	0
Replicate 2	1	0	0	0	0	40
Replicate 3	1	0	0	0	0	0
Replicate 1	2	0	0	0	40	0
Replicate 2	2	0	0	20	60	40
Replicate 3	2	0	0	0	20	60
Replicate 1	3	0	0	0	60	40
Replicate 2	3	0	0	20	80	60
Replicate 3	3	0	0	40	40	80
Replicate 1	4	0	0	0	80	40
Replicate 2	4	0	0	0	20	40
Replicate 3	4	0	0	40	40	40
Replicate 1	5	0	0	0	60	40
Replicate 2	5	0	0	40	80	40
Replicate 3	5	0	0	60	80	40
Replicate 1	6	0	0	0	60	100
Replicate 2	6	0	0	0	60	80
Replicate 3	6	0	0	60	80	60
Replicate 1	10	0	0	60	100	100
Replicate 2	10	0	0	0	100	60
Replicate 3	10	0	0	40	40	60
Replicate 1	24	0	0	0	20	40
Replicate 2	24	0	0	0	60	80
Replicate 3	24	0	0	60	60	100

Swimming Style

Percentage of *Daphnia magna* displaying abnormal swimming style

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	0	0	20	60	80
Replicate 2	1	0	0	0	20	60
Replicate 3	1	0	0	0	0	60
Replicate 1	2	0	0	0	60	80
Replicate 2	2	0	0	80	80	80
Replicate 3	2	0	0	0	60	80
Replicate 1	3	0	0	20	100	100
Replicate 2	3	0	0	20	100	100
Replicate 3	3	0	0	40	80	100
Replicate 1	4	0	0	100	100	100
Replicate 2	4	0	0	100	100	100
Replicate 3	4	0	0	40	100	100
Replicate 1	5	0	0	60	100	100
Replicate 2	5	0	0	40	100	100
Replicate 3	5	0	0	60	80	100
Replicate 1	6	0	0	60	100	100
Replicate 2	6	0	0	60	100	100
Replicate 3	6	0	0	60	100	100
Replicate 1	10	0	0	100	100	100
Replicate 2	10	0	0	100	100	100
Replicate 3	10	0	0	60	100	100
Replicate 1	24	0	0	40	100	100
Replicate 2	24	0	0	60	100	100
Replicate 3	24	0	0	100	100	100

Appendix F Daphnia magna 72 Hour Mortality Bioassay TBT Data

Percentage of dead *Daphnia magna* after 72 hour exposure

	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L	100 µg/L
Replicate 1	40	60	100	100	100
Replicate 2	40	40	100	100	100
Replicate 3	40	40	100	100	100

Appendix G Hyalella azteca Motility Bioassay TBT Data

Swimming Events

Percentage of *Hyalella azteca* performing swimming events

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	0	100	100	100	100	100
Replicate 2	0	100	100	100	100	100
Replicate 3	0	100	100	100	100	100
Replicate 1	1	100	100	40	0	0
Replicate 2	1	100	100	80	0	40
Replicate 3	1	100	100	60	0	0
Replicate 1	2	100	100	0	20	0
Replicate 2	2	100	100	0	20	0
Replicate 3	2	100	100	0	20	0
Replicate 1	3	100	100	0	40	0
Replicate 2	3	100	100	0	0	0
Replicate 3	3	100	100	0	0	0
Replicate 1	4	100	100	40	20	0
Replicate 2	4	100	100	0	0	0
Replicate 3	4	100	100	0	0	0
Replicate 1	5	100	100	20	0	0
Replicate 2	5	100	100	0	0	0
Replicate 3	5	100	100	0	0	0
Replicate 1	6	100	100	20	0	0
Replicate 2	6	100	100	0	0	0
Replicate 3	6	100	100	0	0	0
Replicate 1	12	100	100	0	0	0
Replicate 2	12	100	100	0	0	0
Replicate 3	12	100	100	0	0	0
Replicate 1	24	100	100	0	0	0
Replicate 2	24	100	100	0	0	0
Replicate 3	24	100	100	0	0	0

Substrate Crawling

Percentage of *Hyalella azteca* performing crawling behaviour on substrate

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	0	100	100	100	100	100
Replicate 2	0	100	100	100	100	100
Replicate 3	0	100	100	100	100	100
Replicate 1	1	100	100	80	0	40
Replicate 2	1	100	100	100	60	40
Replicate 3	1	100	100	80	0	60
Replicate 1	2	100	100	20	40	20
Replicate 2	2	100	100	40	20	20
Replicate 3	2	100	100	0	80	0
Replicate 1	3	100	100	0	40	0
Replicate 2	3	100	100	60	0	0
Replicate 3	3	100	100	60	60	0
Replicate 1	4	100	100	40	100	0
Replicate 2	4	100	100	40	0	0
Replicate 3	4	100	100	40	40	0
Replicate 1	5	100	100	20	20	0
Replicate 2	5	100	100	0	0	0
Replicate 3	5	100	100	40	0	0
Replicate 1	6	100	100	40	20	0
Replicate 2	6	100	100	0	20	0
Replicate 3	6	100	100	40	20	0
Replicate 1	12	100	100	20	0	0
Replicate 2	12	100	100	0	20	0
Replicate 3	12	100	100	0	0	0
Replicate 1	24	100	100	0	0	0
Replicate 2	24	100	100	40	20	0
Replicate 3	24	100	100	0	0	0

Immobilization

Percentage of immobilized *Hyalella azteca*

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	0	0	0	0	0	0
Replicate 2	0	0	0	0	0	0
Replicate 3	0	0	0	0	0	0
Replicate 1	1	0	0	0	100	60
Replicate 2	1	0	0	0	40	40
Replicate 3	1	0	0	0	0	40
Replicate 1	2	0	0	80	40	60
Replicate 2	2	0	0	60	60	100
Replicate 3	2	0	0	0	20	40
Replicate 1	3	0	0	100	0	100
Replicate 2	3	0	0	40	100	100
Replicate 3	3	0	0	40	40	100
Replicate 1	4	0	0	60	0	100
Replicate 2	4	0	0	60	100	100
Replicate 3	4	0	0	60	60	100
Replicate 1	5	0	0	80	80	100
Replicate 2	5	0	0	100	100	100
Replicate 3	5	0	0	60	100	100
Replicate 1	6	0	0	60	80	100
Replicate 2	6	0	0	100	80	100
Replicate 3	6	0	0	60	80	100
Replicate 1	12	0	0	80	100	100
Replicate 2	12	0	0	100	80	100
Replicate 3	12	0	0	100	100	100
Replicate 1	24	0	0	100	100	100
Replicate 2	24	0	0	60	80	100
Replicate 3	24	0	0	100	100	100

Burrowing

Percentage of *Hyalella azteca* burrowed

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	0	0	0	0	0	0
Replicate 2	0	0	0	0	0	0
Replicate 3	0	0	0	0	0	0
Replicate 1	1	0	0	0	0	20
Replicate 2	1	0	0	20	40	20
Replicate 3	1	0	0	20	20	20
Replicate 1	2	0	0	0	0	40
Replicate 2	2	0	0	0	20	60
Replicate 3	2	0	0	0	20	20
Replicate 1	3	0	0	0	0	0
Replicate 2	3	0	0	0	0	20
Replicate 3	3	0	0	0	0	20
Replicate 1	4	0	0	20	0	40
Replicate 2	4	0	0	0	0	40
Replicate 3	4	0	0	0	0	40
Replicate 1	5	0	0	0	0	40
Replicate 2	5	0	0	60	0	0
Replicate 3	5	0	0	40	0	40
Replicate 1	6	0	0	0	0	40
Replicate 2	6	0	0	40	0	100
Replicate 3	6	0	0	40	0	60
Replicate 1	12	0	0	0	20	20
Replicate 2	12	0	0	40	0	80
Replicate 3	12	0	0	40	40	60
Replicate 1	24	0	0	0	0	0
Replicate 2	24	0	0	20	80	80

Replicate 3	24	0	0	40	40	80

Grouping

Percentage of *Hyalella azteca* grouping

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	0	0	0	0	0	0
Replicate 2	0	0	0	0	0	0
Replicate 3	0	0	0	0	0	0
Replicate 1	1	0	0	0	60	0
Replicate 2	1	0	0	0	0	0
Replicate 3	1	0	0	0	0	0
Replicate 1	2	0	0	0	0	0
Replicate 2	2	0	0	0	0	0
Replicate 3	2	0	0	0	0	0
Replicate 1	3	0	0	0	0	0
Replicate 2	3	0	0	60	0	60
Replicate 3	3	0	0	0	0	0
Replicate 1	4	0	0	0	0	80
Replicate 2	4	0	0	60	0	60
Replicate 3	4	0	0	0	0	60
Replicate 1	5	0	0	0	0	0
Replicate 2	5	0	0	60	0	100
Replicate 3	5	0	0	0	0	60
Replicate 1	6	0	0	0	0	40
Replicate 2	6	0	0	40	60	80
Replicate 3	6	0	0	0	40	60
Replicate 1	12	0	0	0	0	80
Replicate 2	12	0	0	60	80	60
Replicate 3	12	0	0	0	60	60
Replicate 1	24	0	0	0	0	60
Replicate 2	24	0	0	0	100	60
Replicate 3	24	0	0	60	60	60

Body Length

Percentage of *Hyalella azteca* displaying shortened body length

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	0	0	0	0	0	0
Replicate 2	0	0	0	0	0	0
Replicate 3	0	0	0	0	0	0
Replicate 1	1	0	0	40	80	0
Replicate 2	1	0	0	0	0	0
Replicate 3	1	0	0	20	40	80
Replicate 1	2	0	0	40	40	60
Replicate 2	2	0	0	0	40	100
Replicate 3	2	0	0	20	40	60
Replicate 1	3	0	0	40	40	100
Replicate 2	3	0	0	20	100	100
Replicate 3	3	0	0	20	60	60
Replicate 1	4	0	0	60	80	100
Replicate 2	4	0	0	40	100	100
Replicate 3	4	0	0	40	80	60
Replicate 1	5	0	0	60	80	100
Replicate 2	5	0	0	80	100	100
Replicate 3	5	0	0	60	80	100
Replicate 1	6	0	0	80	60	100
Replicate 2	6	0	0	40	80	100
Replicate 3	6	0	0	60	80	100
Replicate 1	12	0	0	80	80	100
Replicate 2	12	0	0	60	100	100
Replicate 3	12	0	0	60	80	100
Replicate 1	24	0	0	60	100	100
Replicate 2	24	0	0	60	80	100
Replicate 3	24	0	0	60	80	100

Body Orientation

Percentage of *Hyalella azteca* displaying abnormal body orientation

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	0	0	0	0	0	0
Replicate 2	0	0	0	0	0	0
Replicate 3	0	0	0	0	0	0
Replicate 1	1	0	0	0	0	0
Replicate 2	1	0	0	0	0	0
Replicate 3	1	0	0	0	0	0
Replicate 1	2	0	0	0	20	40
Replicate 2	2	0	0	0	0	0
Replicate 3	2	0	0	0	20	0
Replicate 1	3	0	0	20	20	40
Replicate 2	3	0	0	0	0	0
Replicate 3	3	0	0	20	20	20
Replicate 1	4	0	0	0	0	60
Replicate 2	4	0	0	20	0	0
Replicate 3	4	0	0	20	40	40
Replicate 1	5	0	0	20	0	20
Replicate 2	5	0	0	0	0	0
Replicate 3	5	0	0	20	40	40
Replicate 1	6	0	0	20	0	20
Replicate 2	6	0	0	20	0	0
Replicate 3	6	0	0	20	40	40
Replicate 1	12	0	0	20	0	20
Replicate 2	12	0	0	20	0	0
Replicate 3	12	0	0	20	40	40
Replicate 1	24	0	0	0	40	40
Replicate 2	24	0	0	0	0	0
Replicate 3	24	0	0	20	40	40

Appendix H Hyalella azteca Motility Bioassay Atrazine Data

Swimming Events

Percentage of *Hyalella azteca* performing swimming events

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	100	100	40	40	40
Replicate 2	1	100	100	100	20	40
Replicate 3	1	100	100	0	60	40
Replicate 1	2	100	100	0	0	0
Replicate 2	2	100	100	40	20	20
Replicate 3	2	100	100	0	20	20
Replicate 1	3	100	100	20	0	0
Replicate 2	3	80	60	20	20	0
Replicate 3	3	100	100	100	40	40
Replicate 1	4	60	80	20	20	0
Replicate 2	4	100	100	0	20	0
Replicate 3	4	80	60	0	0	0
Replicate 1	5	100	100	20	0	0
Replicate 2	5	100	100	0	0	20
Replicate 3	5	100	100	0	0	20
Replicate 1	6	100	100	20	0	0
Replicate 2	6	100	100	0	0	0
Replicate 3	6	80	80	0	20	0
Replicate 1	10	60	60	0	0	0
Replicate 2	10	0	20	0	0	0
Replicate 3	10	0	20	0	0	0
Replicate 1	24	0	20	0	0	0
Replicate 2	24	60	60	20	0	0
Replicate 3	24	60	40	0	0	20

Substrate Crawling

Percentage of *Hyalella azteca* crawling on substrate

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	100	100	20	60	80
Replicate 2	1	100	100	100	20	100
Replicate 3	1	100	100	0	60	80
Replicate 1	2	100	100	0	0	0
Replicate 2	2	100	100	100	80	40
Replicate 3	2	100	100	0	40	40
Replicate 1	3	100	100	20	0	0
Replicate 2	3	100	100	40	20	0
Replicate 3	3	100	100	0	60	40
Replicate 1	4	100	100	20	0	0
Replicate 2	4	100	100	40	20	0
Replicate 3	4	100	100	0	20	20
Replicate 1	5	100	100	20	0	0
Replicate 2	5	100	100	20	20	20
Replicate 3	5	100	100	20	0	20
Replicate 1	6	100	100	20	0	0
Replicate 2	6	100	100	0	0	0
Replicate 3	6	100	100	0	20	0
Replicate 1	10	100	100	0	0	0
Replicate 2	10	100	100	20	0	0
Replicate 3	10	100	100	20	0	0
Replicate 1	24	40	0	0	0	0
Replicate 2	24	100	100	40	0	0
Replicate 3	24	100	100	0	0	20

Immobilization

Percentage of immobilized *Hyalella azteca*

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	0	0	60	40	60
Replicate 2	1	0	0	0	80	60
Replicate 3	1	0	0	100	60	60
Replicate 1	2	0	0	100	100	100
Replicate 2	2	0	0	0	60	60
Replicate 3	2	0	0	100	60	60
Replicate 1	3	0	0	80	100	100
Replicate 2	3	0	0	60	80	100
Replicate 3	3	0	0	100	40	60
Replicate 1	4	0	0	80	100	100
Replicate 2	4	0	0	60	80	100
Replicate 3	4	0	0	100	80	80
Replicate 1	5	0	0	80	100	100
Replicate 2	5	0	0	80	80	80
Replicate 3	5	0	0	80	100	80
Replicate 1	6	0	0	80	100	100
Replicate 2	6	0	0	100	100	100
Replicate 3	6	0	0	100	80	100
Replicate 1	10	0	0	100	100	100
Replicate 2	10	0	0	100	100	100
Replicate 3	10	0	0	80	100	100
Replicate 1	24	100	80	100	100	100
Replicate 2	24	0	0	60	100	100
Replicate 3	24	0	0	100	100	80

Burrowing

Percentage of *Hyalella azteca* burrowed

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	0	0	60	40	40
Replicate 2	1	0	0	0	20	0
Replicate 3	1	0	0	100	0	0
Replicate 1	2	0	0	80	100	60
Replicate 2	2	0	0	0	40	20
Replicate 3	2	0	0	100	0	0
Replicate 1	3	0	0	80	80	80
Replicate 2	3	0	0	40	60	80
Replicate 3	3	0	0	100	0	20
Replicate 1	4	0	0	80	80	80
Replicate 2	4	0	0	40	60	40
Replicate 3	4	0	0	80	80	40
Replicate 1	5	0	0	80	80	60
Replicate 2	5	0	0	20	40	40
Replicate 3	5	0	0	80	100	40
Replicate 1	6	0	0	80	100	100
Replicate 2	6	0	0	40	60	80
Replicate 3	6	0	0	60	40	60
Replicate 1	10	0	0	100	100	60
Replicate 2	10	0	0	80	100	80
Replicate 3	10	0	0	60	60	60
Replicate 1	24	60	20	60	60	20
Replicate 2	24	0	0	60	80	60
Replicate 3	24	20	20	20	60	40

Grouping

Percentage of *Hyalella azteca* grouping

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	0	0	60	0	0
Replicate 2	1	0	0	0	0	0
Replicate 3	1	0	0	80	0	0
Replicate 1	2	0	0	100	100	100
Replicate 2	2	0	0	0	0	60
Replicate 3	2	0	0	100	0	60
Replicate 1	3	0	0	80	100	100
Replicate 2	3	0	0	80	80	80
Replicate 3	3	0	0	100	0	0
Replicate 1	4	0	0	80	100	100
Replicate 2	4	0	0	60	100	100
Replicate 3	4	0	0	100	60	80
Replicate 1	5	0	0	80	100	100
Replicate 2	5	0	0	80	0	60
Replicate 3	5	0	0	80	100	0
Replicate 1	6	0	0	80	100	100
Replicate 2	6	0	0	100	80	100
Replicate 3	6	0	0	80	60	80
Replicate 1	10	0	0	100	100	80
Replicate 2	10	0	0	100	100	100
Replicate 3	10	0	0	60	100	80
Replicate 1	24	0	100	80	100	60
Replicate 2	24	0	0	60	80	100
Replicate 3	24	0	0	60	100	80

Body Length

Percentage of *Hyalella azteca* displaying shortened body length

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	0	0	60	20	20
Replicate 2	1	0	0	0	40	40
Replicate 3	1	0	0	100	40	40
Replicate 1	2	0	0	100	100	100
Replicate 2	2	0	0	20	80	80
Replicate 3	2	0	0	100	80	60
Replicate 1	3	0	0	80	100	60
Replicate 2	3	0	0	80	80	100
Replicate 3	3	0	0	100	60	80
Replicate 1	4	0	0	80	80	80
Replicate 2	4	0	0	40	100	100
Replicate 3	4	0	0	100	100	100
Replicate 1	5	0	0	80	80	100
Replicate 2	5	0	0	100	100	80
Replicate 3	5	0	0	100	100	80
Replicate 1	6	0	0	80	100	100
Replicate 2	6	0	0	100	100	100
Replicate 3	6	0	0	100	100	80
Replicate 1	10	0	0	100	100	80
Replicate 2	10	0	0	80	100	100
Replicate 3	10	0	0	100	100	100
Replicate 1	24	60	40	80	100	40
Replicate 2	24	0	0	60	100	100
Replicate 3	24	0	0	60	100	80

Body Orientation

Percentage of *Hyalella azteca* displaying abnormal body orientation

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	0	0	0	0	0
Replicate 2	1	0	0	0	0	0
Replicate 3	1	0	0	0	0	0
Replicate 1	2	0	0	0	0	0
Replicate 2	2	0	0	0	0	0
Replicate 3	2	0	0	0	0	0
Replicate 1	3	0	0	0	0	0
Replicate 2	3	0	0	0	0	0
Replicate 3	3	0	0	0	0	0
Replicate 1	4	0	0	0	0	0
Replicate 2	4	0	0	0	0	40
Replicate 3	4	0	0	0	0	40
Replicate 1	5	0	0	0	0	0
Replicate 2	5	0	0	40	0	20
Replicate 3	5	0	0	0	0	0
Replicate 1	6	0	0	0	0	0
Replicate 2	6	0	0	0	0	0
Replicate 3	6	0	0	0	20	40
Replicate 1	10	0	0	0	0	0
Replicate 2	10	0	0	0	0	0
Replicate 3	10	0	0	0	40	60
Replicate 1	24	0	0	0	0	0
Replicate 2	24	0	0	0	0	0
Replicate 3	24	0	0	0	20	20

Time (Hours)	0	0.25	0.5	0.75	1	1.25	1.5	1.75	2	2.25	2.5	2.75	3	3.25	3.5	3.75	4	4.25	4.5

Appendix I Lumbriculus variegatus Burrowing Behaviour TBT Data

Time (Hours)	0	0.25	0.5	0.75	1	1.25	1.5	1.75	2	2.25	2.5	2.75	3	3.25	3.5	3.75	4	4.25	4.5
Number of *Lumbriculus variegatus* Burrowed (/10)																			
Reference																			
Replicate 1	0	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Replicate 2	0	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Replicate 3	0	7	8	7	8	8	7	8	7	7	8	8	9	8	7	6	9	10	10
Replicate 4	0	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Replicate 5	0	6	5	6	7	6	7	7	8	8	8	8	9	8	10	10	10	10	10
Average	0	8.6	8.6	8.6	9	8.8	8.8	9	9	9	9.2	9.2	10	9.2	9.4	9.2	10	10	10
0.1% DMSO																			
Replicate 1	0	5	4	4	6	6	5	7	9	6	7	8	8	9	9	8	8	9	9
Replicate 2	0	7	6	5	5	6	6	7	7	7	7	7	7	7	8	7	7	7	8
Replicate 3	0	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Replicate 4	0	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Replicate 5	0	9	10	10	10	10	10	9	9	10	10	10	10	10	9	9	9	9	9
Average	0	8.2	8	7.8	8.2	8.4	8.2	8.6	9	8.6	8.8	9	9	9.2	9.2	8.8	9	9	9.2
0.1 µg/L TBT																			
Replicate 1	0	9	10	10	10	9	9	10	9	10	10	10	10	10	10	10	10	10	10
Replicate 2	0	7	9	9	10	10	10	10	10	9	9	9	10	10	10	10	10	10	10
Replicate 3	0	8	9	9	9	8	7	8	8	8	7	7	7	7	7	7	8	9	9
Replicate 4	0	9	10	10	10	9	8	8	9	10	10	10	10	10	10	10	10	10	10
Replicate 5	0	8	7	8	8	7	8	7	7	7	7	7	8	8	8	8	8	8	8
Average	0	8.2	9	9.2	9.4	8.6	8.4	8.6	9	8.8	8.6	8.6	9	9	9	9	9	9.4	9.4

1.0 µg/L TBT																			
Replicate 1	0	8	8	8	8	9	9	8	8	8	8	8	9	9	8	9	9	9	9
Replicate 2	0	10	10	10	10	9	9	9	8	8	10	10	10	10	10	10	10	10	10
Replicate 3	0	3	8	8	8	10	10	8	7	8	8	8	8	8	8	7	8	8	8
Replicate 4	0	6	7	9	8	9	8	7	7	10	10	10	10	10	10	10	10	10	10
Replicate 5	0	7	6	7	8	7	9	8	9	9	8	9	9	7	9	10	10	10	10
Average	0	6.8	7.8	8.4	8.4	8.8	9	8	8	8.6	8.8	9	9	8.8	9	9.2	9	9.4	9.4
10 µg/L TBT																			
Replicate 1	0	4	5	6	8	8	8	9	8	8	8	8	9	10	9	10	10	10	10
Replicate 2	0	4	7	9	8	8	9	9	8	7	7	8	10	10	10	10	10	10	10
Replicate 3	0	3	5	7	7	6	6	8	8	9	9	10	9	8	7	8	8	8	8
Replicate 4	0	8	9	10	8	9	10	9	10	10	9	10	9	8	9	10	10	10	10
Replicate 5	0	2	1	2	7	4	5	4	9	8	8	9	9	10	9	10	9	9	10
Average	0	4.2	5.4	6.8	7.6	7	7.6	7.8	9	8.4	8.2	9	9	9.2	8.8	9.6	9	9.4	9.6

Appendix J Lumbriculus variegatus Burrowing Behaviour Atrazine Data

Time (Hours)	0	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6
Number of *Lumbriculus variegatus* Burrowed (0/10)													
Reference													
Replicate 1	0	4	4	4	5	5	9	5	4	4	6	8	8
Replicate 2	0	4	5	7	7	8	9	9	9	10	10	9	9
Replicate 3	0	7	9	8	8	9	9	9	9	9	10	10	9
Replicate 4	0	4	7	5	10	10	5	5	7	7	7	7	7
Replicate 5	0	1	6	4	5	6	6	6	7	7	7	7	9
Average	0	4	6.2	5.6	7	7.6	7.6	6.8	7.2	7.4	8	8.2	8.4
0.1% DMSO													
Replicate 1	0	9	7	8	8	9	9	9	9	9	9	9	8
Replicate 2	0	4	4	4	6	7	8	8	8	7	9	6	6
Replicate 3	0	4	4	5	4	5	5	7	6	6	6	6	6
Replicate 4	0	4	5	4	5	7	7	7	7	8	8	8	8
Replicate 5	0	7	8	8	8	9	6	8	7	8	8	6	7
Average	0	5.6	5.6	5.8	6	7.4	7	7.8	7.4	7.6	8	7	7
5 µg/L Atrazine													
Replicate 1	0	1	1	1	4	4	4	4	7	7	8	8	7
Replicate 2	0	4	4	7	4	4	4	4	4	4	8	8	6
Replicate 3	0	2	3	3	4	4	4	4	4	4	4	6	3
Replicate 4	0	4	5	3	7	7	7	7	3	5	3	3	4
Replicate 5	0	4	5	5	5	5	5	5	7	4	8	9	10
Average	0	3	3.6	3.8	5	4.8	4.8	4.8	5	4.8	6.2	6.8	6
50 µg/L Atrazine													
Replicate 1	0	6	6	6	6	9	10	10	9	9	10	10	10
Replicate 2	0	3	5	5	6	6	6	6	9	8	8	7	7
Replicate 3	0	2	0	3	3	4	5	4	2	5	4	6	6
Replicate 4	0	4	6	5	2	5	5	5	6	6	9	5	6
Replicate 5	0	4	4	2	5	7	7	7	7	7	7	7	7
Average	0	3.8	4.2	4.2	4	6.2	6.6	6.4	6.6	7	7.6	7	7.2
100 µg/L Atrazine													
Replicate 1	0	3	3	2	4	6	6	8	6	6	6	6	6
Replicate 2	0	3	7	6	7	7	8	8	8	6	7	8	8
Replicate 3	0	5	7	7	7	7	7	9	8	8	8	8	10
Replicate 4	0	4	4	4	4	4	6	6	5	4	5	6	6
Replicate 5	0	1	1	5	5	5	5	5	8	8	7	9	7

Average	0	3.2	4.4	4.8	5	5.8	6.4	7.2	7	6.4	6.6	7.4	7.4

Appendix K *Lumbriculus variegatus* Grouping Behaviour TBT Data

Number of *Lumbriculus variegatus* Grouping (/10)																		
Time (Hours)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**	**9**	**24**	**30**	**42**	**48**
Reference																		
Replicate 1	0	0	0	2	0	6	10	8	7	9	7	8	8	9	9	5	8	9
Replicate 2	0	0	2	0	3	8	9	8	7	7	7	8	0	8	4	7	7	9
Replicate 3	0	0	0	3	3	3	9	10	9	10	4	8	8	7	4	8	6	9
Replicate 4	0	0	0	2	2	0	5	7	6	9	6	8	10	6	10	6	9	7
Average	0	0	0.5	1.75	2	4.25	8.25	8.25	7.25	8.75	6	8	6.5	7.5	6.75	6.5	7.5	8.5
0.1% DMSO																		
Replicate 1	0	0	2	0	2	7	8	5	7	7	4	8	4	4	5	3	10	6
Replicate 2	0	0	0	0	0	4	8	7	7	6	4	9	9	7	9	10	9	7
Replicate 3	0	0	0	2	2	4	0	0	5	8	8	9	6	4	10	7	6	7
Replicate 4	0	0	0	0	2	7	10	10	10	2	0	9	10	5	5	6	9	7
Average	0	0	0.5	0.5	1.5	5.5	6.5	5.5	7.25	5.75	4	8.75	7.25	5	7.25	6.5	8.5	6.75
10 µg/L TBT																		
Replicate 1	0	0	2	0	2	7	5	6	7	6	3	8	8	3	7	9	4	10
Replicate 2	0	0	0	0	3	2	6	6	9	7	0	3	0	5	8	7	8	0
Replicate 3	0	0	0	2	7	9	8	0	6	10	10	10	0	7	9	7	8	5
Replicate 4	0	0	0	3	0	0	6	8	9	7	2	5	8	2	9	0	5	10
Average	0	0	0.5	1.25	3	4.5	6.25	5	7.75	7.5	3.75	6.5	4	4.25	8.25	5.75	6.25	6.25
50 µg/L TBT																		
Replicate 1	0	0	3	0	0	9	4	7	0	2	4	0	0	0	2	7	0	10
Replicate 2	0	0	0	0	6	3	8	8	10	8	2	4	3	9	2	7	6	10
Replicate 3	0	0	0	0	4	7	3	0	10	9	5	8	9	3	10	0	6	10
Replicate 4	0	0	0	0	4	3	5	9	10	7	8	9	5	9	4	8	4	10
Average	0	0	0.8	0	3.5	5.5	5	6	7.5	6.5	4.75	5.25	4.25	5.25	4.5	5.5	4	10
100 µg/L TBT																		
Replicate 1	0	0	0	0	4	7	2	3	7	8	5	7	7	6	9	0	7	5
Replicate 2	0	2	2	2	2	3	2	5	6	8	5	4	4	4	9	5	4	10
Replicate 3	0	0	0	3	2	0	0	4	2	4	5	5	7	2	4	4	8	3

Replicate 4	0	0	0	3	3	0	4	2	0	3	8	8	3	4	4	5	5	8
Average	0	0.5	0.5	2	2.8	2.5	2	3.5	3.75	5.75	5.75	6	5.25	4	6.5	3.5	6	6.5

Appendix L Lumbriculus variegatus Grouping Behaviour Atrazine Data

Number of *Lumbriculus variegatus* Grouping (/10)													
Time (Hours)	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**5.5**	**6**
Reference													
Replicate 1	0	0	0	0	2	2	4	8	10	10	7	10	10
Replicate 2	0	5	7	7	6	8	8	5	9	10	2	0	0
Replicate 3	0	0	4	5	5	4	7	10	9	0	6	10	8
Replicate 4	0	7	10	7	6	8	8	9	8	10	10	7	0
Average	0	3	5.25	4.75	4.75	5.5	6.75	8	9	7.5	6.25	6.75	4.5
0.1% DMSO													
Replicate 1	0	0	0	0	0	0	0	0	9	9	8	0	0
Replicate 2	0	5	7	7	8	7	9	9	7	9	10	10	10
Replicate 3	0	9	7	8	9	6	9	10	10	8	7	10	10
Replicate 4	0	0	0	0	0	0	0	0	0	4	0	4	0
Average	0	3.5	3.5	3.75	4.25	3.25	4.5	4.75	6.5	7.5	6.25	6	5
10 µg/L TBT													
Replicate 1	0	0	0	0	0	0	0	0	0	9	5	0	5
Replicate 2	0	0	9	9	10	10	10	9	10	9	9	9	9
Replicate 3	0	0	0	0	5	8	10	10	10	10	10	9	8
Replicate 4	0	2	9	6	7	8	6	9	5	9	10	10	9
Average	0	0.5	4.5	3.75	5.5	6.5	6.5	7	6.25	9.25	8.5	7	7.75
50 µg/L TBT													
Replicate 1	0	3	6	6	8	9	10	9	10	10	7	5	7
Replicate 2	0	0	0	0	6	7	8	5	7	8	9	5	0
Replicate 3	0	8	5	5	5	6	6	7	7	8	7	9	8
Replicate 4	0	2	3	3	3	5	5	8	8	9	10	10	10
Average	0	3.25	3.5	3.5	5.5	6.75	7.25	7.25	8	8.75	8.25	7.25	6.25
100 µg/L TBT													
Replicate 1	0	8	6	6	6	7	8	10	8	9	10	10	9
Replicate 2	0	4	8	5	7	6	5	6	8	10	9	3	0
Replicate 3	0	8	7	7	6	7	7	6	9	8	7	8	5

Replicate 4	0	9	9	8	6	6	4	10	9	10	10	10	0
Average	0	7.25	7.5	6.5	6.25	6.5	6	8	8.5	9.25	9	7.75	3.5

Appendix M Lumbriculus variegatus Motility Bioassay TBT

Vessel Position

Percentage of *Lumbriculus variegatus* in middle of bioassay vessel

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	1	10	0	0	10	10
Replicate 2	1	0	20	10	0	10
Replicate 3	1	10	0	0	0	10
Replicate 4	1	30	0	20	10	30
Replicate 1	3	0	0	10	0	0
Replicate 2	3	0	0	0	0	0
Replicate 3	3	0	20	0	0	0
Replicate 4	3	0	0	0	0	20
Replicate 1	5	0	0	0	50	0
Replicate 2	5	0	0	0	50	0
Replicate 3	5	0	0	0	0	0
Replicate 4	5	0	0	0	0	0
Replicate 1	24	0	0	0	0	0
Replicate 2	24	0	0	0	0	0
Replicate 3	24	0	0	0	0	0
Replicate 4	24	0	0	0	0	0

Locomotion Style

Percentage of organisms displaying abnormal movement style

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	1	0	0	0	0	0
Replicate 2	1	0	0	0	0	0
Replicate 3	1	0	0	0	0	0
Replicate 4	1	0	0	0	0	0
Replicate 1	3	0	0	40	50	100
Replicate 2	3	0	0	20	0	67
Replicate 3	3	N/A - all in groups	0	0	100	100
Replicate 4	3	0	N/A - all in groups	25	50	100
Replicate 1	5	0	0	33	100	100
Replicate 2	5	0	0	100	100	100
Replicate 3	5	0	0	N/A - all in groups	100	100
Replicate 4	5	0	0	100	100	100
Replicate 1	24	0	0	100	100	N/A - all in groups
Replicate 2	24	0	N/A - all in groups	100	100	100
Replicate 3	24	0	N/A - all in groups	100	N/A - all in groups	100
Replicate 4	24	0	10	100	N/A - all in groups	N/A - all in groups

Immobilization

Percentage of organisms immobilized

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	1	0	0	0	0	0
Replicate 2	1	0	0	0	0	0
Replicate 3	1	0	0	0	0	0
Replicate 4	1	0	0	0	0	0
Replicate 1	3	0	0	40	0	100
Replicate 2	3	0	0	20	0	67
Replicate 3	3	0	0	20	0	80
Replicate 4	3	0	0	25	50	80
Replicate 1	5	0	0	33	40	33
Replicate 2	5	0	0	0	20	60
Replicate 3	5	0	0	0	25	60
Replicate 4	5	0	0	10	100	80
Replicate 1	24	0	0	0	10	100
Replicate 2	24	0	0	0	80	100
Replicate 3	24	0	0	100	N/A - all grouping	50
Replicate 4	24	0	10	100	N/A - all grouping	80

Body Length

Percentage of organisms displaying shortened body length

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	1	0	0	0	0	0
Replicate 2	1	0	0	0	10	0
Replicate 3	1	0	0	0	0	0
Replicate 4	1	0	0	0	0	0
Replicate 1	3	0	0	0	0	100
Replicate 2	3	0	0	0	0	67
Replicate 3	3	N/A - all in group	0	0	0	80
Replicate 4	3	0	0	25	0	80
Replicate 1	5	0	0	17	40	100
Replicate 2	5	0	0	0	40	75
Replicate 3	5	0	0	0	25	75
Replicate 4	5	0	0	0	100	100
Replicate 1	24	0	20	40	30	N/A - all in groups
Replicate 2	24	0	N/A - all in groups	0	0	100
Replicate 3	24	0	N/A - all in groups	100	N/A - all in groups	100
Replicate 4	24	100	10	100	N/A - all in groups	N/A - all in groups

Body Orientation

Percentage of organisms displaying abnormal body orientation

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	1	0	0	0	0	0
Replicate 2	1	0	0	0	0	0
Replicate 3	1	0	0	0	0	0
Replicate 4	1	0	0	0	0	0
Replicate 1	3	0	0	20	0	100
Replicate 2	3	0	0	20	0	67
Replicate 3	3	N/A - all in groups	0	0	100	80
Replicate 4	3	0	0	25	50	80
Replicate 1	5	0	0	17	40	33
Replicate 2	5	0	0	0	40	25
Replicate 3	5	0	0	0	25	25
Replicate 4	5	0	0	20	100	0
Replicate 1	24	0	0	0	10	N/A - all in groups
Replicate 2	24	20	N/A - all in groups	0	0	100
Replicate 3	24	20	N/A - all in groups	100	N/A - all in groups	50
Replicate 4	24	0	10	100	N/A - all in groups	N/A - all in groups

Group Movement

Percentage of organisms displaying movement in, out and within groups

	Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	1	N/A - no grouping	N/A - no grouping	100	N/A - no grouping	100
Replicate 2	1	N/A - no grouping	N/A - no grouping	100	100	100
Replicate 3	1	100	N/A - no grouping	N/A - no grouping	100	100
Replicate 4	1	N/A - no grouping	100	100	N/A - no grouping	N/A - no grouping
Replicate 1	3	100	100	0	100	80
Replicate 2	3	100	100	100	100	100
Replicate 3	3	100	100	100	100	N/A - no grouping
Replicate 4	3	N/A - no grouping	100	100	60	N/A - no grouping
Replicate 1	5	100	100	75	100	0
Replicate 2	5	100	100	N/A - no grouping	100	17
Replicate 3	5	N/A - no grouping	100	20	67	17
Replicate 4	5	N/A - no grouping	100	100	0	0
Replicate 1	24	100	N/A - no grouping	100	N/A - no grouping	0
Replicate 2	24	100	80	15	0	0
Replicate 3	24	N/A - no grouping	100	0	50	0
Replicate 4	24	80	N/A - no grouping	0	50	20

Appendix N Lumbriculus variegatus Motility Bioassay Atrazine

Vessel Position

Percentage of organisms in middle of bioassay vessel

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	0	0	0	0	20
Replicate 2	1	0	0	0	0	0
Replicate 3	1	0	0	0	0	0
Replicate 4	1	0	10	0	0	0
Replicate 1	3	0	0	0	0	0
Replicate 2	3	0	0	0	0	0
Replicate 3	3	0	0	0	0	0
Replicate 4	3	0	0	0	0	0
Replicate 1	6	0	20	20	0	40
Replicate 2	6	0	0	0	0	0
Replicate 3	6	0	0	0	0	0
Replicate 4	6	0	0	0	20	0
Replicate 1	9	0	0	0	0	0
Replicate 2	9	0	0	0	0	0
Replicate 3	9	0	0	0	0	0
Replicate 4	9	0	0	0	0	0
Replicate 1	24	0	0	0	0	10
Replicate 2	24	0	0	0	0	0
Replicate 3	24	0	0	0	0	0
Replicate 4	24	0	0	0	0	0

Locomotion Style

Percentage of organisms displaying abnormal movement style

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	0	0	0	0	40
Replicate 2	1	0	0	0	0	40
Replicate 3	1	0	0	0	20	20
Replicate 4	1	0	0	0	20	n/a - all in group
Replicate 1	3	0	0	0	n/a - all in group	100
Replicate 2	3	0	0	n/a - all in group	100	100
Replicate 3	3	0	0	10	100	100
Replicate 4	3	n/a - all in group	0	n/a - all in group	100	n/a - all in group
Replicate 1	6	n/a - all in group	0	50	50	100
Replicate 2	6	0	n/a - all in group	n/a	100	100
Replicate 3	6	0	n/a - all in group	n/a	100	100
Replicate 4	6	0	0	100	100	50
Replicate 1	9	0	0	100	n/a - all in group	100
Replicate 2	9	0	0	100	100	100
Replicate 3	9	0	0	0	100	100
Replicate 4	9	n/a - all in group	0	n/a - all in group	80	100
Replicate 1	24	0	n/a - all in group	n/a - all in group	80	100
Replicate 2	24	0	n/a - all in group	100	100	100
Replicate 3	24	n/a - all in group	0	100	100	100
Replicate 4	24	10	0	100	100	100

Immobilization

Percentage of immobilized organisms

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	0	0	0	0	0
Replicate 2	1	0	0	0	0	10
Replicate 3	1	0	0	0	0	0
Replicate 4	1	0	0	0	0	0
Replicate 1	3	0	0	0	0	2
Replicate 2	3	0	0	0	0	0
Replicate 3	3	0	0	10	10	20
Replicate 4	3	0	0	0	20	70
Replicate 1	6	0	0	20	10	0
Replicate 2	6	0	0	0	10	10
Replicate 3	6	0	0	0	0	20
Replicate 4	6	0	0	10	10	30
Replicate 1	9	0	0	0	0	40
Replicate 2	9	0	0	0	30	40
Replicate 3	9	0	0	0	30	20
Replicate 4	9	0	0	0	20	30
Replicate 1	24	10	0	20	30	60
Replicate 2	24	0	0	20	10	50
Replicate 3	24	0	20	30	20	20
Replicate 4	24	0	0	10	40	40

Body Length

Percentage of organisms displaying shortened body length

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	10	0	0	20	20
Replicate 2	1	0	0	0	0	10
Replicate 3	1	0	0	0	0	0
Replicate 4	1	0	0	0	0	0
Replicate 1	3	n/a - all in group	0	0	n/a - all in group	100
Replicate 2	3	n/a - all in group	0	n/a - all in group	0	0
Replicate 3	3	0	0	0	25	100
Replicate 4	3	n/a - all in group	0	n/a - all in group	n/a - all in group	n/a - all in group
Replicate 1	6	n/a - all in group	0	10	10	100
Replicate 2	6	0	n/a - all in group	n/a - all in group	20	0
Replicate 3	6	0	n/a - all in group	n/a - all in group	33	100
Replicate 4	6	0	0	30	100	60
Replicate 1	9	0	0	40	n/a - all in group	100
Replicate 2	9	10	0	30	30	100
Replicate 3	9	0	0	0	25	80
Replicate 4	9	n/a - all in group	0	0	30	43
Replicate 1	24	10	n/a - all in group	n/a - all in group	40	100
Replicate 2	24	0	n/a - all in group	40	10	50
Replicate 3	24	n/a - all in group	20	30	20	100
Replicate 4	24	50	0	100	70	40

Body Orientation

Percentage of organisms displaying abnormal body orientation

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	10	0	0	0	20
Replicate 2	1	0	0	0	0	10
Replicate 3	1	0	0	0	0	0
Replicate 4	1	0	0	0	0	0
Replicate 1	3	n/a - all in group	0	0	n/a - all in group	30
Replicate 2	3	n/a - all in group	0	n/a - all in group	0	0
Replicate 3	3	0	0	0	0	0
Replicate 4	3	n/a - all in group	0	n/a - all in group	n/a - all in group	n/a - all in group
Replicate 1	6	n/a - all in group	10	0	0	0
Replicate 2	6	0	n/a - all in group	n/a - all in group	0	0
Replicate 3	6	0	n/a - all in group	n/a - all in group	0	0
Replicate 4	6	0	0	0	0	0
Replicate 1	9	0	0	0	n/a - all in group	0
Replicate 2	9	0	0	0	20	33
Replicate 3	9	0	0	n/a - all in group	n/a - all in group	20
Replicate 4	9	n/a - all in group	0	0	20	20
Replicate 1	24	10	n/a - all in group	n/a - all in group	0	67
Replicate 2	24	0	n/a - all in group	10	10	0
Replicate 3	24	n/a - all in group	20	0	10	0
Replicate 4	24	0	0	100	10	20

Group Movement

Percentage of organisms moving in, out and within groups

	Time	Reference	0.1% DMSO	5 µg/L Atrazine	50 µg/L Atrazine	100 µg/L Atrazine
Replicate 1	1	100	100	100	100	100
Replicate 2	1	100	100	100	100	100
Replicate 3	1	100	100	100	100	100
Replicate 4	1	100	n/a - no groups	100	100	50
Replicate 1	3	100	100	100	100	100
Replicate 2	3	100	100	100	100	100
Replicate 3	3	100	100	100	90	70
Replicate 4	3	n/a - no grouping	100	100	80	30
Replicate 1	6	100	100	80	90	100
Replicate 2	6	100	100	70	90	90
Replicate 3	6	100	100	70	90	80
Replicate 4	6	n/a - no grouping	n/a - no groups	n/a - no grouping	100	100
Replicate 1	9	n/a - no grouping	n/a - no groups	100	100	60
Replicate 2	9	n/a - no grouping	n/a - no groups	n/a - no grouping	90	90
Replicate 3	9	n/a - no grouping	100	100	90	n/a - no grouping
Replicate 4	9	100	n/a - no groups	100	n/a - no grouping	100
Replicate 1	24	n/a - no grouping	100	80	n/a - no grouping	0
Replicate 2	24	100	100	n/a - no grouping	n/a - no grouping	0
Replicate 3	24	100	n/a - no groups	n/a - no grouping	100	100
Replicate 4	24	100	100	0	n/a - no grouping	n/a - no grouping

Appendix O Lumbriculus variegatus 72 Hour Mortality (TBT) Data

Percentage dead organisms after 72 hour exposure

	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
Replicate 1	0	0	0	10	30
Replicate 2	0	0	10	20	20
Replicate 3	0	0	0	20	40
Replicate 4	0	0	0	20	60
Average	0	0	2.5	17.5	37.5

Appendix P Daphnia magna Respiration Data (TBT)

Time	Dissolved oxygen content (umol/L water)				
	Reference	DMSO	10 µg/L	50 µg/L	100 µg/L
0	173.733697	183.04086	176.8360848	175.28489	179.938472
0	181.489666	175.28489	179.9384722	181.48967	169.080116
0	186.143247	178.38728	183.0408597	178.38728	178.387278
1	186.143247	178.38728	175.284891	152.01699	167.528922
1	183.04086	175.28489	175.284891	186.14325	165.977729
1	179.938472	172.1825	170.6313098	173.7337	164.426535
2	175.284891	175.28489	169.0801161	165.97773	165.977729
2	169.080116	172.1825	173.7336973	161.32415	161.324147
2	170.63131	181.48967	158.22176	167.52892	150.465791
3	167.528922	172.1825	169.0801161	176.83608	183.04086
3	170.63131	179.93847	170.6313098	181.48967	184.592053
3	161.324147	176.83608	164.4265349	178.38728	152.016985
4	172.182504	167.52892	173.7336973	170.63131	167.528922
4	170.63131	164.42653	173.7336973	176.83608	183.04086
4	162.875341	156.67057	167.5289224	169.08012	162.875341
5	178.387278	183.04086	187.6944408	186.14325	179.938472
5	139.607435	147.3634	152.0169851	155.11937	173.733697
5	162.875341	156.67057	181.4896659	165.97773	152.016985
6	159.772954	167.52892	173.7336973	145.81221	161.324147
6	162.875341	169.08012	176.8360848	176.83608	183.04086
6	156.670566	164.42653	167.5289224	156.67057	148.914598
12	150.465791	145.81221	173.7336973	181.48967	139.607435
12	147.363404	150.46579	162.8753412	167.52892	155.119373
12	142.709823	139.60744	170.6313098	145.81221	136.505048
24	111.685948	122.5443	116.3395294	89.969236	94.6228173
24	108.583561	113.23714	141.1586291	77.559686	105.481173
24	105.481173	116.33953	131.8514667	94.622817	85.3156549

Appendix Q Daphnia magna Respiration Data (Atrazine)

	Dissolved oxygen content (umol/L water)				
Time	**Reference**	**0.1% DMSO**	**5 µg/L Atrazine**	**50 µg/L Atrazine**	**100 µg/L Atrazine**
0	258.0811	263.2119	255.5156	263.2119	256.0287
0	261.6727	262.6988	263.2119	256.5418	260.6465
0	257.568	257.568	261.1596	262.1857	258.5942
2	205.2335	214.469	196.511	195.4849	188.8148
2	207.2858	203.6942	210.3643	189.3279	189.8409
2	202.155	206.7727	214.982	211.9035	179.0662
4	187.7886	189.8409	161.1083	160.5952	147.255
4	184.197	174.4484	178.5531	158.5428	158.0298
4	180.0924	180.6054	191.8933	195.4849	132.8887
6	164.6998	169.3176	129.8102	133.4017	111.8522
6	161.6213	152.8989	153.412	126.2186	106.7214
6	157.5167	158.0298	172.3961	142.6372	83.11955
10	104.156	109.7999	73.37096	68.75321	45.15136
10	109.2868	99.53822	102.6167	54.38687	56.4392
10	101.5906	99.02514	135.4541	95.43356	24.11493
24	11.80092	13.34017	7.696254	3.078502	1.539251
24	12.82709	8.722422	22.0626	1.539251	9.235505
24	9.748589	12.31401	9.235505	16.93176	1.026167

Appendix R Hyalella azteca Respiration Data (TBT)

Time	Dissolved Oxygen Content (umol/L)				
	Reference	DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
0	181.4897	179.9385	184.5921	176.8361	189.2456
0	179.9385	181.4897	189.2456	186.1432	184.5921
0	187.6944	186.1432	183.0409	189.2456	189.2456
2	173.7337	178.3873	172.1825	162.8753	165.9777
2	173.7337	179.9385	175.2849	170.6313	178.3873
2	186.1432	170.6313	159.773	170.6313	155.1194
4	159.773	147.3634	145.8122	142.7098	159.773
4	162.8753	165.9777	161.3241	153.5682	155.1194
4	164.4265	162.8753	158.2218	148.9146	147.3634
6	152.017	147.3634	158.2218	145.8122	142.7098
6	147.3634	144.261	162.8753	147.3634	139.6074
6	152.017	159.773	164.4265	142.7098	136.505
10	139.6074	147.3634	152.017	133.4027	105.4812
10	155.1194	148.9146	159.773	128.7491	116.3395
10	144.261	156.6706	156.6706	134.9539	110.1348
24	93.07162	103.93	127.1979	74.4573	43.43342
24	116.3395	110.1348	134.9539	85.31565	55.84297
24	100.8276	111.6859	139.6074	100.8276	71.35491

Appendix S Hyalella azteca Respiration Data (Atrazine)

	Dissolved Oxygen Content (umol/L)				
Time	**Reference**	**0.1% DMSO**	**5 µg/L Atrazine**	**50 µg/L Atrazine**	**100 µg/L Atrazine**
0	249.4493	248.8987	249.4493	250	243.9427
0	247.2467	248.348	250	248.8987	247.2467
0	249.4493	252.7533	248.348	249.4493	249.4493
2	227.9736	216.9604	219.7137	198.2379	193.2819
2	213.1057	222.467	221.3656	203.1938	202.0925
2	216.4097	224.6696	218.6123	205.3965	213.6564
4	192.1806	192.7313	191.63	164.6476	146.4758
4	185.022	192.1806	194.3833	166.2996	162.9956
4	183.9207	196.0352	196.0352	174.0088	169.6035
6	170.1542	160.2423	165.1982	127.7533	116.7401
6	153.0837	171.2555	181.1674	142.6211	129.9559
6	155.837	166.8502	179.5154	150.3304	136.5639
10	129.9559	115.6388	128.8546	75.44053	61.67401
10	117.2907	133.8106	147.5771	96.36564	77.64317
10	107.9295	142.0705	148.6784	117.8414	101.3216
24	24.77974	6.057269	18.17181	9.911894	2.753304
24	16.51982	20.92511	84.2511	8.259912	1.101322
24	20.37445	38.54626	45.15419	5.506608	1.101322

Appendix T Lumbriculus variegatus Respiration Data (TBT)

Dissolved Oxygen Content (umol/L)					
Time	Reference	0.1% DMSO	10 µg/L TBT	50 µg/L TBT	100 µg/L TBT
0	206.3088	204.7576	201.6552	209.4112	193.8992
0	209.4112	203.2064	209.4112	221.8207	214.0647
0	220.2695	218.7183	220.2695	198.5528	200.104
1	201.6552	198.5528	193.8992	201.6552	192.348
1	206.3088	214.0647	200.104	212.5135	201.6552
1	209.4112	212.5135	212.5135	215.6159	206.3088
2	201.6552	197.0016	206.3088	207.86	190.7968
2	207.86	197.0016	212.5135	210.9623	189.2456
2	212.5135	198.5528	204.7576	186.1432	197.0016
3	186.1432	183.0409	195.4504	206.3088	189.2456
3	189.2456	190.7968	187.6944	187.6944	189.2456
3	183.0409	189.2456	200.104	193.8992	181.4897
4	169.0801	200.104	193.8992	181.4897	214.0647
4	190.7968	206.3088	183.0409	173.7337	212.5135
4	206.3088	201.6552	175.2849	178.3873	189.2456
5	189.2456	197.0016	200.104	165.9777	203.2064
5	214.0647	204.7576	189.2456	214.0647	187.6944
5	190.7968	198.5528	206.3088	179.9385	206.3088
6	221.8207	198.5528	210.9623	228.0255	214.0647
6	204.7576	203.2064	189.2456	175.2849	223.3719
6	198.5528	198.5528	198.5528	200.104	212.5135
12	190.7968	189.2456	209.4112	212.5135	206.3088
12	195.4504	190.7968	193.8992	206.3088	212.5135
12	198.5528	204.7576	207.86	209.4112	203.2064
24	176.8361	173.7337	169.0801	161.3241	181.4897
24	175.2849	179.9385	172.1825	181.4897	173.7337
24	179.9385	183.0409	179.9385	179.9385	179.9385

Appendix U Lumbriculus variegatus Respiration Data (Atrazine)

Dissolved Oxygen Content (umol/L)					
Time	Reference	0.1% DMSO	5 µg/L	50 µg/L	100 µg/L
0	254.9651	261.4063	254.9651	257.649	256.5754
0	253.8916	256.0386	254.4283	255.5019	257.1122
0	256.5754	256.5754	257.649	259.796	260.3328
2	223.8325	224.9061	213.6339	214.7075	198.0676
2	221.6855	222.759	212.5604	197.5309	200.2147
2	228.6634	214.7075	218.4648	221.1487	202.3618
4	207.7295	192.6999	174.4498	177.1337	172.3027
4	193.7735	191.6264	186.2587	174.4498	166.9351
4	206.6559	184.6484	180.3543	178.744	183.5749
6	184.1116	169.6189	147.6114	155.1261	140.6334
6	154.0526	168.0086	157.81	127.7509	138.4863
6	180.3543	148.6849	155.6629	150.2952	163.7144
10	142.2437	130.4348	92.86098	127.2142	92.86098
10	113.795	128.8245	104.1331	71.39023	92.32421
10	156.7364	92.86098	105.7434	135.2657	119.1626
24	34.88996	30.59581	23.61782	27.3752	8.05153
24	25.22813	27.91197	18.7869	17.71337	11.80891
24	39.18411	27.3752	27.3752	23.08105	12.34568

Appendix V Biomass of Test Organisms

Daphnia magna

	Weight (mg) 8 *Daphnia*	Weight (mg) 15 *Daphnia*
Replicate 1	21.92	51.375
Replicate 2	24.88	58.3125
Replicate 3	20.08	47.0625
Replicate 4	20	46.875
Replicate 5	19.76	46.3125
Replicate 6	22.96	53.8125
Replicate 7	21.28	49.875
Average	21.55428571	50.51785714

Hyalella azteca

	Weight (mg) 3 *Hyalella azteca*	Weight (mg) 8 *Hyalella azteca*
Replicate 1	23.475	62.6
Replicate 2	27.45	73.2
Replicate 3	25.8	68.8
Replicate 4	23.925	63.8
Replicate 5	24.525	65.4
Average	25.035	66.76

Lumbriculus variegatus

	Weight (mg) 10 worms	Weight (mg) 20 worms
Replicate 1	84.9	178.29
Replicate 2	90.6	167.61
Replicate 3	89.95	188.895
Replicate 4	79.5	178.875
Replicate 5	93	176.7
Average	87.59	178.074

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