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# Assessment of Behavioural Parameters of Chironomus Tentans and Lumbriculus Variegatus for the use in a New Early Warning Biomonitoring System for Drinking Water

Jason Ryan Solnik
*Ryerson University*

# ASSESSMENT OF BEHAVIOURAL PARAMETERS OF *CHIRONOMUS TENTANS* AND *LUMBRICULUS VARIEGATUS* FOR THE USE IN A NEW EARLY WARNING BIOMONITORING SYSTEM FOR DRINKING WATER

By: Jason Ryan Solnik

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Jason Ryan Solnik

**Abstract**

Jason Ryan Solnik
Master of Applied Science 2012
Environmental Applied Science and Management
Ryerson University

*Assessment of behavioural parameters of Chironomus tentans and Lumbriculus variegatus for the use in a new early warning biomonitoring system for drinking water*

Behavioural parameters of *Chironomus tentans* and *Lumbriculus variegatus* were investigated using time-lapse photography for the use in an early-warning biomonitoring system for drinking water. The Multispecies Freshwater Biomonitor (MFB) was used to quantify these behaviours and its utility and integration into such a system was evaluated. The contaminants tributyltin, atrazine, copper, and ciprofloxacin were used to elicit stress responses. Time-lapse photography established foraging and swimming behaviours of both species as model behaviours for use in non-visual bioassays. The MFB identified stress responses from both organisms exposed to 100µg/L TBT ($p = 0.008$) but not under exposure to 500µg/L of Cu and 10% EtOH ($p = 0.120$ and 0.286 respectively). The MFB was not successful in detecting behavioural deviations on a consistent basis and was concluded that it was not suitable for integration into an early-warning biomonitoring system. The study concluded with suggested modifications and future work with the MFB.

## Acknowledgements

I would like to take this time to thank Dr. Bostan, my supervisor, for his constant and reliable input, guidance, and encouragement over these two years. I would also like to thank Dr. Laursen for his assistance with the multiple data analysis required with this study, and Dr. McCarthy for her enthusiasm and passion for the project which has boosted my own excitement for this field.

Special thanks go out to fellow graduate student Vivian Fleet. Her initial work with the MFB allowed for a smooth and comfy ride with the multiple technicalities involved in the operation of the MFB. Without her original 'dirty work' concerning the endless complications my own work could not have proceeded. Further thanks go out fellow to Isabelle Netto, whose support and companionship proved invaluable. Thanks guys for making KHE 322A a great place to work.

Finally, and with all due tribute, I would like to thank my family. Their support, encouragement, and pride through all these years have given me so much and has made this journey all the more worthwhile. Thank you.

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# 1.0 INTRODUCTION

## 1.1 Purpose

The purpose of the following study is to investigate the use of the aquatic organisms *Lumbriculus variegatus* and *Chironomus tentans* behaviour as a biological indicator for the detection of foreign contaminants in drinking water. Using behavioural biomonitoring offers many benefits over past established biological and chemical analysis such as quick response time, sub-lethal concentration detection, and broad-spectrum detection. It is believed that with the implementation of a behavioural-based monitoring regime it will be possible to detect foreign contaminants entering drinking water supplies before it is distributed to the public. It is the goal of this study to establish three distinct and observerable behaviours of *L. variegatus* and *C. tentans* that are susceptible to stress under toxic conditions. The behaviours will then be visually monitored while the organisms undergo exposure to varying concentrations of 4 different contaminants abundant in Southern Ontario waterways. Deviations in behaviour, or 'stress' responses, will be classified. In addition, this study will be using the Multispecies Freshwater Biomonitor (MFB) to analyze and quantify theses changes in behaviour. The implementation of systems such as the MFB into a drinking water biomonitoring regime will allow for rapid, automated, and sensitive monitoring subject to low maintenance and minimal supervision. This study is part of a large NSERC project taking place at Ryerson University. In collaboration with several other graduate students it is the goal of this project to create a behavioural biomonitoring system which incorporates several different analytical technologies such as the MFB, Ecotox, and DapniaTox organisms representative of the different trophic levels within an aquatic ecosystem. This approach will allow for a holistic grand overview of the effect foreign contaminants have on the aquatic ecosystem.

## 1.2 Canadian drinking water

As global populations increase so does the demand for potable drinking water. Axiomatically, as populations grow so do the anthropogenic affects on the natural environment. When focusing on the aquatic environment it is evident that these anthropogenic effects have inflicted much damage. Industrial dumping, agricultural runoff, urban sewage, and spills are some of the significant pathways in which foreign contaminants can enter aquatic systems. For these reasons it is of the utmost importance that the managing of drinking water, which is most often obtained from neighboring freshwater systems, encompasses sufficient monitoring and treatment technologies for the community it serves. In Canada, the size of the community and its water source determines the type of monitoring and treatments.

A 2008 study (Clarke 2008) investigated the differences in drinking water management in Canadian cities. Large municipal communities such as Toronto, Vancouver, and Edmonton do not have problems maintaining access to clean drinking water. In 2008 Toronto, with complete confidence in its drinking water treatments, banned both the sale and distribution of bottled water on city premises while increasing access to tap water in all city facilities (Clarke 2008). Fortunately for large communities such as Toronto monitoring drinking water is of great importance to both municipal and provincial governments. The monitoring of these large municipal drinking water supplies involve substance specific chemical testing at both treatment and distribution sites. These tests can be done by the city, or, as is the case with most large municipalities, by out-sourced laboratories. According to the city of Toronto's website, these laboratories test for over 160 chemical, biological, and radiological substances on a continuous basis.

However, upon further investigation, important information such as sampling locations, testing times, tests durations, and tests regularity are not available to the public. Going back to the provincial legislation, it is demanded that large municipal residential systems sample their water at least once a week for a specific parameter (*Safe Drinking Water Act* 2002). Unfortunately, this degree of monitoring is a 'best-case scenario'. When the focus shifts to small municipal residential systems, i.e. non-municipal seasonal residential systems, the frequency of drinking water monitoring decreases from once a week to once every 36 months (*Safe Drinking Water Act* 2002). The situation becomes even more ominous when looking at the drinking water monitoring practices of First Nation communities. Due to the ambiguity of regulatory authority over First Nation communities they are under supported and often overlooked. They lack proper medical treatment centers, affordable housing, and in the interest of this study, safe drinking water. As of November 30$^{th}$, 2010, there are currently 120 First Nation communities across Canada under a Drinking Water Advisory, an advisory which falls under one of two categories: a boil water advisory or a do not drink advisory (Health Canada 2010). In Canada, a nation considered to be 'first world', clean drinking water is not available to all its citizens.

The limitations of current chemical monitoring practices are evident. The plausibility of catching a toxic agent in drinking water before it enters the distribution channels is very low, with chances decreasing with the increase of community population. Laboratory-run chemical specific tests take time, expertise, and large funding. Also, with the time needed to perform the chemical tests for over 160 parameters, it is impossible for these testing laboratories to relate results back to the city in time for there to be any hope of stopping the toxicant from entering the distribution system. However this is only a scenario for the communities that have adequate monitoring facilities. Due to these inadequacies this current system of monitoring is becoming

antiquated and obsolete. To protect the Canadian population from the ever-increasing amount of diverse foreign toxicants entering Canadian waterways and to provide clean, safe drinking water to the nation a new monitoring paradigm must be introduced.

## 1.3 New Drinking Water Monitoring Regime

### 1.3.1 Previous Applications of Biological Early Warning Systems (BEWS)

This study, along with parallel studies by accompanying graduate students, attempts to establish a new drinking water monitoring regime centered on the concepts of behavioural analysis. Behavioural analysis involves the monitoring of aquatic organisms behaviour to indicate exposure to a contaminant. An organism's behaviour integrates many cellular processes and is essential to not only the organism's survival, but population levels and community structure. Impacts on an organism's behaviour can impact important behavioural activates such as predator avoidance, sexual interaction, feeding, and habitat construction, all resulting in numerous impacts on population dynamics. By utilizing behaviour as a test parameter it is possible to obtain results at environmental relevant concentration, typically lower than lethal concentrations, which are not always detectable in typical biological assays such as mortality and accumulation tests.

A contaminant, or stressor, is any foreign agent, be it biological, chemical, physical, or radiological, which induces stress upon introduction into a system. The idea of using organisms to assess the cleanliness of the environment is not new. Rationales and use of organisms in environmental testing initially arose during the 1980s, with studies such as Batac-Catalan and White (1983) and Giesy and Hoke (1989). These studies were among the firsts to address and

compare the use of different species for ecological testing and confirmed the use of these species for the analyses of environmental conditions. Some of these species include *Daphnia magna, Hyalella azteca, L. variegatus*, and *C. tentans*. Since then, these species have increased in popularity for their use in the testing of environmental parameters. These tests have focused around endpoints such as bioaccumulation, reproduction, growth, mortality, emergence, and mass (Bartlett *et al* 2007).

While these efforts have been successful in analyzing environmental conditions using whole organisms, the use of an organism's behaviour to evaluate environmental conditions has not been addressed to the same extent and applicability in Canada. European studies and applications of biomonitoring date back to 1986 when in Basel, Switzerland, a Sandoz chemical storage building experienced a large release of insecticides into the Rhine River, approximately of 40 tonnes along with 400 kg of Atrazine (Fleet 2010). Water ecology and drinking water were greatly affected due to spill, demonstrating a need to identify the location of the plume of contaminants. Fortunately, 500 km downstream of the spill site an automated biomonitoring system was in place, the Dynamic Daphnia, which sounded an alarm after registering altered swimming patterns of the *Daphnia magna* (Fleet 2010).

Since then, cross-border waterways such as the Rhine have seen the implementation of biomonitoring systems such as the Dynamic *Daphnia* test, the bbe *Daphnia*Toximeter as seen in figure 1.1, which also monitors the swimming pattern of

**Figure 1.1** bbe Daphnia Toximeter

*Daphnia magna*, the Mosselmonitor, an automated analysis system which measure the opening and closing behaviour of mussels, and the Dreissena-monitor, another system which monitors the valve motions of zebra mussels (Borcherding 2006). These technologies have spearheaded biological early-warning systems with real world applications.

### 1.3.2 Benefits and Limitations of BEWS

The benefits of such monitoring systems over chemical analyses are many. Intermittent, substance-specific chemical analyses tests are labour intensive, long in duration, and ignore any synergistic affects numerous chemicals may have - an increasing area of debate in ecotoxicology (Townsend *et al* 2008). Biomonitoring systems are becoming increasingly cost-effective, in both set-up and maintenance, and require minimal human operation (Lechelt *et al* 2000). The majority of these systems are of limited size, allowing for site-specific placement and replacement if needed as these systems are placed adjacent to waterways. Finally, these systems are continuous (over a set duration), supplying the user with a full perspective of the quality of the water, as opposed to irregular, time independent testing which occurs with chemical analyses.

While the benefits of the biomonitoring systems greatly outweigh the benefits of chemical analysis, there are some limitations that these systems have yet overcome. The Dynamic *Daphnia* Test, developed in 1978 by Knei (Fleet 2010) and which was used in the 1986 insecticide spill in Basel, Switzerland, operates using infrared (IR) beams. The daphnia swim past multiple IR beams, and the systems records an average of swimming behaviour (Fleet 2010). Upon stress behaviour induced by a contaminant, the daphnia's swimming rate increases resulting in an increase of IR beam interference (Fleet 2010). However the system is unable to identify individual daphnia and cannot define the number of IR beams crossed by a single

organism. So when swimming rates increase due to a stressor, the system cannot distinguish between a single daphnia crossing multiple IR beams or numerous daphnia crossing IR beams. This is important to distinguish as each individual organism will have a somewhat different behavioural response to environmental conditions (Fleet 2010). Also, the Dynamic *Daphnia* Test is not behaviour specific, meaning that it is unable to distinguish between different behaviours that may result in an increase in IR beam interference (Fleet 2010).

Another daphnia based system developed in 1997 by bbe Moldaenke is the bbe *Daphnia* Toximeter. Unlike the Dynamic *Daphnia*, this system uses image analysis, employing a camera frame grabber to digitize the image and plot their trajectory. Their trajectory is then analyzed and compared with base readings. The average trajectories of up to 25 organisms can be tracked simultaneously (Fleet 2010). The established behaviours that effect swimming trajectory are characterized as (i) velocity measured as an average of all organisms in the chambers, (ii) turning and circling of the daphnia which the program calls fractal dimension, (iii) number of whole organisms moving, (iv) average height of all organisms in the chambers, (v) the distance between the organisms, (vi) and a parameter called V-class index, which is a comparison of velocity ranges of the daphnia under exposure to varying stressors (Fleet 2000; Green *et al* 2003).

These parameters have been successful in detecting behavioural changes of the daphnia (Green *et al* 2003). Nevertheless, the bbe *Daphnia* Toximeter has several limitations. Similar to the Dynamic Daphnia, individual daphnia cannot be recognized. Due to this, the increased swimming velocity of a portion of the daphnia may be offset by the regular or decreased swimming velocity of the remaining daphnia, resulting in a delay or failure to raise an alarm. Different from the previous daphnia system, this system relies on image analysis, demanding a clear field-of-vision in order for the monitoring to operate. In waters that exhibit high levels of

turbidity this system will be unable to characterize the movement of the daphnia in the sample. Additional filtering of sample to increase light penetration may result in the removal of contaminants, resulting in the Daphnia Toximeter relaying a false negative.

The above two biomonitoring systems are examples of a non-optical system and an image analysis system. Within the last twenty years there have been several systems, either of which are non-optical or image analysis, which have been developed to monitor source drinking water or industrial effluents. The organisms of choice for these systems include fish, algae (in the use of flowcytometers), bivalves (i.e. Mosselmonitor), and invertebrates such as *D. magna.* No matter the species these systems use, there is one inherent flaw within each. Each system relies on the behavioural indicators of one organism. Be it an invertebrate, a fish, or a bivalve, each system relies upon the response of a single species. This is where accurate detection of stressors may vary. Due to the fact that species react differently to different stressors and concentrations, it is possible that these systems may not be 'painting the entire picture' of contamination. In order to determine the full extent of contamination it is necessary to expose several different species to the same tests and to monitor them simultaneously to observe for varying degrees of behaviour change. Where these systems fall short this study attempts to strengthen the scientific rigor and applicability of behavioural biomonitoring systems with the use of multi-species analysis.

### 1.3.3 Introducing Early Warning Biomonitoring Systems (EWBS): A Multi-Species Approach

As previously mentioned this study is part of a collaborative work to create a biomonitoring system utilizing organism's behaviour as sub-acute indications of contamination stressor in Ontario drinking water. Initial work on this study included several graduate works from previous years. The first step in the direction of a multi-species behavioural monitoring system was to identify sub-acute stress behaviours in the organisms of choice. This began with my previous undergraduate work (2009) and Gill Marshall's graduate work (2009). Marshall studied stress behaviour in *D. magna, H. azteca*, and *L. variegatus* and attempted to identify repeatable, sub-acute, non-lethal stress behaviours. My own work focused on *C. tentans* and *L. variegatus*, efforts which began with the culturing and identification of normal ecological behaviour of each species.

Marshall exposed each of the three species to varying levels of TBT and atrazine and recorded behavioural responses. It was concluded that the behaviour with which to observe for the indications of stress in *D. magna* were (i) swimming ability; ability to maintain height within water column, (ii) swimming style, (iii) immobilization, (iv) secondary antennae use, and (v) spinning movements (Marshall 2009). Marshall's work with *H. azteca* concluded with the identification of the behaviours of (i) immobility, (ii) substrate crawling, (iii) and body length (Marshall 2009). Finally, Marshall identified up to six parameters of *L. variegatus* behaviour. The three most influential behaviours, which were later acknowledged as crucial stress identifiers upon collaboration with my own work, were (i) swimming or locomotion patterns, (ii) immobilization, (iii) and group movement or colony formation (Marshall 2009). This work was

later referred back to by other graduate students whose own work focused on the use of these behaviours in biomonitoring technologies.

Vivian Fleet and Isabelle Netto continued to study multi-species behavioural biomonitoring with the use of the biomonitoring systems ECOTOX, Daphnia Tox, and the Multispecies Freshwater Biomonitor (MFB). Fleet worked with the MFB (discussed in section 2.4) using *D. magna*, *H. azteca* and *L. variegatus* in an attempt to use the behavioural endpoints previously indicated by Marshall to detect the presence of a contaminant in an automated, non-optical system. Netto studied the feasibility of using ECOTOX and Daphnia Tox in the suite of system. ECOTOX is an image analysis system that tracks several movement parameters of the flagellate *Euglena gracilis* in an automatic, real-time, static bioassay (Netto 2010). Daphnia Tox, similar to ECOTOX, is also an image analysis system that operates in real-time analyzing locomotory behaviour of *D. magna* (Netto 2010). Daphnia Tox was also shown to have the potential to use other marcoinvertebrates in its analysis, such as rotifers, and *H. azteca* (Netto 2010).

Fleet's work with the MFB concluded with varying success. Previous studies (Gerhardt et al, 2003), illustrated the successful use of the MFB in the detection of contaminants in waters from the Rhine River using species such as *Gammarus pulex* and *D. magna*. The automated system was capable of detecting behavioural changes of the organisms and relaying an alarm to operators (Gerhardt *et al* 2003). Once the MFB was set up by Fleet and housed with *D. magna* and *H. azteca*, the system was set to detect stress behaviours upon exposure to varying concentrations of TBT and atrazine. Once completed the data relayed from the MFB was analyzed for statistical variability. Fleet discovered a slight discrepancy between her results and the results obtained by Gerhardt. While the MFB was capable of detecting immobility with both

*D. magna* and *H. azteca* it was not capable of detecting minute behavioural changes, such as ventilator motions or leg and antennae movement (Fleet 2010). As discussed in Marshall's study, immobility, while a key behavioural indicator of stress, is not the most sensitive behavioural response of *D. magna* and *H. azteca* (Marshall 2009; Fleet 2010). Immobility was shown to be caused by high concentrations of contaminant and/or during extended periods of exposure (Fleet 2010). Both Marshall and Fleet identified swimming height within water column and swimming patterns to be more sensitive behavioural parameters in which to measure shot-term, sub-acute exposure to stressors. Due to the systems inability to analyze these sensitive behaviours Fleet concluded that the MFB was not suitable for integration into an early-warning biomonitoring system for drinking water. Fleet indicated that further studies were needed to identify which *D. magna* behaviours are both sensitive enough to elicit a sub-acute response to a contaminant and which sensitive behaviours can actually be detected by the MFB. Continuing from where Fleet left off, this study will be conducting tests on the ability of the MFB to detect sub-acute behavioural changes in the benthic invertebrates *C. tentans* and *L. variegatus*.

Netto's work with Daphnia Tox suffered multiple mechanical and software difficulties. As a result no data was gathered from this system. However, Netto was fortunate to meet with an operator of the bbe Daphnia Toximeter in Zurich, Switzerland. The Hardhof groundwater has had the system in operation since 2006 and has experienced no difficulty in operation and maintenance. Netto describes its operation and application in the monitoring of groundwater drinking water as an example of how early warning biomonitors can eventually be successfully implemented into a drinking water monitoring system in Ontario (Netto 2010).

Netto's work with ECOTOX provided results for discussion on its incorporation into an the biomonitoring system. Although the ECOTOX system was found to be less sensitive then

visually observed stress behaviours under exposure to copper, the system was successful in detecting changes in the gravitatic behaviour of *E. gracilis* during a 24h exposure test to TBT and atrazine. Although even with these results Netto concluded that it is still unknown if ECOTOX is a system that has the required sensitivity for its inclusion into a suite of automated systems. It was concluded that further study was needed (Netto 2010).

### 1.3.4 Creation of Response Library and Statistical Modeling

The final goal of these past works as well as this study is the amalgamation of all behavioural responses from the multitude of organisms into a single digital library. This library will house all data gathered from all the behavioural tests and will be used as a reference databank. Once a behavioural parameter (i.e. swimming height) of an organism, (i.e. *D. magna)* under contamination (i.e. TBT) at a certain concentration (i.e. 100µg/L) is establish and quantified, it will be stored in the library and be used in future behavioural tests to identify unknown stressors in sample water. It will be used as a reference guide that will correctly identify the contaminant and its concentration according to the behaviour it elicits from the organisms. Since the final goal of this and other studies is the inclusion of multiple species using several automated behavioural biomonitoring systems, identification of stressor and concentration will be based on numerous data sources. Due to this large number of variables, multivariate analysis will be utilized in order to perform studies and groupings across these multiple dimensions.

This analysis will take into account the effect of all variables of interest (i.e. the numerous behavioural endpoints). From this host of variables, factor analysis will be used to identify latent dimensions and therefore reduce the attribute space form this large number of

variables to a small number of factors. Factor analysis will provide this reduction of variables for data modeling. This reduced set of correlated variables will then be transformed into a set of uncorrelated variables called principal components, the analysis of which is termed principal component analysis (PCA). PCA creates components that are linear combinations of the original data which account for as much as the variance in the multidimensional data as possible. The number of components that will be used for further cluster analysis will be based on the number of components that explain between 70-90% of the variation among the components (Yeung & Ruzzo 2001).

Once the components are chosen, cluster analysis will determine which groups of observations are uniform and are separate from different groups. This can be done using many different types of clustering procedures of which will be chosen once data sets have been collected. All aspects of these statistical protocols will be handled by fellow graduate student, Aryo Maradona whose part in this study is the collection and integration of gathered data into a statistical model.

## 1.4 The Multispecies Freshwater Biomonitor

As mentioned above this study will be utilizing the MFB in the attempt to quantify behavioural responses of *C. tentans* and *L. variegatus* to TBT, atrazine, ciprofloxacin, and copper. Initially developed in Germany by A. Gerhardt the MFB is an automated, online biomonitor that operates in real-time to provide statistically transformed data on the behavioural changes of aquatic organisms (Gerhardt *et al* 2003). It is a non-optical system that utilizes an electric field inside enclosed chambers that house aquatic organisms. An electric field is spread across the inside of each chamber and the interference from the organism's movement is detected by the disturbance in the field and recorded as movement (Gerhardt *et al* 2003).

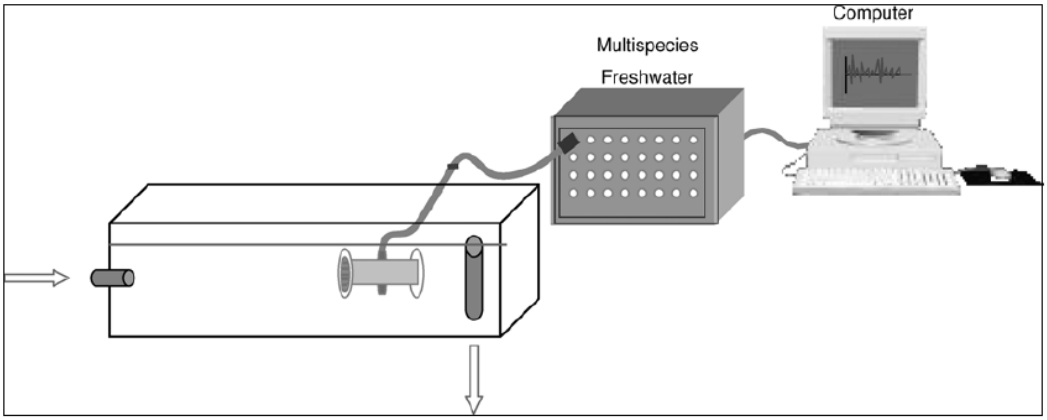



**Figure 1.2** Schematic of the Multispecies Freshwater Biomonitor (Fleet 2010)

The system is composed of 8 -96 individual chambers and associated channels which can be used and read by the system simultaneously (Fleet 2010). Inside each chamber the electric field is generated by two set of stainless steel plate electrodes located on the walls of the chamber. These electrodes produce a high frequency signal of alternating current of up to 50 kHz (Fleet 2010).

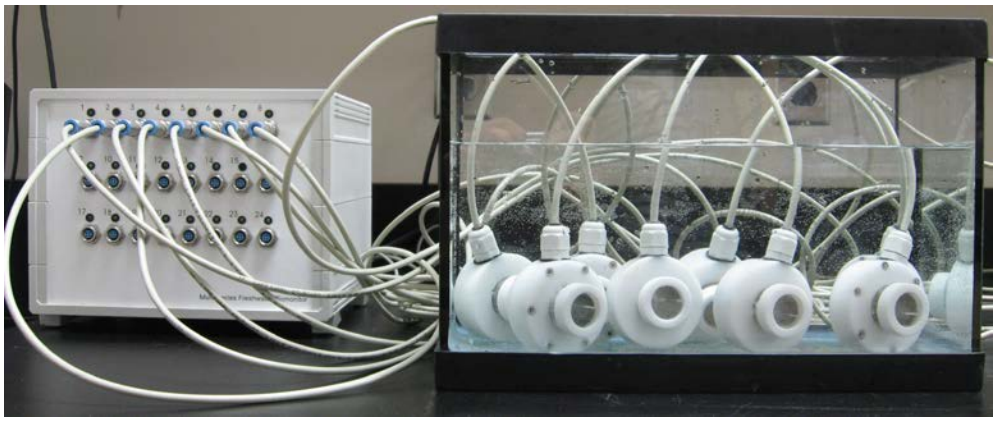


**Figure 1.3** The Multispecies Freshwater Biomonitor

The movement of the organisms housed in the chamber is detected by a second pair of electrodes that are non-current carrying and read as an impedance signal within the field (Fleet 2010). Due to the high number of chambers capable of running parallel test in concert, interference between chambers must be avoided. This is done by each chamber sending signals to the MFB impedance recorder in synchronicity (Fleet 2010; Gerhardt *et al* 2003). The amplitude of the signals generated within the chambers change in relation to the size of the movement allowing for differentiation between behaviours in accordance with amplitude (Fleet 2010). The size of the housed organism in relation to the chamber also plays a role in the amplitude of the signal. Amplitude readings, in volts, are taken for 240 seconds (4 minutes), followed by 360 seconds (6 minutes) of analysis. Each series of measurements (each 240 seconds) are taken every 10 minutes. Readings are plotted as volts in real-time against time (in seconds).

These readings are put through a Fast Fourier Transformation (FFT) by the MFB. This converts amplitude readings into frequencies in Hertz (Fleet 2010; Gerhardt *et al* 1994). This transformation relates high amplitude readings to low frequency values, and low amplitude readings to high frequency values. Frequency readings are on a scale of 0.0 to 8.0 Hz with 0.5 Hz

intervals. Slow, large movements elicited by the organism, such as swimming and other behaviours attributed to locomotion, are registered in the low frequencies, from 0.5 to 2.5 Hz. Rapid, small movements by the organism such as ventilator behaviour and forelimb/antennae movement are registered in the higher frequencies of 3.0 to 8.0 Hz (Fleet 2010).

### 1.4.1 Benefits and Limitations to using MFB

The advantages of the MFB allow for quick deployment and rapid readings, both of which are important in real-time monitoring (Green *et al* 2003). The relative small size needed for the MFB, approximately $1m^2$ (Fleet 2010) gives the operator freedom to place the MFB in a variety of locations such as adjacent to riverbanks or effluent discharge areas. The ease of deployment of chambers and rapid relaying of data permits for time sensitive readings occurring in areas of low contact time with water, i.e. rapid moving rivers. The multi-chambered MFB allows for the allocation of numerous species for testing simultaneously. No filtration or pre-treatments of incoming waters is necessary as the MFB is a non-optical system that is not reliant on visual determination of behaviour. The chambers can be placed in a flow-though system or set up in a static test, depending on the toxin of concern and assessments desired.

A key advantage over other behavioural biomonitoring systems, both non-optical and image analysis, is the opportunity of testing on benthic organisms. Since the MFB does not need to establish visual contact with the housed organism, benthic organisms are free to occupy their preferred habitat while undergoing continuous monitoring. The area within the chambers permits the placement of substrate on the bottom for benthic organisms without impeding the two sets of electrodes. Since benthic organism prefer low light dark conditions (Phipps *et al* 1993) tests can

be performed in little to no light; this also stands for other aquatic organisms who elicit behavioural responses in low light conditions.

The MFBs limitations, as were stated in the discussion of Fleets graduate work, do not place the same restrictions on the current study as it did on Fleet's. This study focuses on the detection of sub-acute stress behaviours in benthic invertebrates. As such, the inability of the MFB to pick up the smaller movements of *D. magna* and the proclivity of *H. azteca* to settle in a small niche within the chamber will not affect behavioural responses of the two benthic invertebrates.

## 1.4 Contaminants

The four contaminants used in this study represent a wide class of both legacy and emerging contaminants of interest within the Great Lakes water shed. Similar contaminants were used by previous graduate student Vivian Fleet and Isabelle Netto. This was done in the interest of conformity and in the attempt to create a response library of these contaminants from the various organisms and monitoring technologies used.

### 1.4.1 Tributyltin

Tributyltin is an organotin compound and is generally represented by the formula RxSnL4−x (where x = 1–4) (Environment of Canada 2009). “R” is representative of a typical organic group, such as methyl, propyl, or butyl, which is bonded covalently to the tin atom by a carbon-tin bond (Environment of Canada 2009). Halogens usually make up the anionic moieties on tin, represented in the formula by “L”. These may also be other sulphur or oxygen based organic moieties such as –SR’, -OR’, -OC(O)R’, -S-, -O-, etc. (Fleet 2010). Tributyltin is designated tri due to the number of carbon-tin bonds in the molecule, containing a $Sn(C_4H_9)_3$ grouping. Other designations are mono, di, or tetrasubstituted (Environment of Canada 2009). TBT and its derivatives have low water solubility’s ranging from 1 to 10 mg/l (Focardi *et al* 2000; Alzieu, 1998), and a high octanol/water partitioning coefficient ($K_{ow}$ = 5000-7000), suggesting a high potential for bioaccumulation (Focardi *et al* 2000).

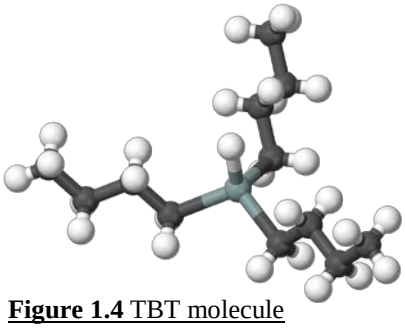


**Figure 1.4** TBT molecule

Since the discovery of its biocidal properties in the 1950s TBT has been used for a variety of purposes. It was discovered that TBT was the most toxic of the triorganotins against

gram-positive bacteria and fungi, with biocidal properties against a wide range of aquatic species (Fleet 2010). It has been used a general lumber preservative, a deterrent of Schistosomiasis in Africa (in the use of a molluscide), a disinfectant in circulating industrial cooling systems, breweries and leather processing plants, a preservative in textile mills, and a slimicide in paper mills (Maguire 2000; Yang *et al* 2000).

*TBT in the environment*

However it is its primary function as a biocide agent used in antifouling paints applied to the hulls of ships that has been the focus of international regulatory debates since detrimental environmental effects were observed in the 1970s.

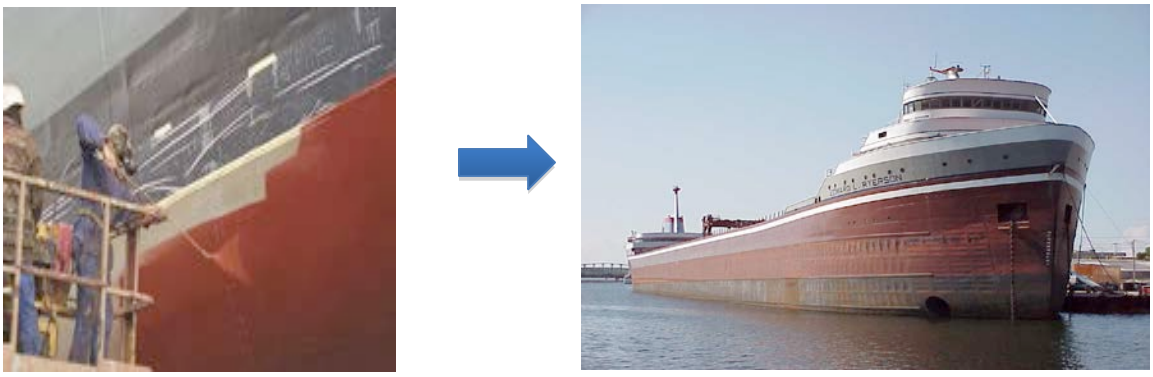


**Figure 1.5** Application of TBT-incorporated antifouling paints

Large ocean-faring vessels accumulate biological growth on their hulls during their contact with the water and as a result this growth, a mix of barnacles, bacteria, tubeworms, mussels and algae, can amass to amounts that increase the drag coefficient of the ship. This results in reduced speed and increased fuel consumption. Before the introduction of antifouling paints, vessels would remove the biological growth by manually scraping it off during dry-dock

– an event which increased according to the amount of growth. However, with the introduction of antifouling paints, the rate of dry-docking for the purpose of scraping on biological growth shrank from several months to years (Fleet 2010).

The first application of TBT antifouling paints onto boat hulls was in the form of free association paints. Figure 1.6 illustrates how the TBT molecules, labeled “Biocide”, leaches out while the paint remains intact.

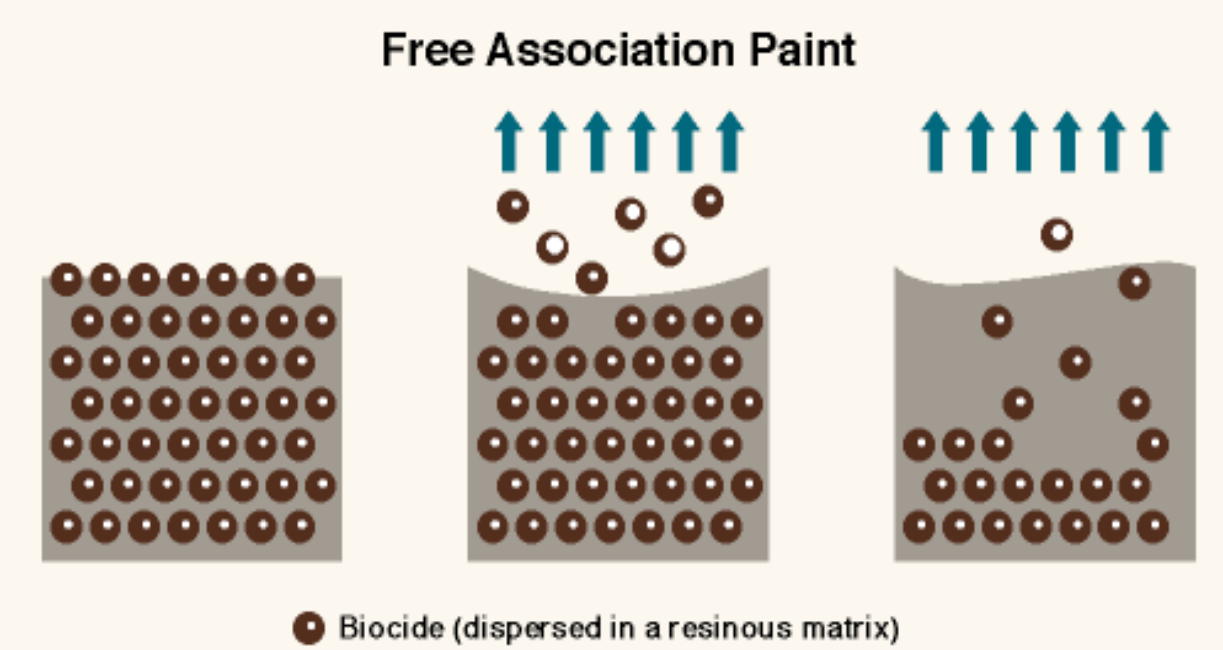
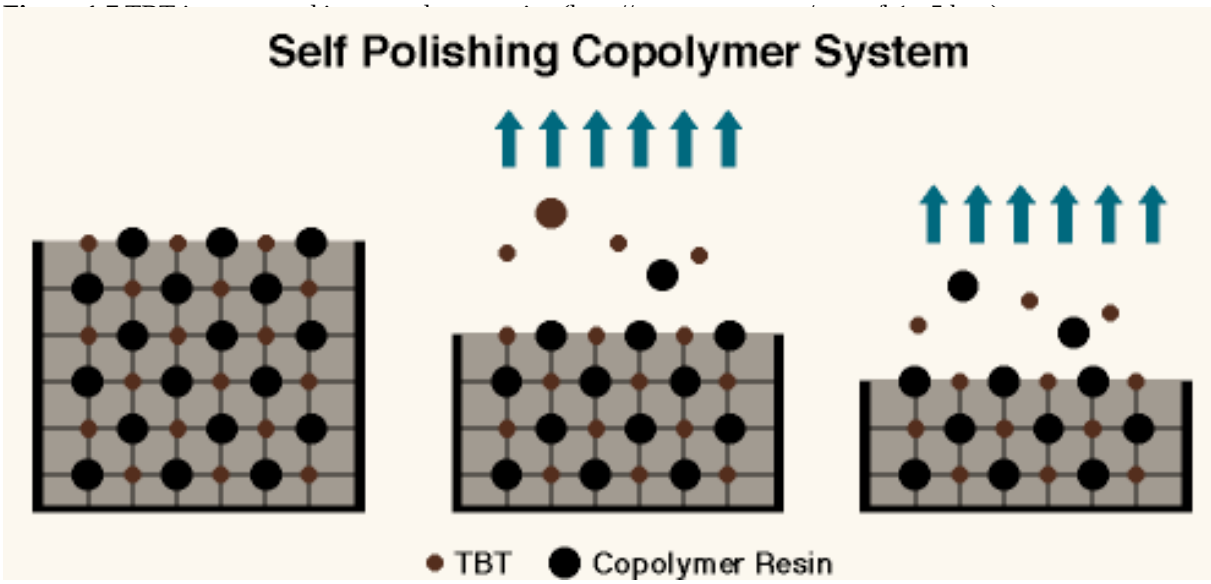



**Figure 1.6** TBT mixed with Free Association Paints (http://www.ortepa.org/pages/b1pt5.htm)

Free association paints are made by dispersing TBT in a resinous matrix into paint, allowing for the slow release of TBT into surrounding environment. This system has been the primary basis of the majority of antifouling paints and has been in use for the better part of the last century. However for numerous reasons its application has decreased over time. This system of mixing cannot control the rate of release of TBT from the paint, nor is the TBTs release evenly distributed throughout the paint. This results in ‘rough’ patches on the hull where the TBT has fully leached out - a ‘rough’ patch where biota can successfully adhere and accumulate.

Another problem with this paint system is the unintentional high initial release rate into aquatic environments, resulting in a highly concentrated TBT 'cloud' forming around the hull when the vessel is stationary. Subsequent release of TBT declines steadily from the matrix such that the anti-fouling performance of the paint diminishes over time (Mora 1996). The biocidial properties of these free association paints last between 12-24 months, at which point all the TBT has leached out leaving behind a simple coat of paint with no biocidal properties. (EXTOXNET, Ortep).

To prevent reduced effectiveness and uncontrollable release rates of the TBT a new mixing system was introduced, the Self Polishing Copolymer Paints (SPC) (Figure 1.7). The figure illustrates how the co-polymer resin, attached to the TBT molecule, remains incorporated within the paint and only disperses into the aquatic system as the paint does. TBT SPC paints are currently used today and show vast improvement over free association paints. This copolymer system is based on a combination of biologically active resins and TBT. The TBT is chemically bonded throughout the copolymer resin system (Ortep).



This paint system will react by hydrolysis with seawater resulting in a slow release of TBT over time into the surrounding environment combating biological attachment. The resulting paint surface of the system is mechanically weak and is eroded over time by moving seawater. This results in an exposure of a fresh surface of the TBT polymer, creating a 'self-polished' smoother surface. This hydrolysis/erosion process is repeated until the TBT-resin polymer is exhausted and no paint remains on the surface of the hull. Due to this controlled release a uniform anti-fouling performance is attained during the life of the paint which is anywhere between 5-6 years (EXTOXNET; Mora 1996).

TBTs effectiveness at pest control in these anti-fouling paints is its constant release into the surrounding water, and thus its main point of entry into the aquatic environment. High concentrations of TBT are generally found close to docks, dry-docks, and shipyards, and high traffic waterways used for international shipping and receiving (Mora 1996). High levels of up to 800 ppt have been found along the US east coast. The west coast has reached a spike of 1000 ppt in San Diego Bay. Levels of up to 840 ppt have been recorded in the Great Lakes.

*TBT toxicity*

Once TBT has been introduced into the aquatic environment there are several metabolic pathways it may go through, all eventually leading to the tin ion (Alzieu 1998). Metabolic degradation is dependent on several factors including temperature and the presence/absence of microorganisms. All metabolites of TBT, such as monobutyltin and dibutyl tin (MBT and DBT respectively) are less toxic than TBT itself. Due to TBTs strong partitioning coefficient and low water solubility it binds strongly to suspended particulate matter – both organic materials and

inorganic sediments. Depending on aerobic/anaerobic conditions the half-life of TBT in sediments is estimated to be 100-800 days (Focardi *et al* 2000). In deep anoxic environments, TBTs half-life can be anywhere from 2-4 years (Focardi *et al* 2000), with a resident time of up to 15 years in sediments (Maguire 2000). In highly aerobic aquatic environments TBT can take up to three months to degrade (Maguire 1987). Freshwater environments are considered to be particularly sensitive to TBT and its metabolites due to the abundant of sensitive species as well as greater accumulation rates due to longer resident time and lower exchange rates compared to open oceans (Netto 2010).

Research in TBTs mode of action has revealed several possible pathways for it toxicity. Oehlmann *et al* (1995) identified a disturbance of steroid hormone on the level of estrogen biosynthesis. Their observed virilization phenomenon was linked to an inhibition of the cytochrome P-450 dependant aromatase. TBT has also been suggested to distruput cytochrome P-450 monooxygenase mediation of testosterone metabolic processes (Oberdoster *et al* 1998; Netto 2010). Physiological expressions of these disruptions in normal cytochrome P-450 enzymatic activities include the imposex phenomena and the reduction of female sex glands and offspring sizes in many marine and fresh water species (Oehlmann *et al* 1995; Davies & Minchin 2002; Oberdoster *et al* 1998; Rank 2009). At extremely low concentrations TBT stimulates production of ATP and inhibits its conversion to ADP, leading to cellular malformations, muscle entropy, and decreased metabolic output (Fent 1996; Fleet 2010).

Earliest observed impacts of TBT on the aquatic environment date back to the 1970s where in France authorities observed decreased growth rates in Pacific oysters (*Crassostrea gigas)* in the Bay of Arcachon (Alzieu *et al* 1989; Champ 1999). Abnormal spatfalls (spawning of oysters and other shellfish), decreased survival rates, and shell malformations were also

observed (Alzieu 1989; Dyrynda 1992). These findings failed to be linked to TBT exposure until early 1980s, where France introduced the first ban on the use of TBT antifouling paints in 1982. Shortly after the Bay of Arcachon findings England discovered similar decreases in growth of the Pacific oyster along the east coast (Ebddon *et al* 1989). As if the discoveries in France and England ignited a spark, abnormalities in aquatic biota all over Europe were being linked to TBT exposure. Findings in Ireland (Minchin *et al* 1997), Scotland (Evans *et al* 1994), the Netherlands (Mensink *et al* 1997), Norway (Folsvik *et al* 1999) all stated that local populations of molluscs were being impacted by TBT compounds. These effects ranged from reduced reproduction, reduced neonate and juvenile growth, abnormal shell formations, poor weight gain, shell calcification, and imposex (Alzieu 1998; Schimdt *et al* 2005; Champ 1999).

Once absorbed, mollusks release TBT very slowly from their bodies (EXTOXNET). Other crustaceans (lobsters) elicited reduced growth rates as well. These effects have been shown to occur at levels as low as 1.0 ppb (EXTOXNET). This high sensitivity has allowed molluscs to act as biological indicators of ambient TBT concentrations.

Snails have by far been the main species of study, specifically *Nucella lapillus* (dogwhelk). As mentioned above, TBT causes imposex, a condition where females develop male sexual reproduction characteristics (penis and/or vas deferens) (Alzieu 1998), illustrating TBTs potent endocrine disruptor properties (Garaventa *et al* 2006; Fleet 2010). Laboratory testing has demonstrated imposex in female dogwhelks at TBT levels of 50 ppt (Alzieu 1998, EXTOXNET).

TBT toxicity has been studies on several freshwater invertebrates such as *Hexagenia limbata* (mayfly larva), *Chironomus tentans* (midge larva), *Hyalella azteca* (amphipod), *Daphnia*

*magna* (cladoceran), and *Lumbriculus variegatus* (oligochaete). These species represent a large range of ecological factors – habitation in water column, life cycle, feeding behaviours, and reproduction. It is important to have a range of feeding behaviours – from algae grazing, detritus grazing on the surface of sediments, and burrowing and ingestion of sediment particles - for exposure to TBT in sediments may occur from passive diffusion of TBT dissolved in the interstitial water or direct ingestion of sedimentary particulate matter with sorbed TBT (Day *et al* 1998). These invertebrates have also been extensively used in toxicity tests since the 1980's due to their widespread distribution, contaminant sensitivity, and ecological importance (Bartlett *et al* 2007; Geisy & Hoke 1989). Studies have shown decrease in percent survivalship *in H. azteca, H. limbata,* and *T. tuberfix* at TBT concentrations of greater than 10.5 µg Sn/g dry weight or 132.0 µg Sn/L in interstitial water (Day *et al* 1998). Day also identified that *Hexagenia* species were the most sensitive to TBT, with am LC50 of 1.7 µg Sn/g dry sediment or 1.2 µg Sn/L interstitial water. *D. magna* have shown reduced survival rates, up to 60%, at TBT levels of 912 ng Sn/L (Bartlett *et al* 2007). At TBT levels of 2.0 µg Sn/g dry weight, *H. azteca* has shown significant deceasing of survival and reproduction capabilities illustrating a larger tolerance to TBT than other invertebrates (Bartlett *et al* 2004).

Chironomus spp. have shown survival and endocrine effects at relatively low concentrations of TBT, < 50ng Sn/L (Fargasova 1998; Bartlett *et al* 2004; Bartlett *et al* 2007). Reduction in growth rates of the three juvenile benthic invertebrates (immature *H. azteca, C. riparus* larva, and *H. limbata* nymphs) was observed as TBT concentrations increased to 0.9, 1.5 and 2.7 µg SN/g dry weight respectively (Day *et al* 1998). Weight lost was observed in *Hexagania* nymphs over the 21-day exposure period. *T. tuberifx* has shown reduced survivorship at 7.9 µg Sn/g dry weight with complete mortality at concentration of 10.5 µg Sn/g dry weight *T.*

*tuberfix* reproduction was also affected as TBT concentrations increased. At concentrations of TBT at 1.2 µg Sn/g dry weight significant differences in reproduction were observed, with % cocoon hatching, average number of cocoons per adult, and average production of young per adult considerably reduced (Day *et al* 1998).

TBT toxicity to benthic freshwater invertebrates may potentially be augmented for two reasons. First, benthic organisms ingest and burrow in the sediment and are therefore exposed to high levels of TBT due to TBTs high partitioning to organic matter in the sediment. Second, they may respond in a similar matter to marine invertebrates which studies have shown are extremely sensitive to TBT at low concentrations (Day *et al* 1998; Fent 1996). Thus their applications in TBT monitoring may be beneficial in detecting environmentally low concentrations of TBT.

Bioaccumulation data has been obtained for the benthic invertebrates *C. tentans* and *L. variegatus*. Larval mortality in *Chironomus* spp. has been shown to be a sensitive parameter to measure toxic levels of TBT at levels around 200 µg/L (Vogt *et al* 2007). Larval emergence was another effective endpoint and was observed to be affected at high levels of TBT concentration (200 µg Sn/g dry weight) along with oviposition (Vogt *et al* 2007). These studies have revealed a concentration-dependant reduction in the growth rate of the population through three separate life stages: larval mortality, emergence and oviposition. While valuable, these studies have only checked for acute toxicity at high concentrations, even though TBT is known to be toxic at acute, sub-acute and chronic levels (Bartlett *et al* 2007).

In mammals, TBT's toxic effects occur in the form of TBT chloride (TBT-Cl) (Focardi *et al* 2000). Toxicity of this compound has been research in laboratory rats (*Rattus norvegicus*). Teratogenic effects in fetal rats have been reported during fetal development. Infertility has also

been revealed upon exposure to TBT-Cl in early pregnancy (Focardi *et al* 2000). A TBT metabolite, DBT, has caused atrophy in the thymus in rats, with subsequent suppression of T-cell mediated immune response (Snoeij *et al* 1988).

Recent studies have focused on the toxicity of TBT on large aquatic mammals, investigating tissue concentrations and distribution of TBT and its metabolites. Dolphins, whales, porpoises and sea lions have exhibited high concentrations ranging from 16 to 1.152 µg/kg in regions from Italy to Thailand (Harino *et al* 2008; Focradi *et al* 2000). TBT has been shown to accumulate and biomagnify in these mammals, with the highest levels of TBT occurring in the liver during juvenile stages of life (Harino *et al* 2008; Focardi *et al* 2000). TBT biomagnification factors (BMF) are in the ranges of 0.6-0.8 for pinnipeds to 6.0 in cetaceans (Focradi *et al* 2000). Relationships between large-scale mortality of cetaceans and immune suppression caused by TBT metabolites have been suggested (Kannan *et al* 1997).

Further immune system abnormalities due to TBT and its dealkylated metabolites accumulation have been investigated in harbor seals (*Phoca vitulina*). TBT concentrations in harbor seals from Bic National Park, located in the St. Lawrence Estuary have been found to be in the range of 0.1 – 0.4 ng Sn/g wet weight (ww) in blood and 1.2 – 13.4 ng Sn/g ww in blubber (Frouin *et al* 2008). Peripheral blood mononuclear cells isolated from adult and pups exposed to varying concentrations of TBT, MBT and DBT in vitro found that phagocytic activity of blood cells significantly deceased at 200nM TBT in adult seal, and 200 nM of DBT inhibited phagocytosis efficiency in pups (Frouin *et al* 2008).

*TBT Regulations*

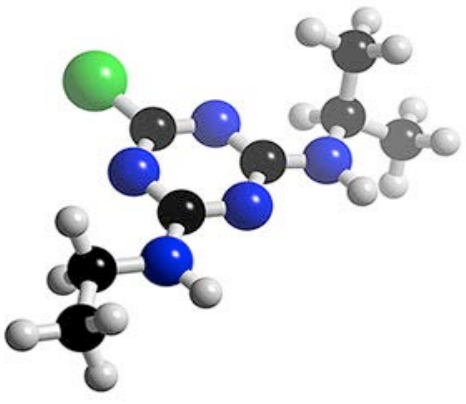
As mentioned above the very first regulatory ban on TBT was in France, when on January 19 1982 it was banned on vessels less than 25m in length (Alzieu 1991). French Ministry of the Environment (MOE) announced a 2 year temporary ban on TBT antifouling paint consisting of more than 3% (wt.) tin travelling both the Atlantic coasts and the English Channel (Champ 1999). An extension was made on September 16 1982, effective through to February 12 1987, banning application of TBT paints on vessels under 25m, exempting vessel with aluminum hulls (Alzieu 91; Champ 1999).

Following Frances example, England's Environment Minister in Parliament announced the first regulatory action to reduce environmental impacts associated with TBT exposure on July 24 1985. This action consisted of 5 regulatory actions; (1) regulations to control retail sale of TBT free associated paints, (2) establishment of a notification regime for all new antifouling agents, (3) the development of guidelines for cleaning and applications of antifouling paints to ships, (4) establishment of a provisional ambient Environment Quality Target (EQT) of 20 ng/L, and (5) further investigation into TBT impacts and monitoring programs allowing government to assess effectiveness of these actions (Champ 1999). Retail sale of TBT antifouling paints was brought under control of the government with the Control of Pollution Regulations of 1985, regulations developed under sections 100 and 104(1) of the Control of Pollution Act of 1974, and brought into force on January 13 1987 (Champ 11999). England subsequently lowered its EQT from 20 ng/L to 2.0 ng/L in 1987 (Champ 1999). These new regulations lowered the maximum allowed tin in copolymer paints from 7.5 to 5.5 % under the Control of Pollution Act of 1986 (COPA), amending allowances under the Control of Pollution regulations of 1985, and coming into effect on May 28 1987. This amended Act prohibited the retail sale and supply of any and all antifouling paints containing tri-tin compounds (Champ 1999). Interestingly, this Act did not

make exceptions to accommodate aluminum-hauled vessels, vessels with outboard motors or lower drives, as did the French and US regulations did.

As these new regulatory Acts were being developed and gaining wide acceptance across Europe, the US and Canada began to investigate and create regulations for the use of TBT antifouling paints. In 1989 Canada set its first regulatory ban on the application of TBT anti-fouling paints on vessels under 25m in length with a leaching rate applicable to larger vessels containing the anti-fouling paint (Fleet 2010). While its use is now regulated under the Canadian Environmental Protection Act (CEPA) and the Pest Control Products Act (PCPA), data on TBT quantities in the aquatic environment is missing. Data is also incomplete on TBT concentrations in water and sediments in the freshwater portion of the Great Lakes St. Lawrence Seaway (GLSLS). Due to these absent studies there is no recognized criterion for sediment quality under TBT toxicity (GLSLS Study 2007). As of June 2000, the Canadian Pest Management Regulatory Agency (PMRA) began a nationwide phase out of TBT antifouling paint and stopped accepting and processing applications to register new TBT antifouling paints (Showalter 2005). PMRA determined that the "use of TBT antifouling paints represent an unacceptable risk" to aquatic environments (Showalter 2005). Anticipating the global ban originating in Europe, Canada banned all sales and applications of TBT antifouling paints in 2002 (Showalter 2005).

### 1.5.2 Atrazine



Atrazine is a selective systematic herbicide with the chemical formula $C_8H_{14}ClN_5$ (Canadian Environmental Quality Guidelines 1999). Its CAS name is 2-chloro-4-ethylamino-6-isopropylamino-1,3,5,-triazine with trades name such as AAtrex, Griffex, Maizine, and Sutazine (Johnson 1986). It is a white crystalline substance with a low solubility of 28mg/L at 27$^{o}$C (Kidd & James 1991), but soluble in many organic solvents at 360 to 183 000 mg/L in such solvents as chloroform, diethyl ether, and DMSO (Mudhoo & Garg 2011; Kidd & James 1991). Atrazine exhibits a high octanol-water partition coefficient (log $K_{ow}$= 2.3404) (Kidd & James 1991). Atrazine is available as material at 99.9% active ingredient and as a manufacturing use product containing 80% for creation of powders, pellets, and tablets (Mudhoo & Garg 2011). It exhibits a high molecular affinity for organic matter in soil-clay complexes (Binet *et al* 2006), an affinity increasing with increasing organic carbon content. This sorption to organic matter decreases atrazine's bioavailability however increases its persistence despite its readily biotic and abiotic degradation (Mudhoo & Garg 2011). Atrazine can be physiochemical or biochemically degraded or mineralized into more than 15 metabolites, with the four main ones being desethylaltrazine (DEA), deisopropylatrazine (DIA), didealkylatrazine (DDA), and hydroxyatrazine (HYA) (Radosevich *et al* 1997). Primary chemical (abiotic) degradation produces HYA, identified by Prata *et al* (2003) as the most important degradation compound with a higher retention in soils over the other degradation compounds. N-dealkylation of Atrazine side chains yields DEA and DIA (Mudhoo & Garg 2011). Both DEA and DIA have similar toxicities with greater water solubility and weaker interactions with organic matter than the parent herbicide (Abate & Masini 2005; Mudhoo & Garg 2011).

**Figure 1.8** Atrazine molecule

Atrazine is a broadleaf and grassy weed herbicide used in agriculture, specifically corn and low brush blueberries, soybean, sugarcane, and sorghum, and as a nonselective weed control on non cropland (i.e. constructed forests, golf courses) (Abate & Masini 2005; Netto 2010). It is applied either pre or post emergence (growth) to the crop at a rate of 1.1 to 1.7 kg/ha (Gaynor *et al* 1998), with increased rates of application associated with finer textured soils. Originally patented in Switzerland in 1958, its use as an agricultural weed killer started in the US in 1959, and it was subsequently introduced in Canada in 1960 (Fleet 2010). Since then atrazine has become the heaviest applied herbicide in the world. In 1990 more than 1.2 million kg were sold in Canada, with more than 90% going to Ontario and Quebec (Agriculture Canada and Environment Canada 1995). Between 1990 and 1993, up to 28 million kg of atrazine was applied annually in the US. In 1993 alone, India applied over 3 million kg as a pre-emergence application for corn (Mudhoo & Garg 2011).

*Atrazine in the environment*

Due to this copious application it is no wonder atrazine has been found in the surrounding environments. The herbicide enters aquatic ecosystems through terrestrial runoff by means of storm events and leaching (deNoyelles *et al* 1982), and to a lesser extent tile drainage (Caux & Kent 1995) where it predominantly remains in its aqueous phase. It may also enter the environment from spillage, accidental discharge during storage, and during disposal (Canadian Environmental Quality Guidelines 1999). Contamination of surface and groundwater has been detected nationwide, from Prince Edward Island to British Columbia (Health Canada 1993; Fleet 2010). During application periods in June atrazine concentrations of 686 μg/L to 3,400 μg/L

have been reported in surface runoff from Ontario watersheds (Gaynor *et al* 1998). Atrazine levels in drinking water have been reported to reach 81 μg/L, exceeding Health Canada's drinking water regulations of 5 μg/L and the 2 μg/L Canadian Water Quality Guidelines (CWQG) to prevent harm to aquatic life (Health Canada 1993). More recently, a US sampling of agricultural lands between 1998 and 2001 found that 57% of the 83 streams sampled had a pesticide/herbicide that exceed one or more regulatory safe levels for aquatic life, 18% of which were atrazine (Pimentel 2005). Another US study found atrazine exceeding the USEPAs drinking water standard in 33% of samples gathered in the Midwest (Rocha *et al* 2008).

*Atrazine toxicity*

Similar to other triazine herbicides, atrazine's mode of action is the inhibition of photosystem II by binding to the plastoquinone-binding protein. This results in starvation and oxidative damage due to breakdown in the electron transport process, and eventual plant death. At high intensities oxidative damage is increased (Canadian Environmental Quality Guidelines 1999; Fleet 2010). Atrazine toxicity to aquatic biota is dependent on exposure time (Gaynor *et al* 1998). Toxicity studies have been performed on numerous organisms, from microbial communities to fish. As atrazine primarily inhibits photosynthesis, aquatic plants have been found to be more sensitive to atrazine than other aquatic organisms. Larsen *et al* (1986) calculated a 24-h EC50 for green algae (*Pseudokirchneriella subcapitata*) of 33-72 μg/L; the algae cultures exposed to a maximum atrazine concentration of 882 μg/L elicited complete arrestment of all photosynthetic activity. The s-triazine herbicide associates with a 32-kDa protein in complex B of photosystem II effectively blocking and inhibiting electron transfer between quinone acceptors (Schulz *et al* 1990), a result coined as the "Kautsky effect". At low

atrazine concentrations of 0.8 μg/L, duckweed *(Lemna minor)* showed similar affects and attempts to overcompensate with an increase in chlorophyll content and overall photosynthetic activity; once atrazine levels reach inhibitory concentrations (>6.9 μg/L), the Kautsky effect predominates (Schulz *et al* 1990). Studies on fish showed that Bluegills (*Lepomis macrochirus*) and fathead minnows (*Pimephaleas promelas*) were not affected by atrazine during an 18-month study at concentrations of 95 μg/L and 213 μg/L respectively (Macek *et al* 1976). Zebra fish (*Danio rerio*) swimming behaviour and motility was affected at atrazine levels of 6 μg/L (Dewey 1986). Bullfrogs (*Rana castabeaina*) showed an LC50 of 410μg/L (Gaynor *et al* 1998), and leopard frogs (*Rana pipiens*) exhibit reduction in growth rates at a 96-h exposure to 310μg/L of atrazine. Atrazine reduces hatching success along with larval deformations in juvenile *C. tentans* at concentrations of 230 μg/L (Dewey 1986).

Atrazine exposure in humans is most commonly from contaminated drinking water with some cases due to exposure during field applications. Atrazine toxicity has been linked to several health issues including both acute and chronic nausea and dizziness. Severe cases of atrazine toxicity have been shown to increase the risk of ovarian cancer, reproductive system tumors, malignant tumors in the uterus and breast, and non-Hodgkin's lymphoma (Health Canada 1993; Fleet 2010). In men, a 2003 study undertaken by Health Canada demonstrated an increase risk of prostate cancer in farmers, leading to the classification by Health Canada of atrazine as a Group 3 carcinogen (a possible cancer-causing agent in humans). In light of these findings Health Canada, along with the World Health Organization (WHO), has recommended a daily maximum intake of atrazine of 0.5 mg per kilogram of body weight.

Although the exact mode of atrazine toxicity is unknown, Wan *et al* (2006) has observed moderate to extreme modifications in behaviour in non-target aquatic organisms. A possible

mechanism of atrazine toxicity may be the suppression of acetylcholinesterase, an enzyme responsible for the breakdown of acetylcholine. This leads to a buildup of acetylcholine causing continual nerve stimulation and muscle activity, resulting in paralysis and finally death (Shimabukuro & Swanson 1969). Liu *et al* (2001) has suggested a link between atrazine toxicity and ATP availability and regulation. Oxidative stress is another mechanism of toxicity suggested by Bhatti *et al* (2011); linked to the increase of reactive oxygen radicals leading to macromolecule and DNA damage and possible varying forms of cancers.

*Atrazine regulations*

The current interim water quality guideline for atrazine for the protection of aquatic life is 2.0 μg/L for freshwater ecosystems and was based on Canadian Drinking Water Quality guidelines. Table 1.1 shows several guidelines for atrazine use. There is currently no marine quality guideline (CCME 1999). The European Union (EU) placed a ban on all Atrazine use and sales in 2004 due to its persistence in groundwater. However no follow-up bans or regulations have been set in Canada or the US.

**Table 1.1** Recommended Canadian water quality guidelines for atrazine

| Water use | Guidline |
|---|---|
| Raw water for drinking water supply | No recommended guideline * |
| Freshwater aquatic life | 2 μg/L |
| Livestock watering | 5 μg/L (interim) ** |
| Irrigation | 10 μg/L (interim) |
| Industrial water supply | No recommended guideline |
| Recreational water quality and aesthetics | No recommended guideline |

* Guidline for Canadian Drinking Water Quality, maximum acceptable concentration (MAC) of 5 μg/L recommended (Health Canada 1993)

** Guidline under development, Guidline for Canadian Drinking Water Quality (MAC) adopted in interim (Caux & Kent 1995)

### 1.5.3 Copper

Copper (Cu) is a transitional metal with the atomic number 29 and an atomic mass of 63.546. It occurs naturally as two ions – cuprous ion (copper (I)) and cupric (copper (II)) ion. It forms the common salt, copper (II) sulfate which has a melting point of 1356$^{o}$K and a boiling point of 2868$^{o}$K (Dort 2010). Copper is ubiquitous and occurs naturally in the environment, in rocks, soil, plants, animals, and water. All living organisms require copper for regular metabolism and survival. Trace amounts in our food in necessary for healthy living. However, some forms of copper and excess amounts of copper can lead to serious health problems.

Copper and its complexes have many uses. It is combined with other metals to make brass and bronze pipes and faucets used in plumbing, electrical wire production, electroplating, and roofing (Dort 2010). A 1981 survey ranked Canada as the fourth largest producer of mined copper worldwide with 8.3 million tonnes mined, and sixth in the world for refined copper production producing 0.477 million tonnes (Dort 2010). Over 36 percent of that mining occurs in Ontario (Health Canada 1992; Dort 2010). The Great Lakes Water Quality Board (GLWQB) has reported metal-contaminated sediments to be a major concern for the Great Lakes, particularly in sites near mining and petrochemical activity (Flemming & Trevors 1989). In the petrochemical industry, copper is used as a catalyst for the removal of mercaptans in oil refining (Health Canada 1992). Copper is also used in ammoniacal solutions to remove carbon monoxide in natural gas before it is used for heating or energy production. Other energy industrial uses copper oxychloride as additions to coal to produce fine granular ash after combustion for easy removal (West 1982). Agriculture also uses copper as biocidal agents in the form of fungicides, algaecides, and insecticides (Health Canada 1992). In these applications copper is used in the form of copper oxide, copper chloride, and copper oxychloride (Dort 2010). Canada's PMRA currently has 96 registered products that contain copper as the active ingredient, with 34 listed

pesticide applications (Dort 2010). Copper-based fungicides are applied to timber and fabrics to protect against fungal infestations. Interestingly, fungicidal copper is also used for disease prevention. Copper sulfate mixed with lime and water, a compound called Bordeaux mixture, is used to prevent over 300 diseases (Dort 2010). Similar to the way tin is used in TBT anti-fouling paints, cuprous oxide flakes and powders are mixed into paints for anti-fouling applications on boat and ship hauls.

*Copper in the environment*

Copper can enter the aquatic environment in many ways. Naturally it can occur through atmospheric deposition via gravitational settling, dry deposition, or in the form of precipitation (Dort 2010). However it is the anthropogenic relapse of copper into the aquatic environment that leads to environmental harm. Waste disposal from industry, highway and agricultural runoff, urban wastewater discharge, and mining waste are all means of copper release into the aquatic environment. Coppers toxicity in the aquatic environment is dependent on its speciation and not solely on concentration and residence time (Dort 2010). The different species of copper released into the aquatic environment determine its bioavailability, and therefore its overall toxicity. The cupric ion predominates over the cuprous ion in aquatic systems, forming organic and inorganic complexes most of which are stable (Mansilla-Rivera & Nriagu 1999) The cupric ion can partition into three different phases: (i) aqueous, (ii) solid, and (iii) biological. The aqueous phase keeps copper in its free ion form and with soluble organic/inorganic complexes. The solid phase binds to particulate matter in the substrate, either floating in the water column or settling in the sediment. Finally the biological phase of copper occurs when copper is absorbed by organisms and incorporated into their systems (Flemming & Trevors 1989). The most toxic form

of copper in the aquatic environment is the soluble form. This form of copper allows for free association throughout the water column and maximum interaction with biota. However, insoluble copper, (mainly oxides, phosphates, and carbonates), and its deposition in the sediment poses implications for benthic toxicity and re-suspension. Therefore this form must also be considered when investigating copper toxicity.

The site of copper deposition within an aquatic environment also depends on environmental physiochemical factors such as temperature, pH, redox potential, soil and sediment size type, elemental composition, hardness, organic content, and trophic status (Dort 2010; Flemmin & Trevors 1989; Health Canada 1992). In a study by Stone and Droppo (1996), extraction data suggested increasing bioavailability potential of copper with decreasing sediment grain size. Interestingly, dissolved organic carbon may pose as a competitive presence for the partitioning of copper. Kramer *et al* (2004) found that as natural organic matter (humric and fulvic acids) increased, partitioning of insoluble copper decreased and dissolved cupric ion content increased - thus decreasing coppers bioavailability and toxicity.

*Copper toxicity*

The mechanism of copper toxicity to aquatic biota is not quite fully understood but it has been suggested by Untersteiner *et al* (2003) that copper may disrupt metabolic processes that regulate internal oxygen concentration in aquatic organisms. With its known becterialcidal properties copper has the potential to alter aquatic microbial community structure, inducing resistance-based selective microbial evolution (Crooks *et al* 2011). Studies done by Edral & Demirtas (2010) showed copper induced enzymatic alterations in several aquatic plants (*Lemna*

*minor*, *Ceratophyllum submersum*, and *Ptamogeton natans*). Antioxidant enzymes superoxide dismutase and peroxidase levels were shown to be significantly increased in *P. natans*, and decreased *in L.minor and C. submersum*. Furthermore, hydrogen peroxide ($H_20_2$) and malondialdehyde levels in all three plants were found to be very high compared to control studies. The study concluded with results indicating that copper toxicity reduced plant biodiversity and number of aquatic plant species. Algae have shown suppressed cell growth, chlorophyll synthesis, photosynthesis, and motility through several of the proposed mechanisms of enzyme inhibition. This had resulted in oxidative damage and replacement of essential metals (Knauert & Knauer 2008). Long-term copper toxicity on *Scenedesmus quadricauda* at concentrations of 250 μg/L revealed sharp increases in polypeptide complexes to chelate copper. Polypeptide numbers gradually decreased over a sample time of 732 hours, indicating this to be a prime mechanism of heavy metal acclimation or adaptation (Busssieres *et al* 1995).

Copper has been demonstrated to cause significant differences in invertebrate community structure with the increase in exotic species (Crooks *et al* 2010). Total native species pool was shown to have a 40% decline over exotic species with increasing copper concentrations, confirming that anthropogenic activities influence the success of invasive species colonization. Copper was found to impair feeding behaviour and inhibit growth and reproduction of *D. magna* (Barata *et al* 2006). Studies identified the 48h LC50 for *D. magna* to be between 5 and 86 μg/L (Flickinger *et al* 1982). Large amounts of Cu in sediments may reach "self defense" thresholds (Niera *et al* 2011), imposing large-scale negative impacts on macro-invertebrate communities. At sites with elevated sedimentary Cu levels, macrobenthic communities have not only been less diverse, but individual total body mass and body size were reduced compared to sites with lower Cu concentrations (Neira *et al* 2011). Other toxicological data for invertebrates include both

freshwater and marine systems. The euryhaline rotifer *Brachionus plicatilis* ("L" strain) show a 48-h LC50 20.8 μg/L Cu (Arnold *et al* 2010). *C. tentans* exhibited delayed emergence under copper concentrations of 49.4 g/kg dry weight (Clement *et al* 2004). Salmon have shown altered growth, reproduction abnormalities, and behavioural alternations under concentrations as low as 5μg/L. Another study performed on fish indicated a total lack of any species of fish in lakes with copper concentrations greater that 60μg/L (Flemming & Trevors 1989). Coppers toxicity to fish is associated with disruptions to sodium transports (Netto 2010). Lethal and sub-lethal effects such as mortality and hatching delay have been found in the freshwater pulmonate snail *Lymnaea luteola* at Cu concentrations of 3.2, 5.6, and 10.0 μg/L (Khangarot & Das 2010).

Copper also exhibits synergistic effects in combination with other heavy metals such as cadmium and zinc (Hermens *et al* 1984). This creates a dilemma when attempting to quantify copper toxicity in the environment, where concentration measurements of copper may not yield the exact representation of coppers toxicity on biota.

While necessary for healthy metabolism, increased copper levels in the body can cause serious health problems. Toxicity due to elevated copper intake can include metallic taste in mouth, nausea, vomiting, diarrhea, jaundice, and haemolysis (Dort 2010). Extreme copper poisoning can lead to anuria, hypotension, coma, and death. While exact does levels of copper per symptom is unknown, levels over 5 mg/day have been shown to result in gastrointestinal cramping (Hancock & Pon 1999). The WHO has established a daily copper intake guideline of 2 mg/day, not including supplemental copper taken through contaminated water (WHO 2004). Ontario Drinking-Water Standards has set a maximum concentration of copper in drinking water of 1 mg/L – however this guideline has been established using the aesthetic quality of the water

(i.e cloudiness and taste) (MOE 2001). Elevated copper in drinking water may be magnified by corrosion of old copper pipelines and fitting.

### 1.5.4 Ciprofloxacin

Ciprofloxacin (cipro) is a fluoroquinolone antibiotic (FQ). It has a solubility in water of 0.088 mg/mL at 25$^{o}$C and a pH of 6.84, with a n-octanol/pH 7.0 buffered solution partition coefficient (logP) of - 1.70 at 25$^{o}$C, with higher solubilities at pH values above 10 and below 5 (Olivera *et al* 2010).

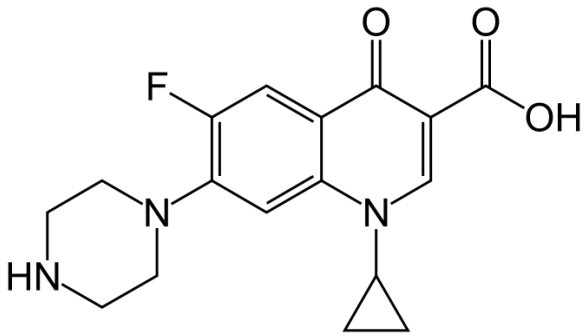



**Figure 1.9** Ciprofloxacin

FQs were first synthesized in 1973 in the form of flumequine. In 1980, floxacins where first created from the original FQ structure with two modifications: a fluorine atom on the sixth carbon and an aliphatic cyclic amine on the seventh carbon. These become the building block for future floxacins, i.e. cipro. FQs are a broad spectrum antibiotic effective against numerous gram-positive bacteria and some gram-negative bacteria. FQs inhibit bacterial growth by interfering with DNA synthesis through inhibition of topoisomerase II and topoisomerase IV in gram-postive and gram-negative bacteria respectively, resulting in impeded replication and eventual death of the cell (Hooper 2001). FQs are licensed for both human and animal use with cirpo as one of the largest globally prescribed antibiotics (Pico & Andreu 2007). The primary mechanism of action of cipro is inhibition of the activity of the A subunit of DNA gyrase (Wolfson & Hopper 1991). Inhibition of bacterial gyrase causes relaxation of the supercoiled DNA, resulting in termination of chromosomal replication and interfering with cellular division and gene expression (Campoli-Richards *et al* 1988). A secondary mode of action is inhibition of the activity of topoisomerase IV, leading to separation of two united DNA stands and subsequent interference with cell replication (Peng & Marians 1993).

Antibiotics as environmental toxicants are rapidly becoming a large area of study in environmental sciences. As health care and pharmaceutical technologies develop, societal uses of drugs in developed countries rises. Its overzealous use has called attention to its presence in household wastewater and its retention in the environment after wastewater treatments (Cordova-Kreylos & Scow 2007). According to the US National Health, in 2003 the US was spending $227.5 billion on prescription drugs, an amount three times larger than in 1997. In Canada, between 1985 and 2001, prescription drug use increased from $3.8 to $16.7 billion, representing over 16% of total health care expenditure in 2001 (Canadian Institute of Health Information 2004). Regulatory authorities are responsible for the licensing of medicinal products and with the rising environmental risk have become epicenter on the emerging matter of increasing public scrutiny and legal requirements. For these reasons the fate of pharmaceuticals in the environment has become an area of increasing importance.

*Ciprofloxacin in the environment*

Pharmaceuticals enter the environment in numerous ways. After administration humans excrete a large amount of un-metabolized antibiotics, eventually ending up in wastewaters (Halling-Sorensen *et al* 2000). Surplus drugs can also be disposed of in household drains, a practice discourage by general practitioners. When these partially degraded or chaste drugs enter wastewater they end up at treatment plants where further degradation occurs through biological processes. If the drugs are not completely metabolized at wastewater treatment plants (WWTPs), they can be released into receiving waters or incorporated into sludge. Furthermore, if WWTP sludge is converted and used for biosolid applications, these drugs enter the topsoil and are

susceptible to uptake by crops and eventually move vertically into ground water (Karnjanapiboonwong 2010). The US has proposed regulations on new medications based on these environmental risks, with environmental risk assessments (ERAs) on new pharmaceuticals being proposed in the European Union. However, there are no regulations or assessment requirements concerning environmental risk on exiting pharmaceuticals.

*Ciprofloxacin toxicity*

After entering aquatic ecosystems pharmaceuticals have a varying effect on biota. Cipro has been shown to have a high persistence with a half life of 1.6-2.5 days in activated sludge reactors (Boxall *et al* 2004). The half lives of FQs in the environment range from 1 month to a year (McClellan & Halden 2010). In the same study cipro showed elevated toxicity to sludge and soil bacterial communities over other antibiotics such as mecillinam and trimethoprim with an EC50 of 0.61 mg/L, over 62.1 and 17.8 respectively. Additionally, cipro was the only antibiotic to have toxic effects on green algae (*P. subcapitata*) eliciting an EC 50 of 2.97 mg/L. In the study by Cordova-Kreylos & Scow (2007) the magnitude of cipro toxicity on microbial communities was found to be inversely correlated to the degree of sorption to the sediments. The study concluded with conclusions identifying that cipro contamination in sediments was found to be selective, appearing to favour sulfate-reducing and gram-negative bacteria.

Although microbial communities are greatly affected by cipro exposure, higher organisms also show toxicity to cipro in aquatic ecosystems. *L. minor* was assessed for number and area of fronds in a study by Kolasińska *et al (*2010). Cipro, and another FQ ofloxacin, were found to be the most toxic out of a total of 8 FQs tested. Cipro $EC_{50}$, $EC_{20}$, and $EC_{10}$ values for

frond numbers were calculated to be 243, 64, and 47 µg/L respectively. Frond area showed similar toxicity data, with a cipro $EC_{50}$, $EC_{20}$, and $EC_{10}$ of 57, 28, and 18 µg/L respectively.

There is little data on the effects of cipro on invertebrates. One study by Halling-Sorensen *et al* 2010 demonstrated a NOEC for *D. magna* up to 60mg/L, and a NOEC at levels up to 100mg/L in zebra fish during a 72-h exposure test..

Once in the environment the fate of cipro and other pharmaceuticals is dependent on their degree of natural attenuation. Release into surface waters results in attenuation to trace levels through dilution, sorption onto suspended particulate matter and sediment, direct and indirect photolysis, and aerobic biodegradation (Pal *et al* 2010; Mompelat *et al* 2009) – all dependent on the properties of the drug and the chemical and biological parameters of the receiving system. In the case where pharmaceutical loaded effluents mix with sea water, the influence of salinity and complex mixing patterns makes calculating the fate becomes difficult. Generally, pharmaceuticals with high log $K_{ow}$ (>5) easily absorb to sediment and can be removed by coagulation. No data exist for natural photolysis and biodegradation of cipro in the environment (Pal *et al* 2010). Other treatment options include UV photolysis and chemical treatment. UV photolysis has no effect on cirpo by UV-C (254nm), however chemical treatment has resulted in a 65% removal success (0.3 mg O3/mg TOC) (Pal *et al* 2010).

Treatment of pharmaceutical contamination in the environment is also important for human health. Indirect exposure through pharmaceutical-contaminated drinking water to date may not be of great importance yet due to relatively low concentration. However, this still poses a risk to human health through increased microbial resistance (Boxall 2004). In a study done by Pomati *et al* (2006), re-uptake of ciprofloxacin at environmentally relevant concentrations poses

the risk of growth inhibition of human embryonic kidney cells HEK293, with the highest effect observed as a 30% decrease in cell proliferation.

Canadian and US drinking water legislation currently places no regulatory bodies on the monitoring and treatment of pharmaceuticals. With increasing inputs of pharmaceutical products and degraded metabolites entering the aquatic environment it is becoming ever more important to have a detection system for these products. With the current high-cost, time-demanding, specific chemical analysis presently occurring at drinking water treatment centers it is unlikely that authorities will add additional pharmaceutical monitoring requirements. This make it all the more vital to have a low-cost, broad-spectrum monitoring regime in place to identify the presence of the un-witnessed contaminants.

### 1.4.5 Dimethyl Sulfoxide

Dimethyl sulfoxide (DMSO) is a common organic solvent used as a carrier in chemical dilutions for chemicals with low water solubility's. For this purpose DMSO was used in the current study as a vector for TBT, atrazine, and cipro. This addition allowed for ubiquitous distribution of contaminant in dilution series. Interestingly, DMSO is also used in human and veterinary therapeutics (Barbosa *et al* 2003; Fleet 2010), ensuring a low toxicity to test organisms. DSMO was chosen over other organic solvents such as methanol, ethanol, acetone, and acetonitrile for its lower toxicity and wide use in biological assays (Bowman *et al* 1981: Hutchinson *et al* 2006; Ren *et al* 2008; Ren *et al* 2009). For this study TBT, atrazine, and cipro were dissolved in a 0.1% DMSO concentration, a low level which has been shown to have no impacts on test organisms (Fleet 2010).

## 1.6 Organisms

The two organisms used in this study are *Chironomus tentans* and *Lumbriculus variegatus*. They are benthic invertebrates ubiquitous throughout North American and European freshwater habitats. They represent a large niche within the aquatic food web and therefore have a large importance when investigating ecology toxicity. Their proliferate use in the area of ecotoxicology, especially behavioural studies, within the past three decades have allowed for significant understanding of their importance as biological indicators for aquatic ecosystems. It is the wish of this study to contribute to the growing accumulation of knowledge regarding these two organisms and to also shed new light on the possible use of their behaviour as part of an early-warning system for drinking water

### 1.6.1 Chironomus tentans

The first organism of focus in this study is the non-biting midge *Chironomus tentans*. Chironomids are a member of the dipteral order, part of the 'true flies' along with mosquitoes, gnats and midges. They begin their lifecycle as an egg incorporated in a large egg-mass. Each egg-mass contains anywhere from 200 to 500 eggs. Upon hatching, the larvae immediately begin to construct a burrow comprised of particulate decaying organic matter.

**Figure 1.10** C*hironomus tentans* larva

The larva lives within its constructed burrow and forages around the opening to feed on microorganisms and organic matter. After several weeks, 4 to 5 for most North American areas, the larva seals itself in its burrow and undergoes a metamorphosis into a pupa (Figure 1.11). The pupa rises to the surface where an adult chironomid emerges. After emerging from its pupa, the flying chironomid reproduces and dies shortly after. The adult life span is anywhere from several hours to 5 days. This multistep lifecycle of chironomids allows for several levels contaminant exposure.

**Figure 1.11** *C. tentans* pupa

Chironomids are most sensitive to toxins as an egg. As larvae, the chironomid can easily ingest a host of toxic agents resulting in delayed growth, failure to mature into an adult, reduced egg count and physiological deformities.

They represent a major niche in the aquatic food web and are a food source for many creatures. Chironomids are ubiquitous and globally distributed among freshwater ecosystems and are the most abundant group of insects found in freshwater ecosystems. This places them in an important position in the aquatic food web and of great interest to studies involving aquatic toxicology. They have been are used extensively in the assessment of environmental contamination in many ways and have a number of characteristics that make them valuable toxicity indicators. Their dual life cycle both an aquatic larva and flying midge (Figure 1.12) allows for both sediment and water contamination studies. Their pupation and emergence events have the ability to reflect both sediment and water quality, effecting both growth and

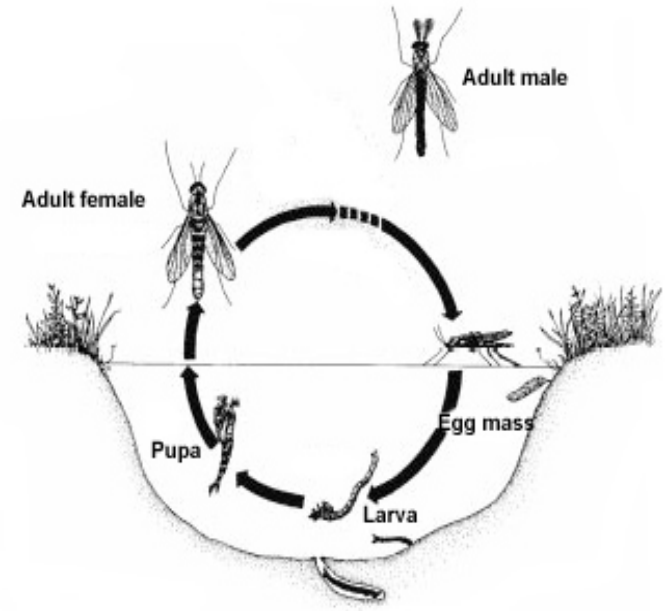



**Figure 1.12** Life cycle of *C. tentans*

reproductive success along with other ecological effects. The ease in life cycle identification and short life-cycle durations has proved advantageous for testing. Their ease in laboratory and detailed life history on several test species (i.e. *tentans*, *riparius*, *dissmilis*) has allowed for wide spread use and appropriate selection for environmental relevance.

Past biological assays have extensively focused on species-assemblage studies pertaining to lake trophication, classification, and paleolimnological identification. Many macro-benthic invertebrates have been used for these studies, however the taxa richness and species abundance of chironomids offered the widest possible spectrum of responses to environmental stress and soon became the ideal indicators of ecosystem-level health (Rosenberg 1992). In the past two decades the steady growth in the use of chironomids in biomonitoring, as measured by Rosenberg (1992) as the number of publications on the subject, have made the study of chironomids in biological monitoring a great and useful tool (Table 1.2).

Since responses to environmental stressors originate on the biochemical and physiological level studies soon began to incorporate direct organism toxicity. Identification of stress indicators provides the earliest possible warning of environmental contamination and since initial conception many biomarkers have been identified. Initial biochemical indicators began

with changes in energy metabolism measured as phosphoadenylate concentrations, enzyme activities (i.e. acetylcholinesterase), RNA, DNA, amino acid, protein content, and iron concentrations (Rosenburg 1992). Morphological deformities have also been used to indicate environmental contamination. Deformities of chironomid mouthparts have been most frequently used with references made to industrial and agricultural pollutants as toxicants. The abundance of morphological deformities in isolated areas are strong indications of high levels of pollutants and have been used to indicate 'hot spots' in need of immediate attention.

**Table 1.2** Locations of identified *C. tentans* deformities and sources (Rosenburg 1992)

| Location | Deformities | Contaminants | References |
|---|---|---|---|
| West end of Lake Erie | Head capsule and mouth-part; thickening of body walls of thorax and abdomen | Industrial and agricultural pollutants | Hamilton and Saether (1971) |
| Parry Sound, Ont. | Mouthpart | Industrial effluents | Hare and Carter (1976) |
| Teltowkanal, West Berlin | Mouthpart | Industrial effluents | Koeh and Frank (1980) |
| Bay of Quinte, Lake Ontario | Mouthpart | Industrial and/or agricultural pollutants | Warwick (1980) |
| Pasqua Lake, Sask. | Mouthpart | Unidentified | Warick (1980) |
| Experimental ponds, Oak Ridge National Laboratory, Tennessee | Mouthpart | Coal liquid | Cushman (1985) |
| 12 Swedish Lakes | Mouthpart | Industrial effluents | Weiderholm (1984) |
| Tobin Lake, Sask. | Antenna | Industrial and agricultural pollutants | Warick (1985) |
| Re-examined material from experiments of Hamilton and Saether (1971). Freshwater Institute, Winnipeg | Antenna | DDT | Warick (1985) |
| Laboratory experiment, University of California, Davis | Mouthpart | Cu | Kosalwat and Knight (1987) |
| Port Hope Harbour, Ont. | Mouthpart | Radiation | Warick *et al* (1987) |
| Tobin Lake, Sask. | Antenna and mouthpart | Industrial and agricultural pollutants | Warick and Tisdale (1988) |
| Murray and Darling rivers, Australia | Mouthpart | Pesticides or heavy metals | Pettigrove (1989) |
| Sites across Canada | Antenna and mouthpart | Organic chemicals, heavy metals sewage, radionucleotides | Warick (1989) |
| Niagara River catchment | Mouthpart | Agricultural, industrial, and domestic pollutants | Dickman *et al* (1990) |

While biochemical, physiological, and morphological toxicity on chironomids has been the focal point, parallel research was also being conducted on the organism's behaviour. Physiological disorders in response to a pollutant will undeniably show early manifestations, providing an 'early warning' which can be used to indicate acute sub lethal toxicity. As early as 1983, studies have looked into using specific behaviours as indications of stress. Batac-Catalan and White (1983) started with using optical-fibers in a light-interruption test to study the behavioural effects of chromium and *C. tentans* larvae. They focused on respiratory movements and found that these movements were suppressed at 0.1 and 1.0 ppm, and were elevated at 10.0 to 1000.0 ppm. As the 48-h EC50 for chromium toxicity on chironomids was 61.0 ppm this study illustrated that behavior could be used to identify sub lethal toxicity at relatively low concentrations. Another behavioural study done by Heinis *et al* (1990) used a technique called impedance conversion to measure deviations in normal feeding patterns. Although this study used *Glyptotendipes pallens* instead of *C. tentans*, results showed measureable deviations from normal feeding behaviour at concentrations greater than 5.0 mg Cd/L, where 'totally aberrant behaviour' was characterized 'by long periods of inactivity and no feeding'. The detection of these behavioural effects, at concentrations as low as 0.5 mg Cd/L. was at a level of 100 times lower than the 144-h LC50 value of 50-100 mg Cd/L (Rosenburg 1992; Hienis *et al* 1990).

Other studies have demonstrated the usefulness in using Chironomid behaviour as toxic indicators. UV radiation tests performed by Bell *et al* (2004) indicate that the burrowing behaviour of chironomid larvae is a protective mechanism for avoidance of stress conditions such as UV exposure. The burrowing effectively decreased photo-enhanced PAH toxicity in 3$^{rd}$ instar larvae resulting in increased survivalship and emergence. In connection to burrow construction, burrow irrigation is a behaviour in which the larvae attempt to increase dissolved

oxygen (DO) levels in surrounding water by 'irrigation motions', described as wave-like undulations along the body. This facilitates increased DO extraction from the surrounding water by reducing the boundary layer covering respiratory surfaces and increasing the flow of water over the body surface (Irving *et al* 2004). Chironomids larvae rely on cutaneuous respiration to receive DO from the water surround their burrows, however some respiration occurs along their four ventral gills and two pairs of anal gills (Irving *et al* 2004; Eriksen *et al* 1988; Nebeker 1972). Chironomids exposed to low DO levels for long periods of time have exhibited decreased growth rates, longer emergence times, and decreased emergence success (Irving *et al* 2004). Deposit-feeding species that are tolerant of short periods of hypoxia, such as chironomids, exhibit irregular irrigation behaviour (Heinies & Crommentuijn 1992). This behaviour increases with DO demand and temperature and imposes great energy demand on larvae. Additionally, the time taken for increased irrigation comes at the expense of other active behaviours such as feeding and burrow-repair (Irving *et al* 2004). This net increase in energy demand and allocation results in less energy available for growth and reproduction (Irving *et al* 2004). Enhanced irrigation behaviour by chironomid species in response to contaminant-induced DO deviations has thus been well documented and its analysis can be used to indicate acute sub-lethal toxicity.

General immobilization of chironomid larvae along with other growth-rate endpoints were examined by Maul *et al* (2008). The organism's ability to move effectively within its surroundings is a demanded for proper feeding, burrow construction, foraging, and other life cycle markers. A body condition index was also used alongside immobilization as an endpoint. While the term BCI was not fully explained it appears to attribute to the larvae morphology and general body positioning. Upon examination under exposure to several pyrethroids it was

discovered that immobilization of the larvae was significantly more sensitive than lethal responses, with EC50s similar to organisms BCI (Maul *et al* 2008).

In nature chironomid larval depend on their ability to anchor to sediment using their posterior assemblages to forage for food and construct burrows. Upon close examination it was discovered that under toxic stress chironomid larva failed to properly orient their body along the sediment, detaching their posterior ends and turning onto the side. This prevented the burrow construction and resulted in exposure to overlaying water increasing predation and decreasing reproduction success. This body positioning was therefore useful in identifying sub-acute toxic conditions, as upon placement into normal conditions, larvae recovered and burrow construction, along with foraging and irrigations behaviours, was observed. Both past and recent studies have identified larval behaviour as early responses to more damaging and life threatening toxic conditions. From such studies, along with my own observations over years of culturing and experimental design, this study will be looking at behavioural deviations of burrow construction, feeding/foraging, and body positioning under exposure to four different contaminants. These behaviours will be organized into three categories; burrow behaviour (which includes construction and irrigation behaviours), body positioning (natural posterior anchoring), and foraging (which includes the presence or absence of feeding and foraging).

### 1.6.2 Lumbriculus variegatus

The oligochaete *Lumbriculus variegatus* is a benthic invertebrate that lives in shallow ponds, marshes and swamps. They feed primarily on dead organic matter, microorganisms and sedimentary material. They are found throughout North America and Europe and represent an important niche in the aquatic food web. *L. variegatus* are made up of repeating body segments each with the capability of regenerating into a whole worm.

**Figure 1.13** *Lumbriculus variegatus*

They have a maximal body size of about 10 cm in length and 1.5 mm in diameter, with approximately 200 – 250 body segments (Ding *et al* 2001). Lumbriculus are tolerant to a wide range of oxygen concentrations (Marshall 2008). In nature lumbriculus undergo sexual reproduction, however under laboratory conditions it is more common to find them reproducing asexually through fragmentation, a process also termed morphallaxis. Once a worm is over 8 segments long it may 'drop' its posterior end, with both ends regenerating into a full worm (Drewes & Fourtner 1989).

Also known as the 'mudworm', lumbriculus has been used in both sedimentary and water toxicity tests since the rise of biological assessmen and have proved advantageous with their ease of culturing and handling, adequate tissue mass for chemical analysis, wide range of tolerance to numerous sediment physiochemical parameters, low sensitivity to contaminants, and long-term

exposure without feeding (Sardo & Soares 2010). Similar to chironomids, lumbriculus have been used in the classification of trophic structures in lakes and in sediment toxicity. A key characteristic of this species is their accumulation of colonies within the sediment. These colonies, or 'clumps', (figure 1.14) can number anywhere between hundreds to thousands of individual worms. This has been used in past bioassays to determine metabolic efficiency, in microcalorimtry test where the heat given off by the colony was analyzed under different toxic conditions (Leppanan & Kukkonen 1998). In nature, clumped worms burrow their anterior ends in the sediment and extend their posterior up into the overlaying water. This orientation allows for gas exchange via dorsal blood vessels (Drewes & Fourtner 1989). This position also allows for rapid response upon stimuli. Lumbriculus contain giant nerve fibers (interneurons), specifically photosensores and chemoreceptors, in their posterior segments with the ability to detect changes in light and water chemistry. These mediate rapid escape response times from the worm in response to passing shadows and contaminant to avoid dangers such as predation and acute toxicity (Drewes & Fourtner 1989, Ding *et al* 2001).

**Figure 1.14** Clump/colony of 10 *L. variegatus*

*Lumbriculus variegatus* move along the sediment using snake-like crawling and wavelike motions (Ding *et al* 2001). In open water lumbriculus use helical swimming motions, where the worm will 'corkscrew' rapidly up into the water for a duration of no longer than several seconds.

This occurs through a rapid shortening of the body length and 'rhythmic waves of helical body bending' (Ding *et al* 2001). It will then slowly settle back onto the sediment and reincorporate into the colony (Ding *et al* 2001).

*Lumbriculus variegatus* crawling motions consists of a series of rhythmic peristaltic contractions of circular and longitudinal muscles along the length of the body. These motions begin at the worm's anterior region and travel down towards the posterior and are assisted by chaetae located along each body segment (Ding *et al* 2001). These movements are generally much slower than the vertical helical swimming and therefore require less energy, providing more efficient movement though the benthos.

The suitability of this organism for short-term bioassays extends from their physiology, sediment habitat, and wide dispersal in the aquatic food web. Their locomotion in water is easily visible and can be used as a biological marker. The effects of introduced toxic agents can visibly impair and/or alter the worms swimming behaviour. Inhabiting the sediment of aquatic systems exposes the organisms to possible agricultural runoff of pesticides, herbicides, insecticides, pharmaceuticals and bio-solid waste from farms. These agents can then be ingested by the oligochaete and lead to bioaccumulation from chronic exposure. Being a food source for a variety of predators, the accumulated toxins transfer to the next trophic level (frogs, fish, wading birds) resulting in biomagnification. Coupled with its environmental based benefits for its use in bio-assays *Lumbriculus variegatus* are also very easily cultured in laboratory settings.

Historically, toxic responses of lumbriculus have been assessed using biological endpoints such as survival, growth, reproduction, bioaccumulation, and avoidance (Ding *et al* 2001; Gerhardt 2006). Sediment toxicity research has focused on ratios of tolerant *tuberfix*

species (i.e. *Limnodrilus hoffmeisteri*) to other tubicifid worms. Contamination levels have also been assed using ratios of lumbriculus to chironomids (Gerhardt 2006).

Recent studies have identified the usefulness of *L. variegatus* behaviour as a sub-lethal indicator of stress. Ding *et al* (2001) has shown that in response to stimuli, lumbriculus response times vary in contaminated sediments. The study also examined swimming frequency, swimming pattern, and crawling pattern under exposure to invermectin. Swimming frequency, as measured by success of failure to swim upon response to stimuli, and swimming pattern, measured as wave-like undulations (crawling) propelling the organism forward, both exhibited their usefulness in detecting sub-lethal stress upon exposure the invermectin. Although both behaviours elicited stress responses it was clear to Ding *et al* (2001) that locomotion movements involved in swimming were inhibited to a greater degree than the peristaltic movements associated with crawling. With the inhibition of swimming the organism's ability to avoid capture is limited, leading to increased predation and affecting the balance of the ecosystem. Locomotion has been found in similar studies to be consistently sensitive to sub-lethal toxic stress induced by a wide range of environmental contaminants (Ding *et al* 2001).

O'Gara *et al* (2004) used locomotion behavioural deviation in lumbriculus to examine toxicity to varying copper concentrations. Digital video analysis was used to examine change in response to posterior stimulation after 0, 1,3, and 8 hours of exposure. Hyperactive locomotion was observers immediately following exposure, expressed as writhing and coiling of the body. These activities rapidly became lethargic ending with zero locomotion movements in response to stimuli. Altered body positions were also observed, where body segments of the worm were observed to constrict and contract upon themselves after 3 hours of exposure. Immobilization, deviations in swimming patterns, and reversal behaviour were all found to be concentration

dependant with significant changes in the organism's response to stimuli and locomotion behaviour rafter 8 hours under all concentrations of copper.

It has been established that locomotion and other behavioural activities are the primary determinant of the physical, chemical, and biological characteristics of the sediment and water (Sardo & Soares 2010). Furthermore, locomotion behaviours are the foundation of vital life functions such as foraging, sexual reproduction, predator avoidance, dispersal, general orientation and response to environmental signals (Drewes & Fourtner 1989). As such, the monitoring and analysis of such behaviour can provide key understanding and identification of acute sub-lethal responses in lumbriculus. In this study locomotion behaviours were the key behavioural characteristics used to identify sub-acute response and were split into three categories: (i) swimming behaviour, as measured by the wave-like undulations along the body used to propel the worm forward, (ii) body positioning, identified as smooth and curved body segments, and (iii) the presence or absence of colony formation.

# 2.0 METHODS AND MATERIALS

The following methods and materials are divided several sections. The first section describes culturing methodologies used to culture *C. tentans* and *L. variegatus* in lab. The second section lists the contaminants and their concentration followed by the experimental procedures of the time-lapse photography and the MFB. The final section details the statistical analysis performed with the data obtained from both experiments.

## 2.1 Cultures

### 2.1.1 *Chironomus tentans*

Culture protocols were obtained and adapted from Ontario ministry to the Environment 2006 culture guidelines. Further assistance was obtained from the Ontario Ministry of the Environment Laboratory Services Branch Aquatic Toxicology Unit, specifically aquatic technician Robert Chongkit.

Start up chironomid cultures were obtained in larval form, with approximately 200-300 first instar larva in the fall of 2008. All vegetable Spirulina flake fish food was also initially supplied by the MOE. Upon acclimation larvae were cultured in 8x11 cm polypropylene rearing trays obtained from VWR. Trays were cleaned according to Ryerson cleaning protocols and rinsed with 10% HCl prior to use. Approximately 50-100 1$^{st}$ instar larvae were housed in one tray. Trays were filled ¾ with municipal drinking water, roughly 3-4 cm in depth that was aerated for at least two days. Silica sand with grain size between 0.0625 and 2 mm was placed on the bottom of trays in a 2mm thick layer to mimic sediment. Silica sand was washed using aerated culturing water to remove small dust particles and debris then autoclaved to remove microbial contaminants. Prior to the introduction of larvae the trays were prepared with culture

water, a thin 2mm layer of silica sand, and 2mL of overlying slurry feed water (described below) and allowed to equilibrate for 24 hours. Care was taken to avoid over feeding at this point as too much food would increase microbial growth and reduce DO levels. Larvae were transferred from travel containers to trays using 2mL transfer pipettes. Larvae were placed below the water surface to avoid contact with air interface. Occasionally larvae would float to the surface and become trapped in the surface tension from the air-water interface. Gently probing with a pipette tip was sufficient to sink larva.

Rearing trays would receive 70% water renewal once a week to account for evaporation and water quality. If water quality was low (e.g. cloudy or odorous), 50-70%would be removed with 1cm aquarium tubing using gravity draining. Renewal water was introduced into trays using similar piping and gravity draining. Fresh water was introduced slowly and away from burrowed larva to avoid disruption of sediments. Upon accumulation of excrement around burrows a 2mL transfer pipette would be used to siphon out the waste.

**Figure 2. 1** *C. tentans* culture rearing tray

Feeding slurry was made using 40mL culture water 1.0g of Nutrafin® Max Spirulina Flakes. Well-mixed, homogenized feeding slurry was fed to cultures below water surface using 2mL transfer pipettes and evenly distributed through tray. Feeding took place once or twice a week depending on brood size and larval instar stage. The feeding slurry contained particulate

spirulina flakes of varying sizes. For feeding, the slurry would be stirred up and allowed to settle to differentiate between flake sizes. Depending on the size and instar stage of larvae, different slurry flake particles were used. This feeding procedure is detailed in table 2.1

**Table 2.1** Feeding rates for *C. tentans*

| Larval growth stage (instar) | Feed Rate | Amount of feed | Feed description |
|---|---|---|---|
| 1 | 1-2 times a week | >2ml | Overlaying slurry water* |
| 2 | 2 times a week | 2 mL | Small floating particulate along with overlaying slurry water |
| 3 | 2 times a week | 2.5mL | Large settled food particulate |
| 4 | 2 times a week | 2.5 – 3 mL | Large settled food particulate |

* Overlaying slurry water describes the overlaying mixture of feed and water after vigorous mixing and settling of large food particles within slurry.

It should be mentioned here that feeding rates were either slightly increased or decreased upon observations made of individual trays. Cultures trays were monitored one day after feed was administered for water quality (cloudiness, odour), larval activity, and amount of food remaining. Amount, rate, and particulate size were all adjusted accordingly. For example, if larvae of the 2$^{nd}$ instar had a particular size of feed remaining untouched but with a good water quality, then the size of the food particulate would be adjusted for smaller size particles. If water was cloudy but no food particles were visible, then the amount or rate of feed administered would be decreased for the following feeding. By monitoring feeding rate and water quality a balance can be obtained with chironomid larva cultures.

Upon maturation into 4$^{th}$ instar stage larvae were transferred from rearing trays using a transfer pipette with the narrow tip cut off and placed in 8L glass aquaria. Larvae were

transferred inside their constructed burrows to reduce stress and prevent energy expenditure in building new burrows upon introduction into adult mating tanks. Occasionally, a larva would pupate in the rearing trays. These individuals were simply transferred to adult breeding aquaria.

All breeding aquaria were cleaned according to Ryerson protocols and rinsed with a 10% HCl solution. Larvae from different rearing trays were introduced into same mating tanks to create a well-mixed adult population. A small layer of silica sand would be placed in bottom of tank, with 3-4 cm of overlaying water. A small amount of feed (0.5-1.0mL) was placed in adult tanks for reduced feeding rates of maturing adults. Silica sand and water conditions were maintained throughout all rearing and breeding trays/tanks. A plastic nylon 5um screen was placed over tanks to prevent adults from escaping. Egg cases were removed from these tanks upon first citing using 10mL pipettes through a hole in the covering mesh screen.

Upon collection egg cases were placed in Petri dishes with culture water from which they were removed. Within days of extraction of eggs from adults tanks first signs of hatching, which was observed as degradation of spiraling egg mass and new swimming larvae was observed. Newly hatched larvae and remaining egg mass were introduced into prepared rearing trays. Newly prepared trays were set up as previously mentioned. Two to three egg masses would be placed per tray to prevent overcrowding. A single egg case would yield approximately anywhere from 100-300 larva. Upon reaching 2$^{nd}$ star level, approximately 100-200 newly burrowed larvae would be visible in each tray. This would give an ultimate yield of 50-100 fully matured larvae for use in experiments and breeding for egg production and establishment of new cultures

### 2.1.2 Lumbriculus variegatus

Start up *L. variegatus* cultures were obtained from the Ontario Ministry of the Environment (MOE) in the fall of 2008 from the Environmental Monitoring and Aquatic Culturing laboratories. Instructions for proper care and maintenance were provided by aquatic technicians Robert Chongkit and Trudy Wetson-Lueng. Approximately 1000 worms were obtained arranged in several clumped colonies. Several times over the 3 years cultures were resupplied with new organisms obtained from the same location during the summer of 2009 and the winter of 2010. Aquarium snails (*Planorbidae* spp.) were also provided and introduced into lumbriculus cultures and used as grazer cleaners.

Cultures were established in two 8L (2 gallon) tanks. Tanks were cleaned using Ryerson cleaning protocols and rinsed with a 10% HCl solution. Cultures were established in different tanks in order to provide a back up should one culture crash. Aerated municipal drinking water was used in tanks. Brown paper towel was used for sediment substrate. The paper towel was shredded into strips 1-2 cm in width and no longer than 10 cm and placed into water. To assist with sinking toweling was stirred with sterile pipits. Brown paper towel strips would settle to the bottom of the tank and act as sediment and a carbon source for *L. variegatus*. Renewal of sediment substrate occurred approximately every two weeks or when it was observed that all paper towel strips were consumed and integrated into colonies. The two tanks were allowed to settle and equilibrate for 48 hours prior to the introduction of organisms. Initial temperature, pH, and dissolved oxygen (DO) readings were 22$^{o}$C, 6.8-7.1, and 80% oxygen saturation, respectively. Temperature was monitored constantly with an aquarium thermometer, while pH and DO were analyzed on a bi-weekly basis (every two weeks). Culture water was changed once a week, with an approximate of 70% exchange. Water was removed out of tanks with 1cm

aquarium tubing using gravity drainage. Parameters of renewal water were measured prior to introduction. New water was introduced gently with 1cm aquarium tubing as to not disrupt lumbriculus colonies. If cultures became cloudy or organisms appeared lethargic with other signs of stress, pH and DO were measured to determine possible causes of disturbance, followed with a 70% renewal of tank water.

Slow-sinking trout feed pellets obtained from local pet supply store was used as feed upon advice from MOE technicians. Pellets were 4mm cubes with an oil content of 20%+ and a protein contents in excess of 45%. Pellet breakdown times varied from 10-45 minutes. Approximately 5-7 pellets were crushed using mortar and pestle and sprinkled into tank twice a week. Depending on culture size and growth/reproduction of organisms, feed rates would increase or decrease. This was determined by monitoring amount of feed left after two days after feeding. If water became cloudy and/or if there was excess food remaining on the bottom of the tank, amount of feed was reduced, and if coupled with cloudy water, a water exchange would be done. Both feed reduction and water exchange prevented growth of anaerobic bacteria and a population boom of snails. However, with proper feeding rates and healthy growth of lumbriculus cultures snail population would occasionally be in need of reduction to prevent competition over space, sediment and food. Adult snails would be removed by hand and placed into separate snail tanks. Lumbriculus and snail waste (excrement) was removed approximately every two weeks waste or when it was observed to be needed.

Cultures were placed away from direct light as lumbriculus are benthic invertebrates and do not live in direct light, although culture laboratory did have large windows and provided natural light exposure allowing for natural circadian rhythms of organisms.

## 2.2 Experiments

### 2.2.1 Contaminants

All contaminant concentrations were derived from environmentally relevant data and remained consistent with prior studies performed by graduate students within this study. The following table lists the concentrations used for each contaminant. To visualize extreme stress behaviour from the organisms it was thought to use a positive control. For this ethanol was chosen due to its known depressant effects on invertebrates (Barker 1973; Kerschbaum & Hermann 1997).

**Table 2.2** Contaminant concentrations

| **Contaminant** | **Concentrations (µg/L)*** |
|---|---|
| TBT | 1 |
| | 10 |
| | 100 |
| Atrazine | 5 |
| | 50 |
| | 500 |
| Cu | 100 |
| | 200 |
| | 300 |
| | 400 |
| | 500 |
| | 5000 |
| Ciprofloxacin | 1 |
| | 10 |
| | 100 |
| EtOH | 0.1% |
| | 1.0% |
| | 10% |

*EtOH in percent concentration

Due to the low solubility's of the contaminants DMSO was used as a carrier. The concentration used was obtained from literature and past experiments performed by Fleet and Netto. Stock dilutions were made in 100% DMSO at 1000X greater concentrations as was used

in experiments. During contaminant tests, 1/1000 of container volume of contaminant was used, creating a 0.1% DMSO dilution. For example, a 100mg/L of TBT in 100% DMSO stock solution was made. During the time-lapse photography experiments, 15mL wells were used. Therefore 15µL from the 100mg/L was pipetted into wells and filled with culture water. The resulting dilution inside the wells would then be 100µg/L in 0.1% DMSO.

### 2.2.2 Photo lapse photography for observing behavioural deviations in *C. tentans* and *L. variegatus*

*Set-up*

A 6-well macro plate was placed on top of a white piece of paper inside the light box. Contaminants were then poured into each well according to the tests being run. Pre-cleaned silica sand was then placed in wells. The camera, attached to the tripod, was placed directly overtop the well inside light box. Connection of the camera to the PSRemote program was then setup. The PSRemote program is a downloadable software program that allows photographs to be taken with a Canon PowerShot camera and viewed within seconds on a computer. After focusing the camera the organisms were placed inside each well. When completed, the PSRemote program began and snapshots were then taken over the four hours of testing. *L. variegatus* test were run using 10 worms per well/exposure with no sediment. *C. tentans* test ran with one 3$^{rd}$ instar larva with a thin 2mm layer of silica sand to mimic sediment and observe for burrow construction.

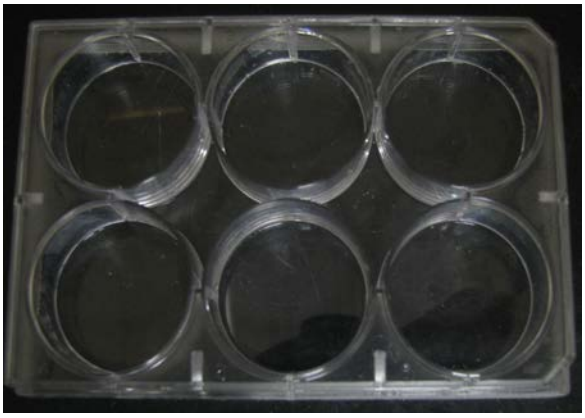


**Figure 2.2** Six-well macro plate

Experimental design and set up was created from detailed observations of lifecycle and normal behaviours over the course of two years.

The 6-well 15 mL macro plate was used for its ease of observation, sufficient depth and large diameter needed for swimming and foraging behaviours of *C. tentans* and *L. variegatus*. It also allowed for six replicates to be captured in a single experiment. Each experiment ran for a total of four hours.

Directly above micro well plate an ISO50 Cannon digital camera was used (figure 2.3). Camera was mounted on a small tripod. A light box was constructed from four 50x75 cm white sided cardboard sheets to prevent glare off water surface and to provide appropriate dark lighting conditions for the benthic organisms. Camera was connected to a computer running the PSRemote program. The program was used to set-up a 4 hour time-lapse photo experiment with snapshots taken every minute. The organism's behavioural reactions to the contaminants were captured in these snapshots.

**Figure 2.3**. Camera mounted on tripod positioned over wells inside light box

After the 4 hour test a total of 240 frames were obtained. Each frame was examined and the behaviour of the organisms was recorded as a numerical value of 1, 2, or 3. Table 2.2 details the how values were assigned. In addition to assigning numerical values to the behaviors of burrow construction and colony formation, the time of first appearance burrow construction and colony formation was recorded.

**Table 2.3.** Evaluating scheme for organism's behaviour

| Numerical Value | Behaviour |
|---|---|
| 1 | No observed deviations from norm |
| 2 | Slight deviation from norm: evidence of stress |
| 3 | Greatest deviation from norm: strong stress indication |

The behaviours that were monitored for *L. variegatus* were swimming behaviour, body positioning, and clumping; behaviours for *C. tentans* were body positioning, foraging, and burrow construction. Behaviours are detailed in the following figures.

**Figure 2.4**. *C. tentans* behaviours used in time-lapse photography experiment: burrow construction (left), foraging(middle), and body positioning (right)

**Figure 2.5**. *L. variegatus* behaviours used in time-lapse photography experiment: Swimming pattern (left), clumping (middle), and body positioning (right)

The selected behavioural parameters were chosen from literature reseach on the ecology of the benthic organisms and studious visual observation over the course of 12 months. It has been long known that *C. tentans* construct burrows to create their own secluded habitat, and that *L. varigatus* congregate itno large colonies. Their foraging and swimming patterns have also been well documented (Drewes & Fourtner 1989; Drewes & Brinkhurst 1990; Gauss *et al* 1985; Geisy & Hoke 1989; Heinies & Crommentuijn 1992; Drewes & Cain 1999). The body positioning parameters were chosen for direct observation of stress under 10% EtOH. *L. variegatus* were observed to lose their smooth-wave-like peristaltic body positions and curl inward on themselves. *C. tentans* were observed to lose their posterior attachment to the sediment and turn onto their side. Without their secure grip on the sediment it was observed that they would undergo a slight curling where the anterior and posterior would draw closer to each others. The shape created by the larva resembled the letter “C”. This body position indicative of stress was therefore coined prone “C” position.

### 2.2.3 Multispecies Freshwater Biomonitor

The next phase of the study was to conduct similar toxicological tests on the organism's behaviour. But instead of relying on visual identification of stress behaviour, an automated monitoring device was used, the Multispecies Freshwater Biomonitor (MFB).

The MFB was previously used by a graduate student working on the same project, Vivian Fleet, whose study was focused on the investigation on the usefulness and possible integration of the MFB into an automated biomonitoring regime. Fleet began by orienting herself with the MFB, discovering its operation capabilities and its limits. Tests were conducted to discover if the chambers, with their small enclosed space, produced any negative effect on the organism and the possibility that the electric field produced within the chambers affected the organism's health and/or behaviour. Fleet concluded that no adverse effects were imposed on the organisms from neither the chambers nor the electrical field produced inside. Furthermore, experiments by Gerhardt *et al* (2006) and deBisthoven *et al* (2004) used *L. variegatus* and *C. tentans* in their behavioural studies using the MFB, and concluded that behaviour was not effect by the MFB. With the safety of the MFB established it was possible to go forth with behavioural testing on *C. tentans* and *L. variegatus*

Ten chambers were used, channels 1 to 11 (channel 2 was not used due to technical difficulties) providing 5 replicates of reference and 5 replicates of toxicant. MFB test ran for 4 hours. All tests took place during the same time of day, between the hours of 12 pm and 5 pm to ensure similar circadian rhythm behaviour.

*MFB set-up with C. tentans*

MFB chambers housed one *C. tentans* larvae each. Square glass containers with a volume of 200mL were used as vessel to hold water and chambers. One end of the chamber was secured with a mesh-covered cap and the other end was placed flat up against the wall of the glass container to allow for visual observations. Once placed in the glass vessel filled with water and contaminant, a larva was inserted inside the chamber below the surface of the water with a 2mL transfer pipette below the

*First round of tests*

The first round of testing was prepared by placing free 3$^{rd}$ instar lava in chambers containing a thin layer of silica sand. Each chamber had one larva and was placed in individual glass containers (figure 2.8). This set up was designed to mimic the set-up of the time-lapse photography test and to use the MFB to analyze larvae's burrow construction and foraging behaviour. However, during the test foraging behaviour could not be distinguished from burrow construction as both behaviours involve large movements from the larva. These large movements elicited similar amplitude readings from the MFB and therefore would be recorded as the same behaviour. After these runs it was concluded that an additional chironomid behaviour end-point would be monitored by the MFB, a behaviour that used smaller movements then foraging so it could be differentiated by the MFB.

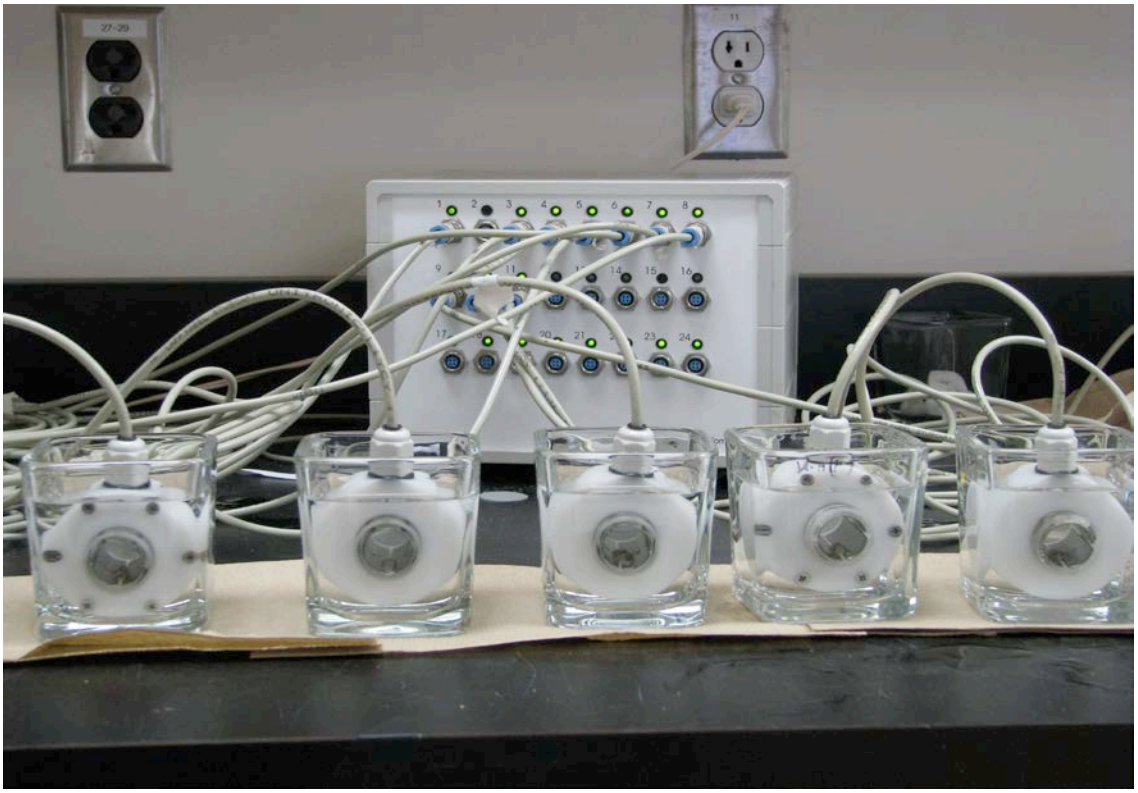


**Figure 2.6** MFB setup with chambers in glass containers

*Second round of tests*

To account for this similarity in the amplitude readings generated by the MFB, it was decided that by placing larva already encased within pre-constructed burrows inside the chambers it would be possible to differentiate and isolate foraging behaviour from burrow construction. Another benefit in using pre-constructed burrows was the possibility in monitoring a behaviour that consisted of small movements and would therefore generate different amplitude readings from the MFB. This smaller behaviour was irrigation behaviour. Burrow irrigation occurs while the larvae are inside their burrows. They elicit peristaltic movements longitudal down their body to create a flow of water through the burrow. This creates an inflow of oxygen-rich water. The slight transparency of the silica sand burrows allowed for this peristaltic movement to be observed and picked up by the MFB. Furthermore, as these contaminants have

the possibility in effecting the organism's respiration, burrow irrigation could prove useful in determining sub-lethal toxicities.

The larvae selected for use in the MFB chambers were removed from rearing trays 24 hours prior to testing and placed in new cultures with new silica sand. This allowed for construction of new burrows with clean silica sand to improve transparency. Larvae were allowed the excess of 24hours to construct burrows to give them enough time to recover from any stress or energy expenditure involved in placement into new trays. Encased larva were transferred from the trays using 2 mL pipettes with cut tips for larger diameters, and placed into the MFB chambers beneath the surface-water interface to avoid air bubble and stress. Chambers were then fully immersed within 15mL glass testing containers. One burrowed larva was used in each chamber as seen in figure 2.9.

**Figure 2.7** *C. tentans* larva encased in burrow inside MFB chamber

*MFB set-up with L. variegatus*

The initial MFB set up as described with *C. tentans* above was unsuccessful with *L. variegatus*. Ten worms were placed below the surface of the water into each chamber. The chamber was sealed with mesh-covered caps and placed in individual glass containers. However the organisms were found to migrate through the nylon micromesh covering and into the side of the screw-top covering, finally finding their way out of the chamber. An alternative setup was constructed to prevent this escape and to provide both sufficient gas exchange and a sealed face to prevent escape. One end of the chamber was covered with a piece of nitrile glove and capped with the chamber screw cap, with the other end of the chamber remaining open (figure 2.6). When situated vertically, the depth of the chamber was too great for the *L. variegatus* to swim up and out.

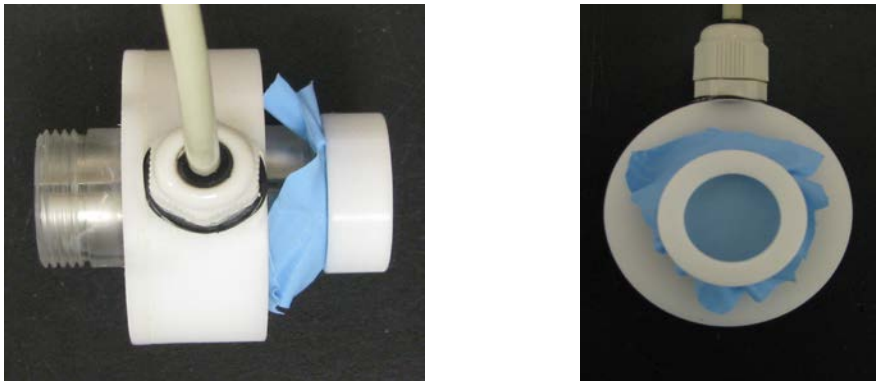


**Figure 2.8** MFB chamber sealed with nitrile glove to prevent escape

The chambers were placed vertically into two 10L glass aquaria tanks, with the covered end on the bottom and the open end on top. Five chambers were house in each with one tank containing reference water and the other containing contaminant as seen in figure 2.7. Ten worms of similar size and health were retrieved from culture tanks and placed into each chamber below the surface of the water. A 2mL transfer pipette was used to transfer worms. Worms

would settle to the bottom of the chamber, resting on the surface of the nitrile glove. A different 2mL transfer pipette was used for each contaminant and for reference water.

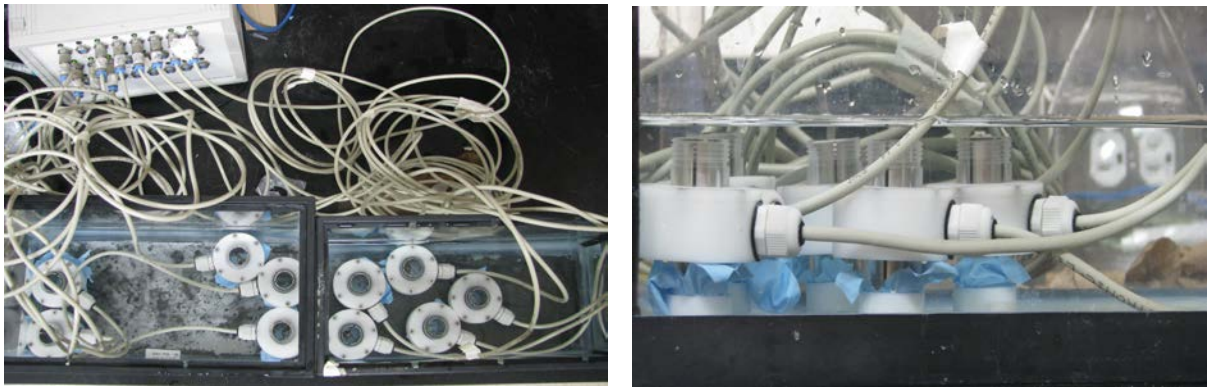


**Figure 2.9** Chambers housing *L. variegatus* placed in tests aquaria. Side view (left); top view (right)

*MFB with Parallel Video*

Prior to using the MFB for behavioural tests information was needed on how the readings generated by the MFB reflect with the observed behaviours of the organisms. In order to do this a reference test was conducted with the MFB. One *C. tentans* larva was placed inside a chamber encased within a pre-constructed burrow. The chamber was placed inside a clear glass beaker filled with culture water. While the test was running the movements of the larva inside the chamber was being captured on video with the ISO50 Cannon camera. With this parallel video it was possible to match the larva's movements to the corresponding amplitude readings generated by the MFB. This permitted electronic interpretation of which behaviour was affected by the contaminants. Once this was completed it was possible to move onto testing. Due to the mechanical difficulties encountered with housing the *L. variegatus* inside the chambers no parallel video was conducted. However due to the singularity of *L. variegatus* behaviour within the chamber (their basic swimming behaviours) the need to differentiate between two distinguishing sized behaviours was not needed as it was with *C. tentans*.

### 2.2.4 Statistics

All statistical analysis was performed using IBM SPSS Statistics 19 accessed through Ryerson University's Virtual Application (VAPPS) website. All graphing was performed using SigmaPlot 12 by Systat Software Inc.

*Time-Lapse Photography*

The numerical values assigned to the behaviours observed during the time-lapse photography experiments required non-parametric analysis. A Wilcoxon Signed T Tests was used to determine if the two related samples (reference and treatment) population's means differed. All three replicates were analyzed for the one with the least variance from reference, which was then used in the Wilcoxon test. If this replicate failed to elicit a significant response, the next replicate with the least amount of variance from reference was used, and so on. Replicate choice and statistical data is presented under each treatment.

In addition a parametric analysis was performed on the burrow construction and colony formation times of *C. tentans* and *L. variegatus,* respectively. Table 3.1 lists burrow times and table 3.3 lists colony formation times. An entry of "NEVER" in any table represents the organism/s failure to construct a burrow or form a colony through the entire tests. A value of 240 was used in statistical analysis, used to represent the total amount of frames taken. Independent T-tests were performed and it was assumed that the data followed a normal distribution with the null hypothesis being that the means of the two data sets (reference and treatment times) were

equal. This is also known as a Student's T-test. Upon completion, if it was found that the two data sets had unequal variances, then the tests would be known as a Welch's T-test.

*MFB*

All frequencies obtained from MFB data were collected into Band 1. Band 1 was then separated into bins (5 for C. tentans and 6 for L. variegatus) according to values (in %time). These bins were put through discriminant analysis to model the differences between the two classes of data, reference and treatment.

Principal component analysis (PCA) used an orthogonal transformation to convert the data sets (% time) of correlated variables into principal components with uncorrelated variables. This was defined that the first principal component (Factor 1) accounted for as much of the variability between the data sets as possible. Each proceeding principal component (Factor 2) had the next highest accounting of variability, in so far that it was orthogonal to (uncorrelated with) the preceding component (Factor 1). Factor analysis, using regression modeling, assigned values to describe variability among components.

## 3.0 RESULTS AND DISCUSSION

The following results are broken up into the two experimental sections: Lapse Photography and MFB. Lapse photography results are based on visual observation of snap shots taken during tests. For brevity only snap shots that clearly identified key behaviours are shown. MFB results were generated from frequency readings from the quadruple impedance converter. Reported results from the MFB are based on a discrete fast Fourier transformation resulting in a histogram of the behavioural frequencies that occurred in the original behavioural signal. Snap shots from parallel video during the mapping experiments are shown to illustrate which behaviours correspond to which frequencies.

It is important to note that prior to experimentation both *C. tentans* and *L. variegatus* were observed during culture for normal baseline behaviours. For three years these cultures were maintained and observed, in which time important ecological data concerning their life stages and behaviours were catalogued. These behaviours, as defined in section 1, were then observed for deviations upon exposure to the 4 chemical contaminants and 1 control stress contaminant. Any such behaviour that deviated from the identified norm was then termed as a stress response.

### 3.1 Lapse Photography

Lapse photography experiments ran for a total of 4 hours, with snap shots taken every minute. Each frame therefore represents each minute, for a total of 240 minutes (4 hours). As described in section 2 each frame was given a value of 1, 2, or 3 depending on the degree of stress as indicated by deviations from normal behaviour, where 1 represents normal behaviour, 2 a slight deviation from normal behaviour, and 3 a large deviation from normal behaviour in each of the three behaviours selected for monitoring in both species.

### 3.1.1 *Chironomus tentans*

*TBT*

Under exposure to 1, 10, and 100 µg/L of TBT there was no deviation from normal foraging behaviour and body positioning observed. Under the largest concentration of 100µg/L delayed burrow construction was observed in two of the three replicates. One replicate failed to construct any burrow during the 4 hours of monitoring (Table 3.1).

All three replicates under all three concentrations had identical values to reference; therefore it did not matter which replicate was chosen for the Wilcoxon Signed Rank Test. The test showed that treatment with 1, 10, and 100 µg/L of TBT did not elicit a statistically significant change in observed foraging, body positioning, and burrow construction behaviours in *C. tentans* larvae ($Z = 0.000$, $p = 1.000$).

Independent T-tests showed that burrowing time under 10, and 100 µg/L TBT did follow a normal distribution with equal variances, with Levene's tests for equality of variances scores of $F = 10.381$and 9.732 and $p= 0.032$ and 0.36 respectively. Burrow time under 1.0µg/L did not follow a normal distribution, failing the Levene's tests for equality of variances scores ($F = 3.502$, $p = 0.135$). With assumed equal variances, there was no statistical significant difference in

burrow time under 10 and 100 µg/L TBT (p = 0.751 and 0.317 respectively). With equality amoung variances not assumed, there was no statically significant difference in burrow times under 1.0 µg/L TBT (p = 0.481).

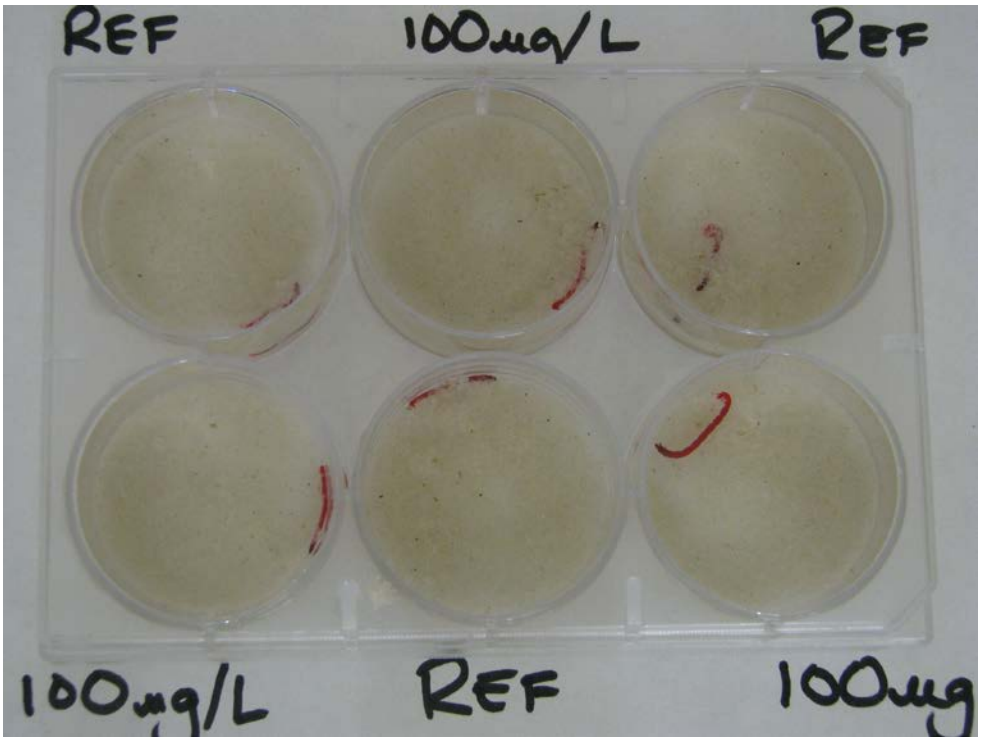



**Figure 3.1** Frame 240 (4 hours) illustrating zero burrow construction from lower right replicate at 100 µg/L of TBT

*Atrazine*

There were no stress indications from any of the three monitored behaviours under atrazine concentrations of 5, 50, and 500 µg/L. Burrow construction was random during exposure to atrazine and under reference conditions.

All three replicates under all three concentrations had identical values to reference; therefore it did not matter which replicate was chosen for the Wilcoxon Signed Rank Test. The test

showed that treatment with 5, 50, and 500 µg/L of atrazine did not elicit a statistically significant change in observed behaviour in *C. tentans* larvae (Z = 0.000, p = 1.000).

Independent T-tests showed that burrowing time under 5, 50, and 500µg/L of atrazine did not follow a normal distribution with equal variances, with Levene's tests for equality of variances scores of F = 4.795, 0.949, 0.048 and p= 0.094, 0.385, 0.838 respectively. With equal variances not assumed, there was no statistical significant difference in burrow time under all three concentrations of atrazine (p = 0.248, 0.942, and 0.749nrespectively).

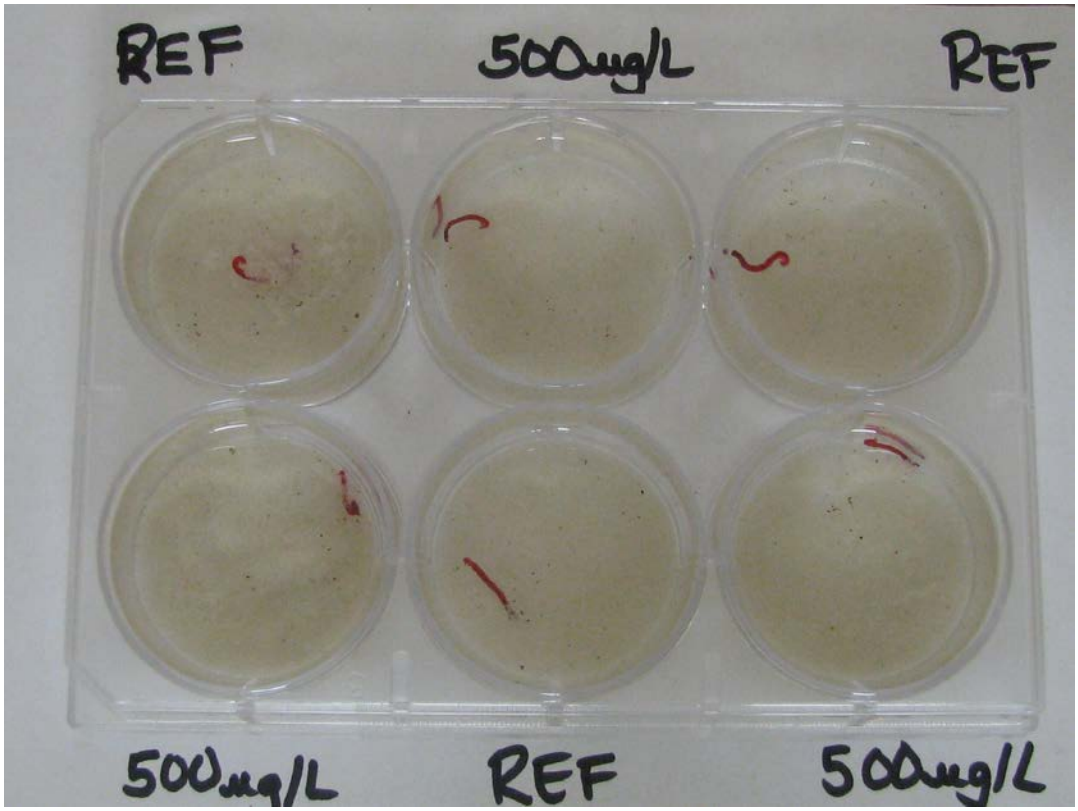



**Figure 3.2** Frame 210 (3 hours 30 minute) illustrating zero burrow construction from two larva (upper middle and upper right) at 500 µg/L of atrazine and reference

*Copper*

There were no deviation in foraging behaviour and body positioning observed under copper concentrations of 100, 400, 500 and 5000µg/L. Experiments conducted with concentrations of 200 and 300 µg/L encountered technical difficulties and are not presented. Burrow construction appeared to be delayed under the largest Cu concentration of 5000 µg/L (Table 3.1)

All three replicates under all concentrations of Cu used had identical values to reference; therefore it did not matter which replicate was chosen for the Wilcoxon Signed Rank Test. The test showed that treatment with 100, 400, 500, and 5000 µg/L of Cu did not elicit a statistically significant change in observed behaviour in *C. tentans* larvae ($Z = 0.000$, $p = 1.000$).

Independent T-tests showed that burrowing time under 100, 400, 500, and 5000µg/L of Cu did not follow a normal distribution with equal variances, with Levene's tests for equality of variances scores of $F = 1.442, 6.206, 0.242, 5.439$ and $p= 0.296, 0.067, 0.648, 0.080$respectively. With equal variances not assumed, there was no statistical significant difference in burrow time under all three concentrations of atrazine ($p = 0.590, 0.290, 0.780$ and $0.377$ respectively).

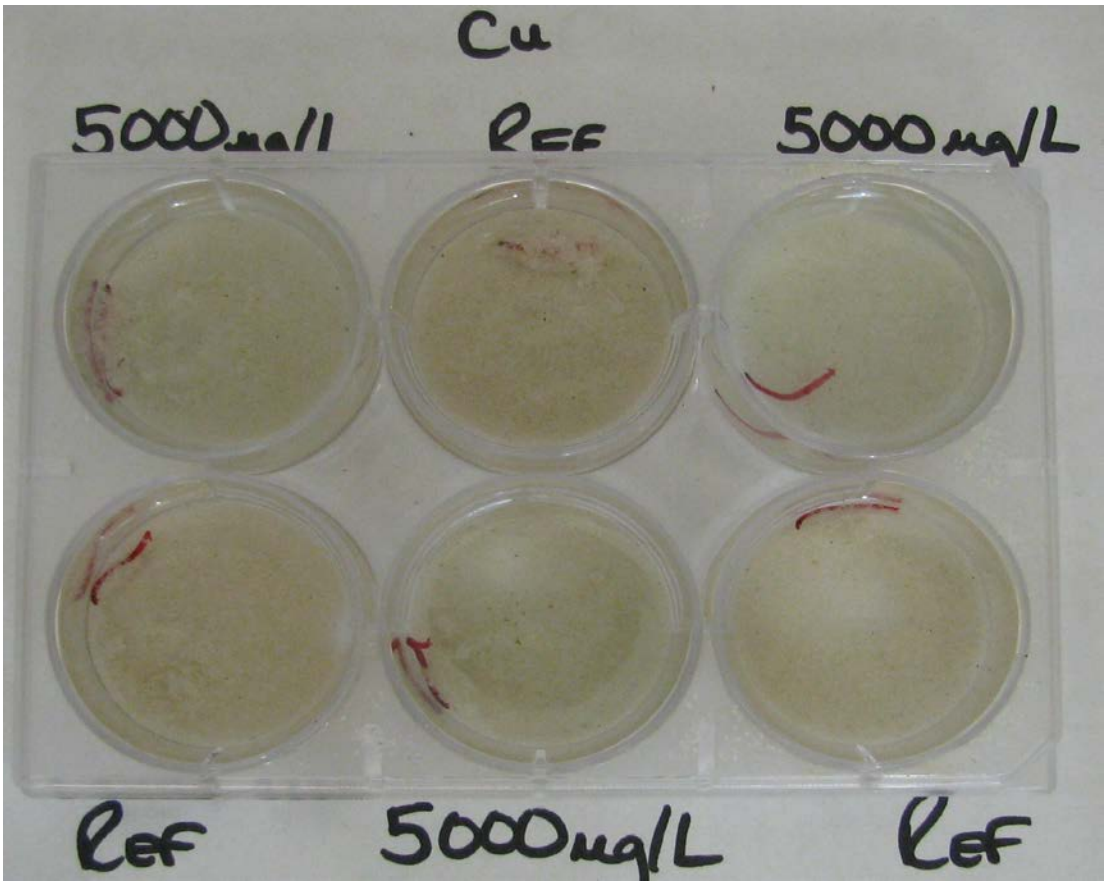



**Figure 3.3** Frame 240 (4 hours) with upper right larva failing to construct burrow under 5000 µg/L of Cu

*Ciprofloxacin*

Under exposure to 1, 10, and 100 µg/L of the antibiotic ciprofloxacin there was no deviations in foraging behaviour and body positioning. Burrow construction appeared comparable to each other and to reference conditions under 1 and 10 µg/L. At a concentration of 100µg/L burrow construction was delayed in one replicate.

All three replicates under all concentrations of ciprofloxacin had identical values to reference; therefore it did not matter which replicate was chosen for the Wilcoxon Signed Rank Test. The test showed that treatments of 1, 10, and 100µg/L of ciprofloxacin did not elicit a statistically significant change in observed behaviour in *C. tentans* larvae ($Z = 0.000$, $p = 1.000$).

Independent T-tests showed that burrowing time under 1 and 100µg/L of ciprofloxacin did follow a normal distribution with equal variances, with Levene's tests for equality of variances scores of F = 8.673 and 13.559, and significance of p= 0.42 and 0.021 respectively. With equal variances assumed, there was no statistical significant difference in burrow time under all three concentrations of atrazine (p = 0.185 and 0.320 respectively).

Ciprofloxacin treatment of 10µg/L did not follow a normal distribution and therefore equal variance were not assumed from Levene's tests for equality of variances scores of F = 0.000 and p = 1.000. There was statistical significance found in burrow time under 10µg/L (p = 0.045)

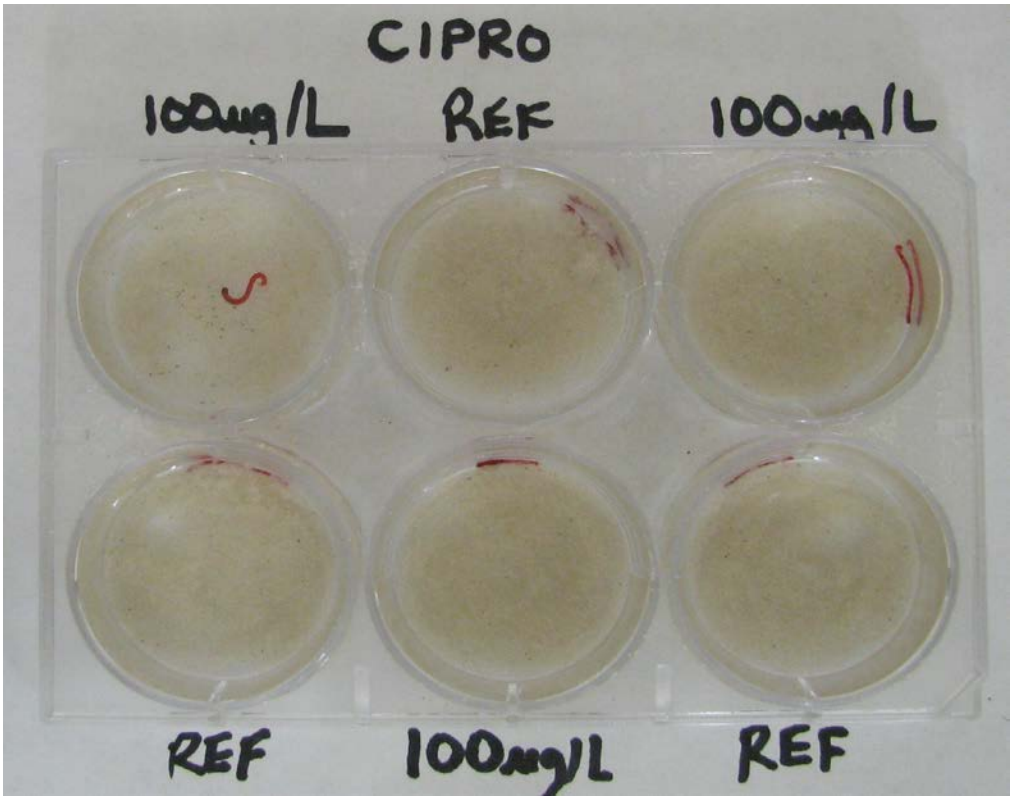



**Figure 3.4** Frame 108 (1 hour 48 minutes) illustrating delayed burrow construction in upper left larva (burrow constructed at frame 117)

*Ethanol*

Ethanol was used as a positive control test. 0.1, 1.0, and 10% concentrations were used. No deviations in behaviour were observed under 0.1%. Under 1.0%, lethargic foraging was evident by comparing larval movements between frames and graded as a 2. There was also no construction of burrows during the 4 hours of testing (Table 3.1). At 10% concentration all larval movements ceased at frame 3 (3 minutes) and given a value of 2. After 3 minutes of exposure larvae lost posterior attachment to substrate and rotated onto their side adopting a prone "C" position and graded as a 3. No burrows were constructed at this concentration as well.

All three replicates under 0.1 and 1% EtOH had identical values to reference; therefore it did not matter which replicate was chosen for the Wilcoxon Signed Rank Test. Replicates under 10% EtOH did elicit a change, all at identical times and therefore did not matter which was used for analysis. The test showed that treatments of 0.1 and 1% EtOH did not elicit a statistically significant change in observed behaviour in *C. tentans* larvae ($Z = 0.000$, $p = 1.000$). EtOH concentrations of 10% did elicit a significant change in behaviour ($Z = -15.428$, $p = 0.000$).

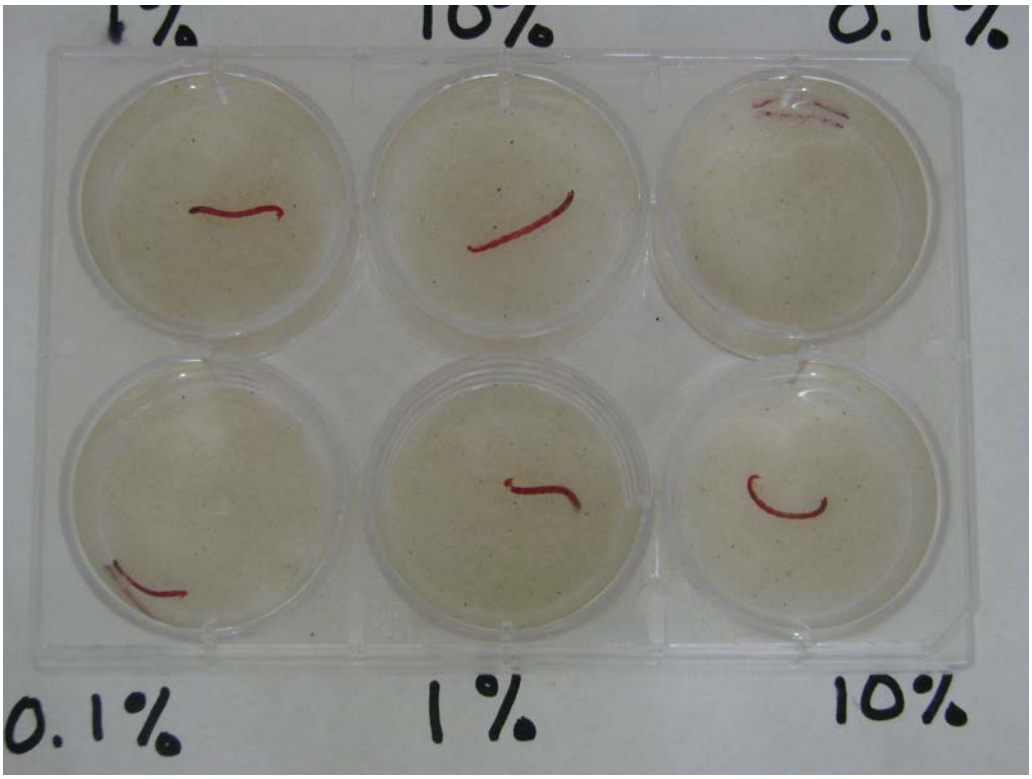



**Figure 3.5** Frame 229 (3 hours 49 minutes) illustrating absence of burrows in 1% (upper left and lower middle) and 10% (upper middle and lower right) wells. Larvae in prone "C' position in both 10% wells

**Table 3.1.** Appearance of burrow construction of *C. tentans* larvae

* Indicates statistical significance in burrow construction times ($p < 0.05$)

| Contaminant Concentrations (µg/L) *EtOH in percent concentration | | Time of Burrow Construction (minutes) | |
|---|---|---|---|
| | | Reference | Contaminant |
| TBT | 1 | 27 | 30 |
| | | 34 | 35 |
| | | 39 | 57 |
| | 10 | 11 | 7 |
| | | 14 | 9 |
| | | 16 | 34 |
| | 100 | 12 | 4 |
| | | 44 | 17 |
| | | NEVER | 38 |
| ATR | 5 | 157 | 40 |
| | | NEVER | 31 |
| | | NEVER | NEVER |
| | 50 | 2 | 6 |
| | | 8 | 35 |
| | | 224 | 172 |
| | 500 | 23 | 8 |
| | | 78 | 192 |
| | | NEVER | NEVER |
| Cu | 100 | 86 | 10 |
| | | 96 | 81 |
| | | NEVER | 120 |
| | 400 | 86 | 21 |
| | | 96 | 16 |
| | | NEVER | 22 |
| | 500 | 86 | 3 |
| | | 96 | 11 |
| | | NEVER | NEVER |
| | 5000 | 7 | 20 |
| | | 25 | 63 |
| | | 65 | 219 |
| CIPRO | 1 | 5 | 12 |
| | | 7 | 13 |
| | | 11 | 35 |
| | 10* | 5 | 13 |
| | | 7 | 13 |
| | | 11 | 18 |
| | 100 | 5 | 7 |
| | | 7 | 19 |
| | | 11 | 127 |
| EtOH | 0.1% | 12 | 15 |
| | | 21 | 18 |
| | 1.0%* | 12 | NEVER |
| | | 21 | NEVER |
| | 10%* | 12 | NEVER |
| | | 21 | NEVER |

*NOTE: One reference test for Cu, CIPRO, and EtOH was used and ran parallel to all corresponding tests.

**Table 3.2** Assignment of numerical values to behaviour of *C. tentans*

| **Contaminant** | **Concentration** | **Behaviour** | | |
|---|---|---|---|---|
| | | Foraging | Body | Burrow |
| TBT | 1 | 1 | 1 | 1 |
| | 10 | 1 | 1 | 1 |
| | 100 | 1 | 1 | 3 |
| Atrazine | 5 | 1 | 1 | 1 |
| | 50 | 1 | 1 | 1 |
| | 500 | 1 | 1 | 1 |
| Cu | 100 | 1 | 1 | 1 |
| | 400 | 1 | 1 | 1 |
| | 500 | 1 | 1 | 1 |
| | 5000 | 1 | 1 | 3 |
| CIPRO | 1 | 1 | 1 | 1 |
| | 10 | 1 | 1 | 2 |
| | 100 | 1 | 1 | 1 |
| EtOH | 0.1 % | 1 | 1 | 1 |
| | 1.0% | 2 | 2 | 3 |
| | 10% | 3 | 3 | 3 |

* Numerical values do not represent at which frame stress behaviour was observed

*Interpretation of foraging behaviours*

The time-lapse photography snap shots were successful in capturing the chironomid larvae's foraging. It was possible to determine all three defined behavioural endpoints within the well plates under exposure to the 4 chemical contaminants and the 1 control stressor. By comparing prior continuous snapshots, the amount and frequency of foraging was determined.

As described in section 2, a numerical value was given to each larva for their foraging behaviours and body position. Decreased foraging, as represented by a 2 or 3, was observed in the higher concentrations of Cu (500 and 5000µg/L), and 1 and 10% of EtOH. These concentrations resulted in slow, lethargic foraging. This was observed as the larvae remaining in the same position over continuous snapshots. This was coupled with zero burrow construction. Under 10% ethanol exposure, the larvae's behaviour quickly became stressed observed as minimal amounts of foraging. Within ten minutes of exposure there was zero foraging occurring and the larvae assumed a prone body position in which there was a ventral rotation onto their side and a curling of the body length into a "C" shape – a body position coined 'prone C' as seen in figure 3.1. The experiment concluded with the death of all larvae in the 10% EtOH treatment.

Reduced foraging rates could impede several larval ecological growth-dependent imperatives upon hatching. Decreased food intake would result from the larvas reduced foraging, increasing mortality and decreasing success of emergence (Maul *et al* 2008). Foraging is mandatory for burrow construction; therefore successful construction of burrows may decrease with reduced foraging behaviour, which may result in increased predation and the larva's ability to maintain body temperatures (Gerhardt *et al* 2003). Foraging, (in essence locomotion behaviours) has been observed to be impaired at concentrations up to 4.3 times lower than LC50s

for some compounds such as bifenthrin, lambda-cyhalothrin, permethrin, fipronil, fipronil-sulfide, and fipronilsulfone, the last two being common fipronil metabolites (Maul *et al* 2008).

Upon hatching, 1st instar larvae immediately begin foraging around for food and particulate matter in which to construct their burrows. A decrease in foraging behaviours would results in delayed burrow construction and decreased food intake. This behaviour is just as important during 3rd instar stages. Upon destruction or disturbance of their habitat larvae it becomes necessary to reconstruct their burrows. This results in higher energy expenditure as the larva forages the sediment for new particulate matter. Essentially foraging behaviour is necessary for all stages of larval development and survival (Lindeberg & Wiederholm 1979; Webb & Scholl 1985).

The lapse-photography was successful in capturing foraging behaviour in all tests. The deviation in foraging behaviour was therefore concluded to be a valuable behavioural endpoint for sub-lethal contamination detection.

*Interpretation of body positioning*

Each individual snapshot allowed for analyses of the larvae's body positioning at that point in time. Larval body positioning, as defined as an elongated body length and attachment of posteriors to sediment, remained normal throughout experiments under all four chemical contaminants. It was only under exposure to 10% EtOH which caused the larva's posterior to detach from the sediment, resulting in a ventral rotation. This caused the larva to free float along the silica sand.

Such changes in body positioning could result in the larva being released from natural sediments and being caught in overlaying water currents. These free-floating larvae would be exposed to increased predation as they are carried away along the sediment, along with increased energy expenditure while they undergo bodily thrashing within the current (Gerhardt *et al* 2003). Also, attempts in re-securing to the sediment would result in excess energy expenditure. A firm attachment to the sediment by natural body positioning is a necessary behaviour for larval chironomids and deviations from this could possibly alter larval survivorship.

*Interpretation of burrow construction behaviours*

Burrow construction varied greatly during both reference and treatments experiments. Burrow construction has been established as a protective mechanism that occurs throughout their entire larval life stage (Bell *et al* 2004). As it grows the larva continuously forages for building materials and increase the size of its burrow to accommodate its own growth in size. This burrow provides a safe habitat for the larva to mature while avoiding predation. It was hypothesized that under stress conditions the larva would deviate from normal burrow construction rate and completion time. It was observed under reference conditions that different larvae construct their burrows as different rates and with varying times of completing and levels of success. Similar observations were recorded during contaminant exposure. TO determine significance between reference and treatment independent T-tests were conducted. As the statistics show, burrow construction was only different from reference conditions under 10ug/L of ciprofloxacin and 1 and 10% of EtOH.

The relative low amount of significance in the difference of mean burrow times between reference and treatments confirms that burrow construction time may not be an ideal parameter for behavioural biomonitoring and/or that greater number of replications are needed to establish a base timeline for larval burrow construction.

Albeit burrow construction was not significantly different in this study deviations from normal burrowing construction may possibly cause harm to larval chironomids. As mentioned earlier burrow construction occurs immediately after hatching. While 4$^{th}$ instar larvae were used for these tests it has been shown that new hatchlings (1$^{st}$ instar) are the most sensitive to pollutants (Gauss *et al* 1985). If burrow constructions was affected in 4$^{th}$ instar larvae it is extremely likely that 1$^{st}$ instar would have increased afflictions. Failure to secure a burrow upon hatching can result in decreased food intake and death by predation or exposure (Pascoe *et al* 1989). It has been suggested by Gauss *et al* (1985) that the use of 4$^{th}$ instar larvae underestimates toxicity to the species. Gauss *et al* found an increasing toxicity to lower level instar larva under exposure to Cu. While the use of 1$^{st}$ instar would prove beneficial it would not prove practical for these types of behavioural biomonitoring tests. The small size of the 1$^{st}$ instars makes it difficult to sample in both the field and laboratory while expressing high sensitivity to transportation and handling stress (deBisthoven *et al* 2004). Moreover, a large cache of ecotoxilogical data has been gathered on 4$^{th}$ instar larvae, allowing for great amounts of comparisons (Taylor *et al* 1991; Gerhardt 1999; Ribeiro *et al* 1999; Heinis *et al* 1990).

### 3.1.2 *Lumbriculus variegatus*

*TBT*

Under all three concentrations of TBT, 1, 10, and 100 µg/L, no deviation in swimming or body positioning was observed. Time of clumping was observed and recorded under each concentration. Delayed clumping was observed in 10 and 100µg/L of TBT. One replicate exposed to 100 µg/L failed to incorporate into a clumped colony even once during the 4 hours of testing (Table 3.3)

All three replicates under all concentrations of TBT had identical values to reference; therefore it did not matter which replicate was chosen for the Wilcoxon Signed Rank Test. The test showed that treatments of 1, 10, and 100µg/L of TBT did not elicit a statistically significant change in observed swimming, body positioning, and colony formation behaviours in *L. variegatus* ($Z = 0.000$, $p = 1.000$).

Independent T-tests showed that time of first colony formation time under 1 and 100µg/L of TBT did not follow a normal distribution with equal variances, failing Levene's tests for equality of variances with scores of $F = 0.099$, 2.427, and $p = 0.768$, 0.194 respectively. With equal variances not assumed, there was no statistical significance in colony formation times under these concentrations ($p = 0.115$ and 0.339)

TBT treatment of 10µg/L did follow a normal distribution with equal variances ($Z = 10.878$, $p = 0.030$). There was a statistical significance in colony formation time ($p = 0.018$).

*Atrazine*

No deviations in swimming patterns or body positioning were observed in any of the worms under exposure to all concentrations of atrazine (5, 50, and 500 µg/L). Clumping of *L. variegatus* occurred randomly throughout the experiment in all wells under all three concentrations of atrazine. Similarity in time of clumping was seen between reference and contaminant tests as seen in table 3.3.

All three replicates under all concentrations of atrazine had identical values to reference; therefore it did not matter which replicate was chosen for the Wilcoxon Signed Rank Test. The test showed that treatments of 5, 50, and 500µg/L of atrazine did not elicit a statistically significant change in observed behaviour in *L. variegatus* ($Z = 0.000$, $p = 1.000$).

Independent T-tests showed that time of first colony formation time under 5, 50, and 500µg/L of atrazine did not follow a normal distribution with equal variances, failing Levene's tests for equality of variances with scores of $F = 0.541, 0.075, 0.008$ and $p = 0.503, 0.798, 0.933$ respectively. With equal variances not assumed, there was no statistical significance in colony formation times under these concentrations ($p = 0.398, 0.365$, and $0.523$)

*Copper*

No stress behaviour was observed in *L. variegatus* swimming patterns or body positioning under exposure to 100, 200, and 300 µg/L of Cu. Under exposure to 400 µg/L, deviations in swimming patterns and body positions were first observed in frame 125, 163, and 177 for each of the three replicates respectively and were graded as a 2. These stress deviations remained the same until the end of the test. Under exposure to 500 µg/L L. variegatus elicited

stressed swimming patterns and body positioning in all three replicates at frames 130, 153, and 213 respectively. Similar to the 400 µg/L test, these deviations were given a value of 2 and continued until the end of the test. These deviations in behaviour under 400 and 500µg/L can be seen in figure 3.9.


**Figure 3.6** Stressed *L. variegatus* under 400 and 500µg/L Cu exposure.

*Note absence of clumping accompanied with deviations in body positioning (kinked, inward isolated curling)

Under exposure to 5000 µg/L of Cu all three replicates of *L. variegatus* elicited stress responses in both swimming and body position in frames 38, 59, and 75 and were graded as a 2. These deviations increased to a value of 3 at frame 50, 72, and 86 respectively. Within minutes of obtaining a value of 3 worms appeared to lose cellular integrity and begin to bleed out, resulting in death of worm. Such results are illustrated in figure 3.10.

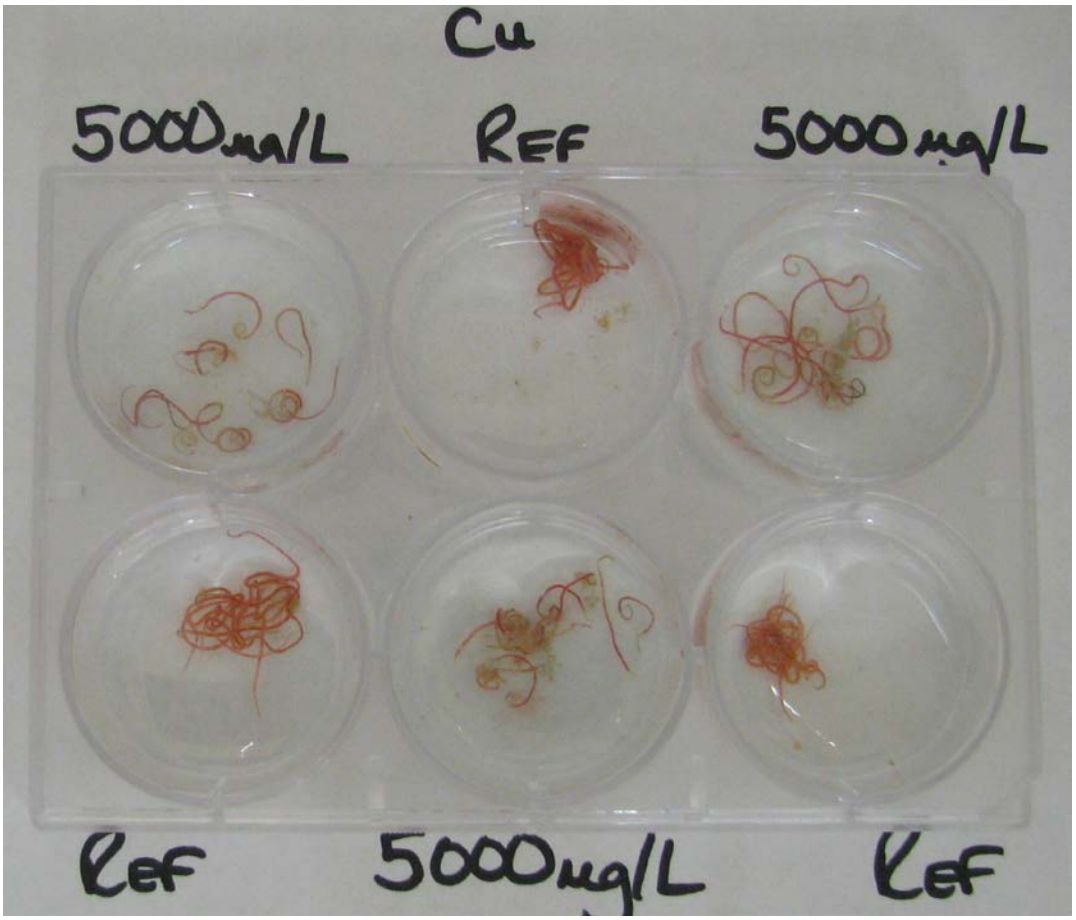



**Figure 3.7** Comparable clumping and body positioning between reference and 5000 µg/L Cu

*Note amount of isolated inward curling in Cu wells

**Also note degradation of cellular membranes in Cu wells and bleeding out of some worms in lower middle well

All three replicates exposed to 100, 200, and 300µg/L of Cu had identical values to reference; therefore it did not matter which replicate was chosen for the Wilcoxon Signed Rank Test. The test showed that these treatments did not elicit a statistically significant change in observed behaviour in *L. variegatus* ($Z = 0.000$, $p = 1.000$).

Replicates under 400µg/L of Cu had different values from reference, and from each other. As described in section 2.2.3, the replicate with the least variance from reference was used for statistical analysis. The two other replicates did not require further analysis, as the replicate

with the least variance from reference elicited a statistical significance in observed behaviours (Z = -8.000, p = 0.000). The same occurred under 500 and 5000µg/L of Cu, with the replicate showing the least amount of variance from reference eliciting a statistically significance change in behaviours (Z = -10.536 and -13.069, p = 0.000 and 0.000 respectively).

Independent T-tests showed that time of first colony formation time exposed to 100, 200, 400, and 500µg/L of Cu did not follow a normal distribution with equal variances, failing Levene's tests for equality of variances with scores of F = 6.779, 2.207, 7.105, 3.818 and p = 0.060, 0.212, 0.056, 0.122 respectively. Treatment of 300 and 5000µg/L of Cu did follow normal distribution with equal variances (F = 8.029, 10.543 and p = 0.047, 0.031).

There was no statistical significance in colony formation time in L. variegatus exposed to 100, 300, 400, and 500µg/L of Cu (p = 0.70, 0.168, 0.397, and 0.969). Statistical significance was found in 200 and 5000µg/L Cu treatments (p = 0.021 and 0.006).

*Ciprofloxacin*

There was no deviation from normal swimming behaviour and body positioning in *L. variegatus* exposed to 1, 10, and 100 µg/L of the antibiotic ciprofloxacin. Clumping events were delayed, as compared to reference, in all replicates of 1 and 10 µg/L, and in one replicate of 100µg/L.

All three replicates under all concentrations of ciprofloxacin had identical values to reference; therefore it did not matter which replicate was chosen for the Wilcoxon Signed Rank Test. The test showed that treatments of 1, 10, and 100µg/L of ciprofloxacin did not elicit a statistically significant change in observed behaviour in *L. variegatus* (Z = 0.000, p = 1.000).

Independent T-tests showed that time of first colony formation time under 1 and 10µg/L of ciprofloxacin did not follow a normal distribution with equal variances, failing Levene's tests for equality of variances with scores of F = 0.425, 2.847 and p = 0.550, 0.121 respectively. With equal variances not assumed, there was found to be a statistical significance in colony formation times under these concentrations (p = 0.0.01 and 0.032).

Treatment with 100µg/L of ciprofloxacin did follow a normal distribution with equal variances (Z = 11.980, p = 0.026). However, no statistical significance was seen in colony formation time (p = 0.436).

*EtOH*

No clumping events were observed under all three concentrations of EtOH. Lethargic swimming was observed from L. variegatus at 10% EtOH exposure. Body positioning was not seen to have deviated from normal.

All three replicates 0.1% EtOH had identical values to reference; therefore it did not matter which replicate was chosen for the Wilcoxon Signed Rank Test. The test showed that treatments of 0.1% EtOH did not elicit a statistically significant change in observed behaviour in *L. variegatus* (Z = 0.000, p = 1.000).

Replicates under 1 and 10% EtOH had different values from reference, and from each other. The least variance from reference was used for statistical analysis. The two other replicates did not require further analysis, as the replicates from both 1 and 10% EtOH with the least variance from reference elicited a statistical significance in observed behaviours (Z = -3.317, -15.232 and p = 0.001, 0.000 respectively).

Since only two replicates were used for EtOH control experiments the Levene's tests for equality of variances was not performed. Independent T-tests performed on time of first colony formation time showed statistical significance ($p = 0.001$) under all concentrations of EtOH.

**Table 3.3** Time of first formation of a clumped colony of *L. variegatus* under exposure to contaminants
*Indicates significant difference in clumping times between reference and treatment ($p < 0.05$)

| Contaminant Concentrations (µg/L) (EtOH in percent concentration) | | Time of first colony formation (minutes) | |
|---|---|---|---|
| | | Reference | Contaminant |
| TBT | 1 | 69 | 93 |
| | | 90 | 101 |
| | | 90 | 121 |
| | 10* | 1 | 116 |
| | | 8 | 131 |
| | | 18 | NEVER |
| | 100 | 80 | 77 |
| | | 103 | 167 |
| | | 125 | NEVER |
| ATR | 5 | 104 | 127 |
| | | 113 | 131 |
| | | 157 | 193 |
| | 50 | 36 | 55 |
| | | 68 | 86 |
| | | 84 | 118 |
| | 500 | 90 | 90 |
| | | 142 | 92 |
| | | 156 | 148 |
| Cu | 100 | 1 | 79 |
| | | 2 | 98 |
| | | 65 | 100 |
| | 200* | 1 | 129 |
| | | 2 | NEVER |
| | | 65 | NEVER |
| | 300 | 1 | 2 |
| | | 2 | NEVER |
| | | 65 | NEVER |
| | 400 | 1 | 23 |
| | | 2 | 33 |
| | | 65 | NEVER |
| | 500 | 1 | 12 |
| | | 2 | 16 |
| | | 65 | 43 |
| | 5000* | 1 | NEVER |
| | | 85 | NEVER |
| | | 112 | NEVER |
| CIPRO | 1* | 1 | 99 |
| | | 1 | 110 |
| | | 30 | 126 |
| | 10* | 1 | 83 |
| | | 1 | 93 |
| | | 30 | 155 |
| | 100 | 1 | 1 |
| | | 1 | 1 |
| | | 30 | 227 |
| EtOH | 0.1%* | 4 | NEVER |
| | | 21 | NEVER |
| | 1.0%* | 4 | NEVER |
| | | 21 | NEVER |
| | 10%* | 4 | NEVER |
| | | 21 | NEVER |

*NOTE: One reference test for Cu, CIPRO, and EtOH was used and ran parallel to all corresponding tests (5000 µg/L Cu excluded)

**Table 3.4** Assignment of numerical values to behaviour of *L. variegatus*

| **Contaminant** | **Concentration** | **Behaviour** | | |
|---|---|---|---|---|
| | | Swimming | Body | Clumping |
| TBT | 1 | 1 | 1 | 1 |
| | 10 | 1 | 1 | 2 |
| | 100 | 1 | 1 | 2 |
| Atrazine | 5 | 1 | 1 | 2 |
| | 50 | 1 | 1 | 2 |
| | 500 | 1 | 1 | 2 |
| Cu | 100 | 1 | 1 | 2 |
| | 200 | 1 | 1 | 2 |
| | 300 | 1 | 1 | 1 |
| | 400 | 2 | 2 | 2 |
| | 500 | 2 | 2 | 2 |
| | 5000 | 3 | 3 | 3 |
| CIPRO | 1 | 1 | 1 | 2 |
| | 10 | 1 | 1 | 1 |
| | 100 | 1 | 1 | 2 |
| EtOH | 0.1 % | 1 | 1 | 3 |
| | 1.0% | 1 | 1 | 3 |
| | 10% | 2 | 2 | 3 |

*Interpretation of swimming behaviour*

Under exposure to 400, 500, and 5000μg/L of Cu and 1 and 10% of EtOH, several organisms retained the same spot through continuous snapshots. The observed deviation from normal swimming behaviour was suggestive of lethargic swimming. *L. variegatus* has shown high sensitivity to Cu in past bioassays, with ranges in behavioural effects such as immobilization and sluggish locomotion (Chapman *et al* 1999; Drews & Binkhurst 1990). As described in section 1, lumbriculus use body undulations for swimming along the sediment. This swimming motion is used to forage for food and to establish large colonies. Under normal conditions, once integrated into a large colony an individual worm remain integrated within the clump. The observed stressed swimming behaviours resulted in reduced foraging and cessation of clumping. Drews and Binkhurst identified that in presence of contaminated sediment *L. variegatus* exhibited marked avoidance of contaminants. If locomotion rates should be reduced due to soluble contaminants in overlaying water, this could interfere with the organism's natural avoidance response.

Similar studies have also observed immobility and reduced locomotion behaviour *in L. variegatus*. Ding *et al* (2000), in a study focusing on the effects of run-off contaminants on non-target species, observed thinner and elongated bodies accompanied with inhibition of swimming, crawling, body reversal, and eventually full paralyses under ivermectin concentrations of 0.3 to 300nM. The same study identified behavioural endpoints as being much more sensitive than other endpoints such as survival. The LC50 at 72h was 560 nM, while their 3h IC50s for swimming, reversal, crawling speed and frequency were 1.1, 16, 51, and 91 nM respectively. Similar studies by Sardo and Soares (2010) identified locomotion as an early warning parameter

of sub-acute toxicity. The decreased locomotion by *L. variegatus* was considered a large risk factor as it limits the organism's ability to avoid capture, resulting in increased predation.

The observed reduced foraging as a result of decreased swimming could result in deceased food intake, limiting energy required for normal day-to-day activities, as well as sexual reproduction (Sardo & Soares, 2010; Ding *et al* 2000). As sexual reproduction is their normal means of reproducing in nature this shift could result in increased asexual reproduction, or fragmentation, with a subsequent reduction in genetic diversity within the lumbriculus population. As genetic diversity is important for the survival of a species, this could pose detrimental changes and have devastating effects for the survivorship of lumbriculus species.

*Interpretation of body positioning*

Similar to the observed lethargic swimming behaviour, deviations from normal body positioning were observed during 400, 500, and 5000μg/L of Cu and 10% EtOH. Inward curling of posterior and anterior body segments was observed. Analogous to changes in swimming patterns under Cu exposure, deviations from normal body lengthening have been observed in exposure to fluoranthene and sub lethal Cu concentrations (Landrum *et al* 2002). Landrums observations indicated an elongation of the body together with thinning of the body length. This prevented normal movement though the water and resulted in isolation of individual worms. There was also evidence of crooked, or kinked, body segments. Instead of maintaining a smooth curvature of the body length the organisms would appear to bend sharply at odd angles. These positions deviated from the worms normal fluidic smooth body lengths and were suggestive of physical stress. Body positions such as these would limit the individuals swimming ability,

resulting in similar cases as previously described. Improper body positioning would also place stress on the worm's respiration, as extended posteriors are used for effective exchanges of gases (Landrum *et al* 2004; Penttinen *et al* 1996)

*Interpretation of clumping/colony formation behaviours*

The noted significance in occurrence of first colony formation may cause harm to *L. variegatus*. In culturing conditions as well as in nature, numerous lumbriculus would congregate and form large integrated clumped colonies of up to 2000 individual worms. This allows for increased metabolism, stabilization of body temperatures, effective gas exchange, and collaborative food sharing. Within the testing wells however, the lumbriculus sampled would continually form and reform clumped colonies. This was assumed to occur because of the limited number of lumbriculus (n=10) and the size of the testing wells. It was therefore established that normal clumping behaviour within the test chambers would consists of the formation of clumped colonies proceeded with continual disintegration and reintegration of individual worms into clumped colonies. This behaviour was observed several times under reference conditions to establish a pattern of normal behaviour. During treatment reduced clumping behaviour was observed under exposure to Cu and EtOH. With the reduction in colony forming many of the requirements for lumbriculus survival may be impeded. This is an essential behaviour and deviation from this behaviour could result in increased mortality. Without the large integration of individual worms into a single mass, body heat cannot be captured and used to establish a balanced homeostatic temperature with their surroundings. Surrounding water temperature could increasingly influence body temperatures and survival at lower temperature would decrease.

Feeding rates could also decrease, as food sharing would not be available to the individual worm. Individual worms would have lower food encounters without the benefit of the size of an entire colony and its increased sediment coverage.

## 3.2 Multispecies Freshwater Biomonitor

### 3.2.1 *C. tentans* MFB data

*Mapping*

Simultaneous filming of *C. tentans* larvae occurred while MFB tests ran with burrowed larvae placed inside the chambers. Foraging and burrow irrigation behaviours were correlated with their respective MFB frequencies. It was found that the lowest frequency (-0.5 Hz) correlated with large foraging events, and smaller body movements involved in foraging and burrow irrigation correlated with slightly higher frequencies ( -1.0 to -2.0).

The following figure illustrates the initial amplitude readings performed by the MFB.

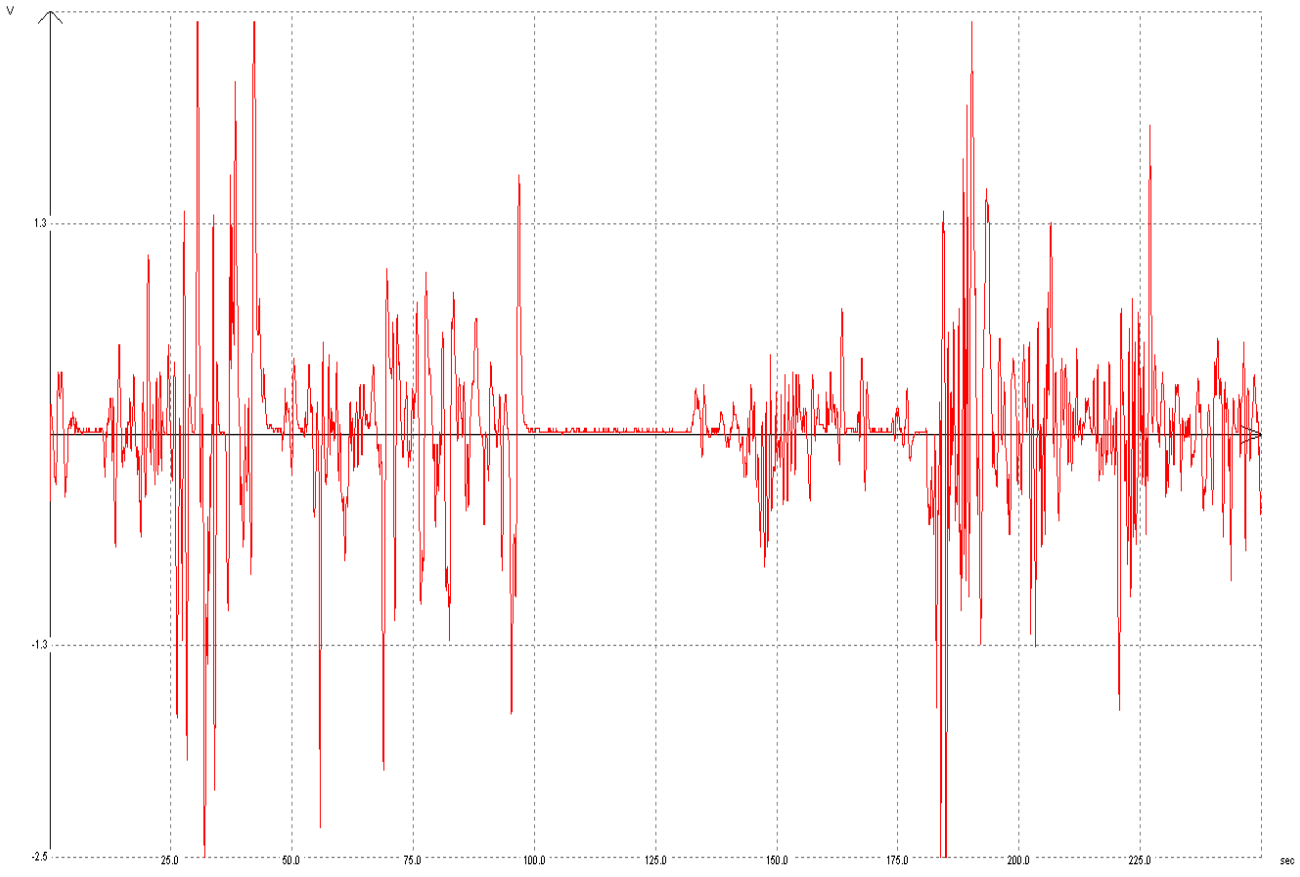



**Figure 3.8** Amplitudes generated by movements of *C. tentans* larva within burrow at 10:00 – 14:00 min

Figure 3.8 depicts the initial amplitude real-time readings (in volts against time in seconds) created by the organism's movements within the chamber. Changes in amplitude readings correlate with the size of the organism's movements. These readings occur for four minutes (240 seconds) every ten minutes. By comparing these amplitudes to video it was observed that the large peaks occurred while the larva withdrew from its burrow and partook in large foraging movements. The smaller amplitudes corresponded with small anterior movements and peristaltic motions occurring within the burrow. Figure 3.9 depicts the amplitude readings generated by large foraging movements, in this case by the larva emerging from its burrow and foraging around. Note the time of the snap shot in the lower right corner of the pictures, 10:32 and 10:47, corresponding to 32.0 and 47.0(seconds) from the circled portion of the graph from figure 3.12. Note the different positions of the larva between the snap shots.


**Figure 3.9**. Amplitudes generated by large foraging movements outside the burrow and the corresponding snapshots

Burrow irrigation was captured at time 13:27, corresponding to time 196 to 208 (seconds) in figure 3.19. A second and third burrow irrigation event was observed at time 20:27 and 23:40 (27 and 220 seconds respectively). Figure 3.10 depicts the low amplitudes generated by burrow irrigation, the first taken from figure 3.11, the last two obtained from the proceeding four minute reading. Since burrow irrigation is visible as peristaltic motions within the burrow it cannot be illustrate in a single snap shot.

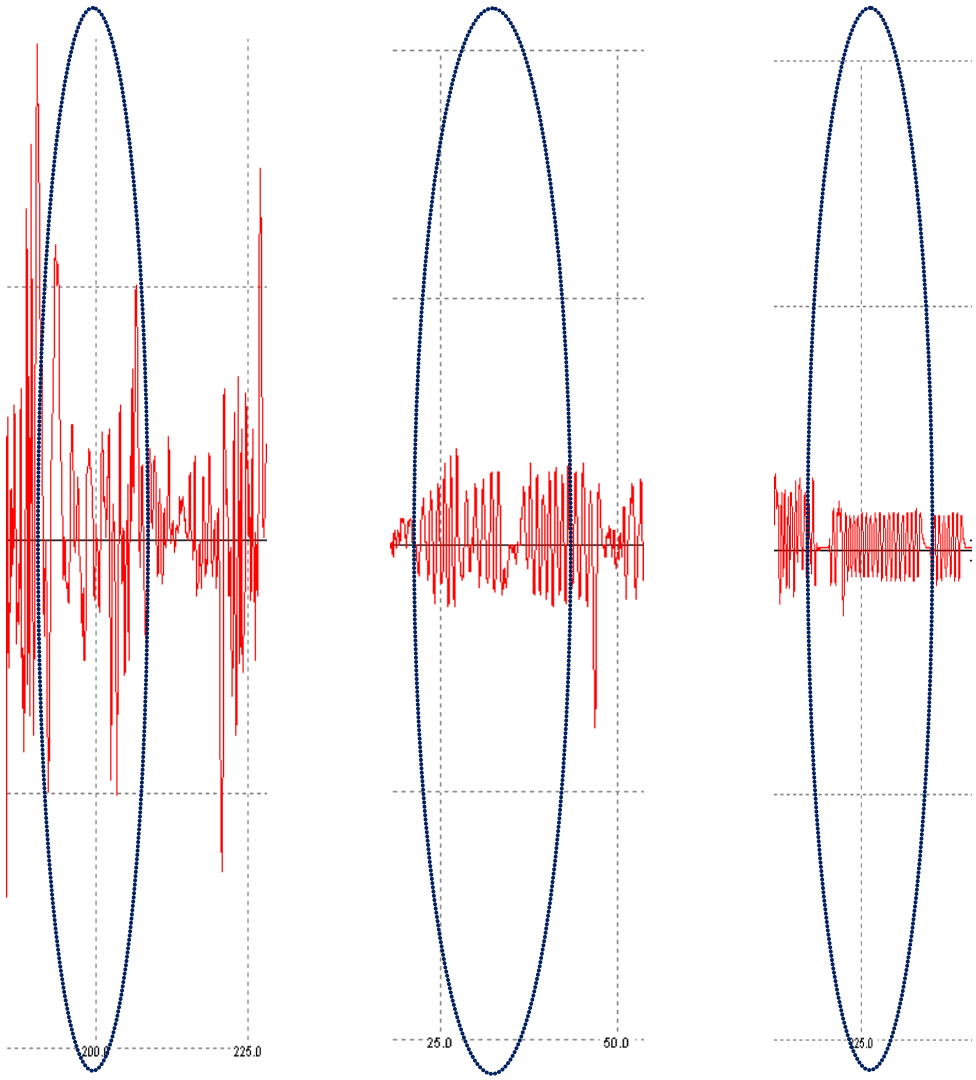



**Figure 3.10** Amplitudes of three distinct burrow irrigation events

The amplitudes are then put through a Fast Fourier Transformation (FFT), converting amplitude into frequencies in hertz (Hz) (figure 3.11)

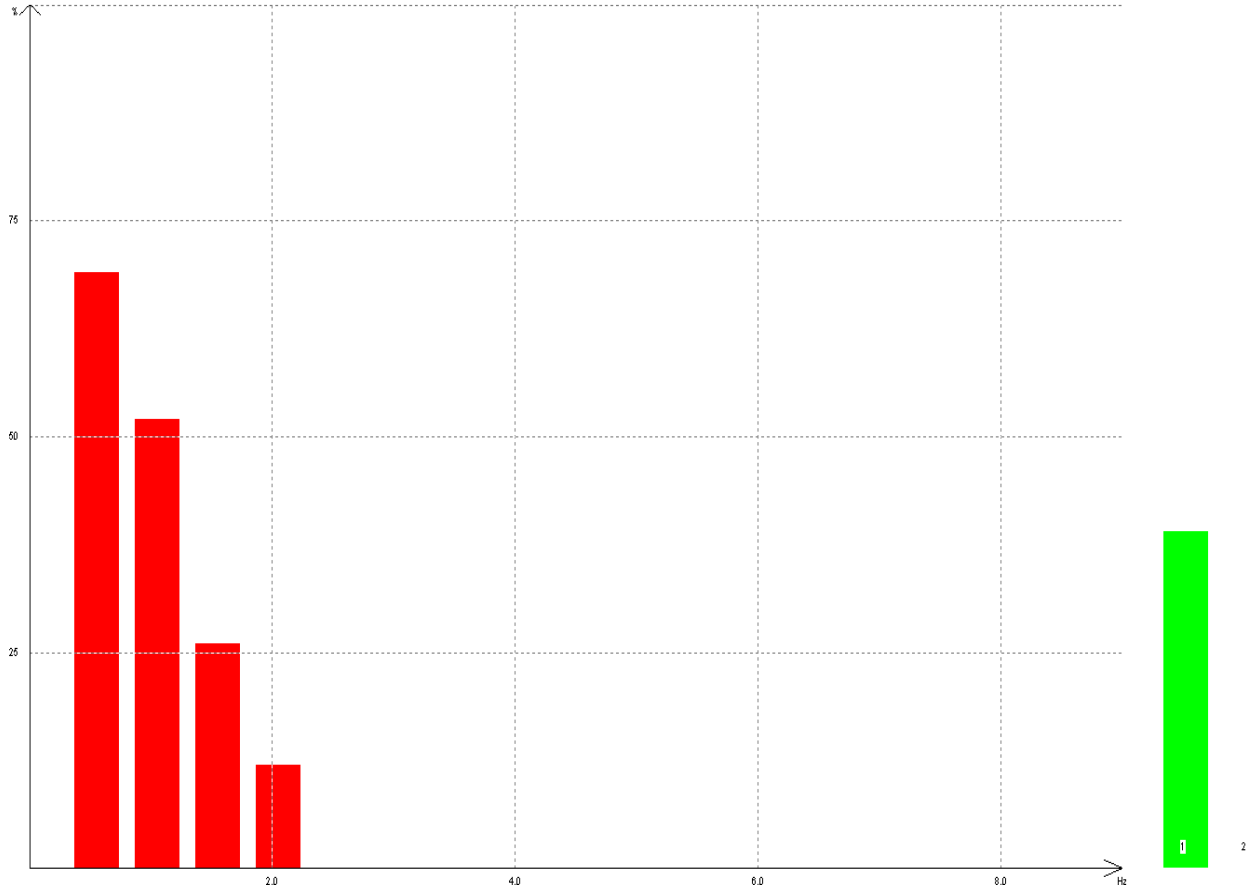



**Figure 3.11** FF transformation: Histogram of time 10:00-14:00 (in % activity time spent)

Figure 3.14 illustrates that all movements from the *C. tentans* larva within the burrow at time 10:00 – 14:00 correspond to Band 1. Note the total absence of a Band 2 bar. This illustrates that the MFB was unable to pick up small behaviours such as ventiliation, as described by de Bisthoven *et al* (2004). However ventilation did occur and was observed during the parallel video. It was identified as burrow irrigation, corresponding to the higher frequencies of 1.5 and 2.0 in the FFT histogram in figure 3.11 (the last two red bars). Therefore ventilation movements

were grouped together with all other movements made by the larva and grouped together in Band 1.

The third graph (figure 3.12) is a long-term reading of % time spent at each frequency.

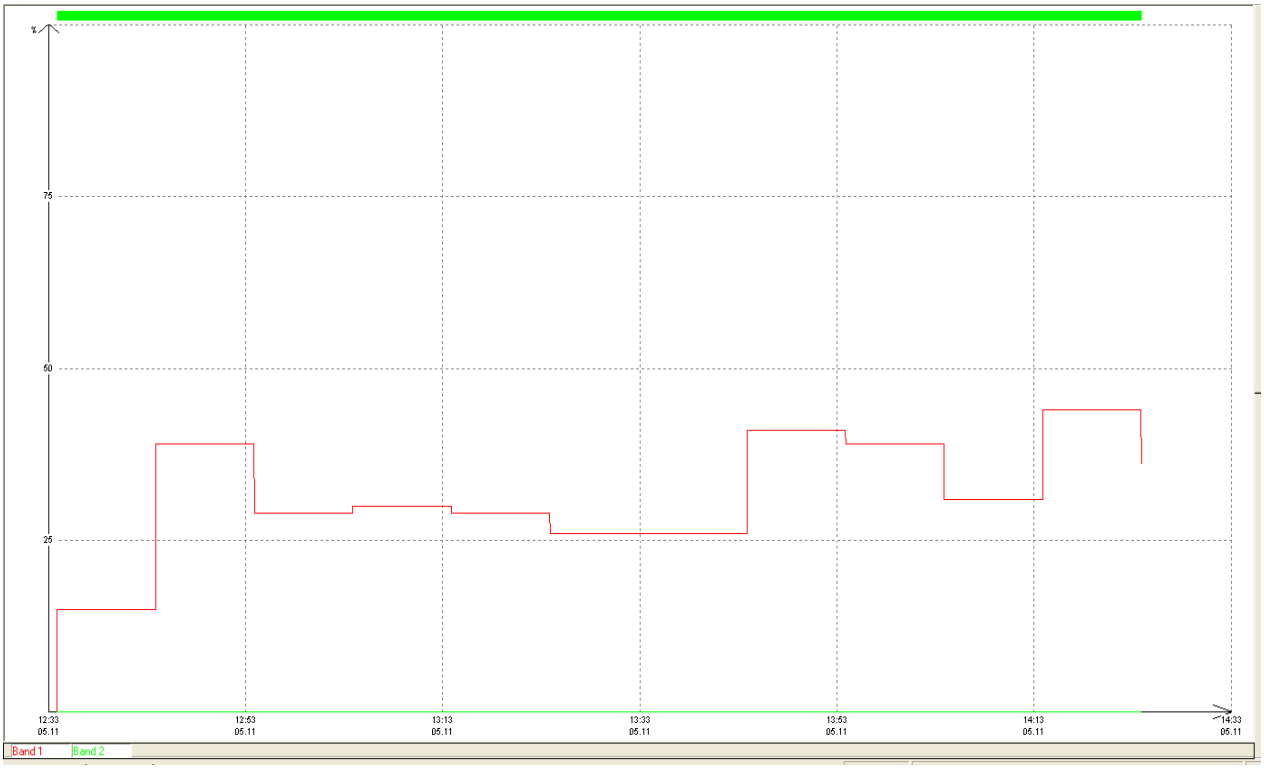



**Figure 3.12** Long-term graph depicting %time spent in activities in Bands 1 and 2 over two hours

The graph depicts the video test running for two hours, however only thirty minutes were captured on video, corresponding to the first three readings. Again note the absences of any Band 2 readings as depicted by the horizontal bright green line along the x axis. Although only the first thirty minutes were captured on video, no Band 2 data was recorded for the full length of two hours.

Due to mechanical difficulties described in section 2, no video map was created for *L. variegatus* tests performed with the MFB.

*MFB data readouts*

MBF tests ran for four hour. The following table represents Band 1 data (% time spent) generated from *C. tentans* larva under exposure to100µg/L TBT. This resulted in a total of 24 Band 1 data readings. Both Cu and EtOH have similar data tables. The channel numbers indicate in which chambers each larva was placed.

**Table 3.5** Band 1 readings for *C. tentans* under exposure to TBT

| Date | Time | | | | REF | | | | | TBT | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Channel | 1 | 3 | 9 | 10 | 11 | 4 | 5 | 6 | 7 | 8 |
| 27.11.11 | 15:00:50 | | 8 | 23 | 31 | 33 | 37 | 17 | 15 | 18 | 38 | 46 |
| 27.11.11 | 15:10:50 | | 33 | 36 | 34 | 29 | 38 | 35 | 38 | 0 | 14 | 13 |
| 27.11.11 | 15:20:50 | | 32 | 17 | 37 | 0 | 10 | 34 | 6 | 35 | 12 | 39 |
| 27.11.11 | 15:30:50 | | 32 | 41 | 37 | 0 | 36 | 2 | 0 | 27 | 36 | 30 |
| 27.11.11 | 15:40:50 | | 28 | 38 | 31 | 7 | 31 | 31 | 0 | 29 | 32 | 36 |
| 27.11.11 | 15:50:50 | | 34 | 29 | 32 | 44 | 37 | 36 | 18 | 28 | 34 | 33 |
| 27.11.11 | 16:00:50 | | 33 | 34 | 34 | 49 | 34 | 32 | 11 | 30 | 18 | 31 |
| 27.11.11 | 16:10:50 | | 32 | 38 | 33 | 40 | 33 | 30 | 21 | 15 | 3 | 31 |
| 27.11.11 | 16:20:50 | | 40 | 33 | 38 | 31 | 33 | 27 | 13 | 0 | 19 | 10 |
| 27.11.11 | 16:30:50 | | 26 | 30 | 32 | 37 | 34 | 28 | 13 | 7 | 33 | 18 |
| 27.11.11 | 16:40:50 | | 31 | 45 | 29 | 32 | 32 | 14 | 18 | 3 | 21 | 41 |
| 27.11.11 | 16:50:50 | | 6 | 33 | 32 | 32 | 34 | 31 | 9 | 0 | 43 | 26 |
| 27.11.11 | 17:00:50 | | 4 | 37 | 44 | 38 | 31 | 22 | 3 | 0 | 12 | 23 |
| 27.11.11 | 17:10:50 | | 31 | 36 | 37 | 32 | 40 | 32 | 2 | 0 | 3 | 18 |
| 27.11.11 | 17:20:50 | | 4 | 38 | 34 | 36 | 31 | 26 | 20 | 7 | 20 | 0 |
| 27.11.11 | 17:30:50 | | 33 | 34 | 38 | 39 | 35 | 25 | 0 | 0 | 7 | 29 |
| 27.11.11 | 17:40:50 | | 31 | 36 | 37 | 44 | 34 | 12 | 2 | 17 | 3 | 5 |
| 27.11.11 | 17:50:50 | | 30 | 40 | 34 | 38 | 33 | 35 | 23 | 4 | 21 | 20 |
| 27.11.11 | 18:00:50 | | 26 | 38 | 47 | 39 | 36 | 30 | 9 | 0 | 26 | 10 |
| 27.11.11 | 18:10:50 | | 15 | 40 | 27 | 9 | 36 | 26 | 7 | 0 | 6 | 11 |
| 27.11.11 | 18:20:50 | | 22 | 33 | 35 | 38 | 33 | 0 | 18 | 0 | 18 | 35 |
| 27.11.11 | 18:30:50 | | 32 | 33 | 33 | 39 | 32 | 18 | 9 | 11 | 6 | 7 |
| 27.11.11 | 18:40:50 | | 35 | 32 | 37 | 38 | 42 | 30 | 13 | 0 | 0 | 31 |
| 27.11.11 | 18:50:50 | | 39 | 35 | 33 | 31 | 33 | 11 | 0 | 0 | 27 | 7 |

Band 1 was sorted into different bins, each bin describing the amount of movement within the chamber. Chironomid larva movements were categorized into five bins. Low readings (which correspond to periods of rest and small amounts of locomotion) were sorted into Bin 1, where high readings (which correspond to large amounts of locomotion) were sorted into Bin 5.

Bins 2-4 were assorted accordingly. The following table is an example of how Band 1 data from the above TBT readings were sorted into bins.

**Table 3.6** Sorting of Band 1 data into bins

| | **Reference channels** | | | | | **TBT channels** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **BIN** | **Ch 1** | **Ch 3** | **Ch 9** | **Ch 10** | **Ch 11** | **Ch 4** | **Ch 5** | **Ch 6** | **Ch 7** | **Ch 8** |
| **10** | 4 | 0 | 0 | 4 | 1 | 2 | 11 | 15 | 7 | 6 |
| **20** | 1 | 1 | 0 | 0 | 0 | 5 | 9 | 4 | 7 | 5 |
| **30** | 5 | 3 | 2 | 1 | 0 | 9 | 2 | 4 | 4 | 4 |
| **40** | 14 | 18 | 20 | 16 | 22 | 8 | 1 | 1 | 5 | 7 |
| **MORE** | 0 | 2 | 2 | 3 | 1 | 0 | 0 | 0 | 1 | 2 |

* Bin MORE indicates readings that were above 40

For example, in channel 1 under reference channels, 4 Band 1 readings from table ? were between 0 and 10, 1 was between 11 and 20, 5 were between 21 and 30, 14 were between 39 and 40, and no Band 1 readings were above 40.

Discriminant analysis showed significance in change in behaviour of chironomid larva exposed to 100 µg/L TBT ($p = 0.008$). EtOH and Cu showed no significant changes in behaviour over treatment ($p = 0.286$ and 0.120). Factor analysis of the TBT treatment identified Bin 1 as having the greatest interpretation of variance between treatment and reference. The following figures show PCA plots with loadings of both TBT and Cu treatments, and EtOH control.

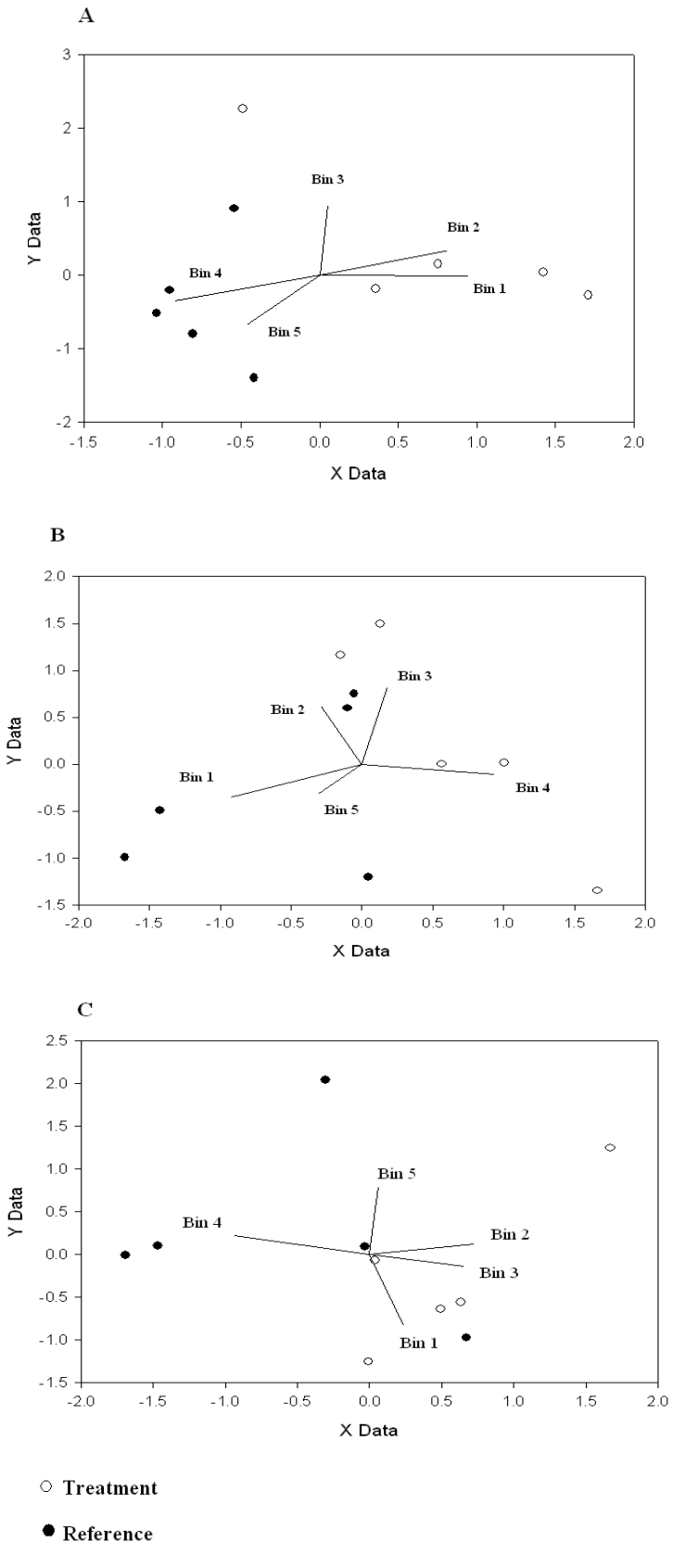



**Figure 3.13** *C. tentans* PCA distributions (A) TBT, (B) Cu, and (C)EtOH

#### 3.2.1.1 Interpretation of MFB data

*Parallel video mapping*

The initial mapping of the MFB responses to the larvae's behaviours allowed for ecological interpretation of the MFB frequencies. It was established that the lower frequencies, -0.5 to -1.5, reflected the desired foraging behaviour. The larva's movements within the burrow, called burrow irrigation, were also detected. While large whole-body movements indicative of foraging were easily identified on the MFB amplitudes graph, having a frequency of -0.5, the small foraging movements produced from the forelegs and mouthpiece were indistinguishable from the similarly small movements of burrow irrigation. Complete segregation of frequencies into the two specific smaller behaviours was not possible due to the duplicity of the behaviours and the limited analyses of the MFB. However in studies performed by de Bisthoven *et al* (2004) undulations of the body were categorized as smaller movements and were expressed as Band 2. This was not found to be the case upon parallel video recording. As seen by the frequencies and paired video timing, burrow irrigation, which is composed of undulations of the body, did not register as any of the higher frequencies. Large undulations of the body within the burrow had a registered frequency of 1.0 – 2.0 Hz. In fact throughout the entire mapping no frequencies above 3.0 Hz were recorded. Therefore burrow irrigation could not be told apart from small foraging movements in the MFB analysis. For this reason the entirety of the lower frequencies, collectively termed Band 1, was used for analyses. Within Band 1, both large and small foraging events were captured, along with burrow irrigation, and was therefore thought beneficial to use this averaged data for further statistical analysis.

*MFB data readings*

Chironomid larva elicited a significant change in locomotion behaviour under exposure to 100µg/L TBT. During time-lapse photography experiments, the only change in behaviour was seen in burrow construction times, with delayed constructions times at 100µg/L of TBT. While burrow construction was not determined a useful endpoint for visual biomonitoring, the activities involved in burrow construction may be measure with the MFB. Since foraging is needed for burrow construction it is possible that the delayed burrowing construction could be due to impaired foraging behaviour that was too varying or too small to assess with the naked eye. However these small movements would be detectable within the electric field of the MFB chamber. Factor analysis determined that bin 1, and to a lesser degree, bin 4, explained the variance in readings between TBT treatment and reference. As described bin 1 was composed of rest periods and small amounts of foraging and burrow irrigation, and bin 4 was large amounts of foraging and burrow irrigation. Therefore the main deviations of behaviour elicited by the chironomid larva under 100µg/L of TBT occurred during rest periods and periods of a lot of foraging and burrow irrigation.

According to the results obtained in the time-lapse photography experiments, significant changes in locomotion were expected in the10% EtOH MFB experiment. Also, while there were no observed changes in swimming behaviour under Cu exposure in the time-lapse photography experiment, it was anticipated to see if there were similar small changes in swimming that were invisible to the naked eye, as there was in the TBT treatments.

Discriminant analysis of Band 1 data indicated that the MFB was not successful in detecting any of these anticipated changes in locomotion under both Cu and EtOH treatments.

Although stress was observed in swimming, body positioning, and burrow construction under Cu and EtOH treatment during the time-lapse photography experiment it was not reflected in the MFB readings. The failure of the MFB to detect these changes in locomotion creates a problematic situation. Why did the MFB fail to detect behavioural changes in the treatments? Especially during the positive control treatment? The absence of readings may be due to the physical set up of the tests there was no changes in behaviour detected by the MFB. As previously established, *C. tentans* construct burrows for several reasons, one of which is avoidance of contaminants. During the visual experiments the larva were forced to reconstruct their burrow while in contact with the contaminated overlaying water. This direct contact without possible escape into burrows may have been responsible for observed lethargic behaviour. In contrast, the MFB tests occurred with the larva placed within pre-constructed burrows. Upon exposure to contamination the larva would already have a route of escape and avoidance from the contaminated overlaying water without the need to expand extra energy under toxic conditions. This pre-established means of escape may have limited the behaviour measured by the MFB. However, as portrayed in the TBT responses, foraging behaviour within a burrow can be detected by the MFB, and would therefore reflect treatment effects.

The unexpected failure of the MFB to show a significant change in behaviours during Cu and EtOH treatments may not be entirely inexplicable when looking at the use the MFB and the inherit plasticity of behaviour. DeBisthoven *et al* (2004) had similar high variations while testing acid-mind drainage (AMD) on chironomid larva. High standard deviations between reference and AMD treatments accompanied inconsistent differences in locomotory behaviour between periods of day and night. Ventilitory behaviour was another source of difficulty, with faster

ventilitory representing on average 6% of activity time with a SD of 15%, data that was excluded from analysis due to its inconsistency.

### 3.2.2 *L. variegatus* MFB data

*MFB data readouts*

Similar data distribution as was used for the *C. tentans* data was used for *L. variegatus*. The only difference is that *L. variegatus* Band 1 readings were distributed into six bins. The following is an example of the bin distribution for *L. variegatus.*

**Table 3.7** Sorting of Band 1 data into bins

* Channel 11 data due to escape of all 10 worms from chamber

| | **Reference channels** | | | | | **TBT channels** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **BIN** | **Ch 1** | **Ch 3** | **Ch 9** | **Ch 10** | **Ch 11** | **Ch 4** | **Ch 5** | **Ch 6** | **Ch 7** | **Ch 8** |
| **10** | 0 | 0 | 0 | 0 | 24 | 0 | 0 | 0 | 0 | 0 |
| **20** | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| **30** | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 3 |
| **40** | 7 | 14 | 14 | 13 | 0 | 11 | 15 | 16 | 14 | 9 |
| **50** | 16 | 10 | 10 | 11 | 0 | 9 | 8 | 5 | 9 | 12 |
| **MORE** | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 |

Discriminant analysis identified no significant change in locomotion behaviour from *L. variegatus* under exposure to Cu and EtOH, with p values of 0.888, and 0.191. Under TBT exposure significance was seen in locomotion ($p = 0.021$). Factor analysis (PCA) identified bin 1 as having the greatest interpretation of variance between treatment and reference.

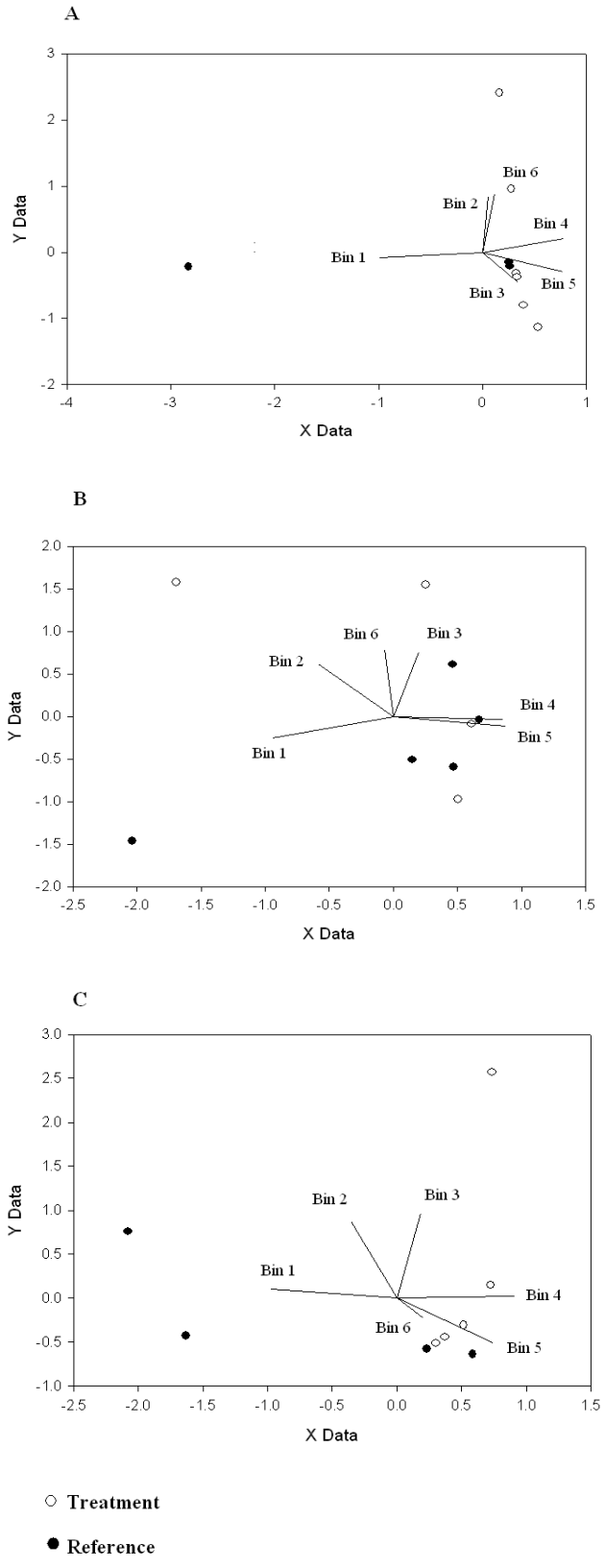



**Figure 3.14** *L. variegatus* PCA distributions: (A)TBT, (B) Cu, (C) EtOH

#### 3.2.2.1.Interpretation of MFB data

*Set-up and Mapping*

Due to numerous mechanical difficulties it was not possible to capture the movement of the lumbriculus on video while there were in the MFB chambers. As described in section 2, the organism's behaviour within the chamber needed to be recorded in order to permit a comparison of behaviour between visual observations and MFB responses – in other words the MFB responses needed to be 'mapped' to the observed behaviour. In order to accomplish this one end cap of the MFB chamber needed to be removed. To keep the organisms within the chamber, the opening was placed flat up against the glass of the experimental container. Despite care taken to assure a solid fitting the worms escaped during the experiment, as mention in section 2. It was assumed that the opening was not flatly placed on the glass side and that the worms escaped through the opening-glass interface. The other end cap was screwed in place and the experiment ran again, this time without a video recording of the behaviour occurring within the chamber. However, the worms once again escape the MFB chamber. The worms were observed to be squeezing though the 5 um Nytex mesh screening covering the end caps.

To prevent escape the mesh was layered in triplicate over the opening. This was successful in preventing escape; however air bubbles would form between the layers and remain inside the chambers. Also, the worm's nature to continuously forage would result in them getting stuck within the triple layers of mesh. Many attempts were made to re-layer the mesh and secure the lumbriculus inside. However all attempts were unsuccessful. As it appeared that the mesh coatings would not prevent the worms from escaping or getting trapped, it was hypothesized to use a solid cover. However, since the chamber needed to be open to the surrounding water and not remain stagnant inside the chambers, the sealing of the end caps would have to be

accomplished in such a way that water would still be able to circulate. This was accomplished by increasing the test container size, from the small cube glass beakers to a 4L glass aquarium, and by rearranging the placement of the chambers to a vertical, 'cap up' position, as opposed to a horizontal position.

The 4L tank would house the chambers while providing enough testing water to overflow into the chambers. The height of the chambers, while placed vertically, was deep enough to prevent the worms form escaping from the open top end. The bottom end cap was sealed with a layer of sterile nitrile glove (figure 2.8). Research was done to evaluate different forms of containment and MBF setup with *L. variegatus*, however such studies (Gerhardt *et al* 2003) failed to detail how the chambers contained the organisms. Gerhardt's works did not include methodologies on *L. variegatus* testing, as her numerous publications and evaluations on the use of the MFB for real-time biomonitoring have focused on *G. pulex* and its suitability for standardization in the field of real-time biomonitoring

*MFB readings*

*TBT*

In all MFB tests the large frequencies associated with locomotion showed high variations between reference and treatment, making it difficult to ascertain significant behavioural changes. However with the data modeling, statistical analysis yielded significance with one contaminant. *L. variegatus* elicited a significant change in locomotion behaviour under exposure to 100µg/L of TBT when monitored with the MFB. Strangely these significant differences in locomotion under TBT exposure were not visible in the lapse photography experiments. The responses elicited by

the worms during the lapse photography experiments were delayed clumping and/or absence of clumping. The observed lack of clumping may have been due to reduced swimming efficiency of which was not visible to the naked eye. These minute small movements would be detectable and measurable with the MFB impedance measurements. These small deviations in swimming behaviour, which accounted for the lack of clumping in the time-lapse photography experiment, may have been picked up MFB and read as stressed locomotry movements.

According to the factor analysis it would appear that bin 1 best explains changes in behaviour between TBT treatment and reference. Recall from before that bin 1 represents periods of rest and small amounts of locomotion. From this it can be deduced that 100μg/L of TBT afflicted the organism's periods of rest and small quantities of swimming and foraging.

*Cu and EtOH*

Recalling results from visual experiments, lethargic swimming movements accompanied with deviated body positioning were observed in *L. variegatus* under 400 μg/L of Cu, which increased as the concentration rose to 500μg/L. Similar deviations in swimming behaviour were observed at 10% EtOH. However similar to results in the MFB experiment with *C. tentans*, these changes that were observed in the time-lapse photography experiments were not reflected in the MFB results. The majority of the MFB readings with *L. variegatus* were in the lower frequencies of 0.5 Hz – 2.5 Hz (Band 1). Higher frequencies, upward to 8.0Hz (Band 2) were not detected. This absences of higher frequencies corresponded with early studies by Gerhardt (2006), where the spontaneous locomotory behaviour *of L. variegatus* was recorded between 0.5 and 2 Hz. The high variation of swimming behaviour captured by the MFB from this study was also seen in

Gerhardt's. Difficulties were encountered with locomotion showing high variation during testing (Gerhardt 2006). High variations in this study along with Gerhardt's may be explained by high genetic variations in the animals tested and various animals responding to stress and other not, each of these dichotomous responses based on the plasticity of behaviour (Gerhardt 2006; Landrum *et al* 2002; Leppänen & Kukkonken1998).

With previous work with L. variegatus and the MFB (Gerhardt 2006) sediment was used and housed within the chambers. Tests ran using spiked and non-spiked sediment. It was found that with unspiked sediment and spiked overlying water, the lumbriculus would increase burrowing into the sediment, a response that may indicate a tendency to withdrawal from and avoid contaminated water. Interestingly there was no decrease in burrowing or withdrawal from the contaminated sediment into clean water. This may explain a preference of sheltering even in contaminated sediments (in this case with sublethal Pb concentrations) over increased predation risks associated with sediment withdrawal. When compared to the work performed in this study it is not possible to draw parallel conclusions. No sediment was used within the MFB chambers during toxicity tests due to the previously discussed mechanical complications. It was therefore impossible to detect am increase or decrease in sediment burrowing or sediment withdrawal. However no mentions of such difficulties were evident in these other studies.

### 3.2.3 MBF and its possible use in the field

The MFB successfully registered significant difference between TBT and reference conditions. At 100ug/L, TBT did elicit a significant change in locomotion behaviours from *C. tentans* and *L. variegatus*. Such stress in locomotion was not observed during lapse photography experiments. In this case the MFB was more successful than visual observations in detecting the presence of a toxic agent. However reversely, the MBF failed to detect a difference in locomotion under exposure to 500ug/L of Cu and the EtOH positive control, even though stressed swimming behaviour was visually observed during the time-lapse photography experiment. In this case, the MFB was not successful in detecting the presence of a stressor known to cause stress behaviour whereas visual observations were successful.

Despite these difficulties it is believed that this present study illustrates the broad utility of the MFB. Its multispecies capability allow for ecological wide assessments of water including sediment dwelling benthic species. Its portability allows for in situ toxicity studies and many chambers let the user house and run many replicates at once. The software lets the user define detection limits and alarm settings. However work must done to correct for high variations among results. Such high variations and difficulties in monitoring appear to arise from the attempt to monitor small non-locomotory behaviours such as ventilation. Future studies with the MFB would require more standardized methodologies that satisfy both the containment issue and practicality of use. It would also be interesting to use the MFB with *G. pulex,* as much research has been published with this organism and would allow for comparison of not only set up designs and other mechanical issue, but comparison of data organization and statistical modeling, both topics of which have shown high variability and proven difficult in Fleets graduate work and this present study.

The dilemma with the positive control presents a difficulty. Neither organism elicited a significant behavioural response under known stress-inducing levels of EtOH. It was decided to use these levels of EtOH as a positive control based on the reactions observed during the lapse photography experiments. The failure of the MFB register any kind of behavioural change poses a problem for integration into an early warning biomonitoring system for drinking water. Since 10% EtOH was known to elicit stress behaviours from these two organisms the absence of a significant readings may be due to problems associated MFB. This is not the only time the MBF has shown high variability in its responses. Fleets work resulted in similar similarities between contaminant and reference, with TBT and reference having a locomotive activity of 9.7 ± 14.7% and 10.3 ± 8.0 % respectively. High deviations were also a problem with Fleets work with the MFB.

The method in which the MFB organizes it data may be causing the problem of high variations. The MFB can only recognize the size of the movement generated by the organisms, registering it as amplitude. Over four minutes these movements are averaged as two values, Band 1 and Band 2. The problem arises when the averaged data cannot differ between sudden sporadic movements and lengthy normal movements of the same amplitude and time duration. If the organism remains at rest for the majority of the four minutes of monitoring and only elicits a few large stress movements such as thrashing, for 30 seconds, this will result in the same % time spent reading if the same organism spent the same 30 seconds performing normal large foraging behavioural movements but spread out over the four minutes of monitoring. Each reading would inform the user that 30 seconds out of 4 minutes where spent on behalf of the organism performing large movements, generating large amplitudes.

These issues have not been addressed in the extensive literature available on the MFB (Gerhardt *et al* 1994; Gerhardt 1998; Gerhardt *et al* 2003; deBisthoven *et al* 2004). Results from these studies have all predominately been performed by Gerhardt and colleagues with minimal outside investigation and analysis of the MFBs fundamentals and have only focused on the end products of Bands 1 and 2. While these bands do give a long-term picture of % time spent in what activity by the organism, it still does not paint the whole picture of responses. Independence readings are inherently simplistic, in such that only magnitudes of disturbance in the electrical field are recorded. Responses can only be designated a scalar value. No other information is registered; information that can enlighten the user to the full behavioural response from the organism. Such information may include body orientation, feeding rates, respiration, metabolism, and avoidance behaviours. If the chambers were able to measure endpoints such as these along with its current motion detection it may be more suitable for use in an early-warning biomonitoring system.

Fleet (2010) came across such difficulties in her attempts in evaluating the MFBs suitability for incorporation into an early-warning biomonitoring system. She identified *H. azteca's* ability to swim through the water column and its style of swimming as two behaviours strongly susceptible to stress. Immobilization, a behavioural parameter strongly registered by the MFB, is caused by higher acutely toxic concentrations and after long exposure periods resulting from exhaustion from the organism (Fleet 2010). Neither swimming style nor water column position are detectable with the MFB, where immobilization appeared to be the only behaviour the MFB registered.

An additional problem occurs with how the MFB records moments of immobilization. Fleet observed the *H. azteca* and *D. magna* spent much of their time immobile in the chambers;

however upon completion of test, organisms showed no signs of stress. While immobile, the organisms may have continued to undergo normal ventilitory behaviours. However, Fleets MFB results did not register any ventilitory responses. If the MBF is unable to differentiate between stationary organisms undergoing normal metabolic process and true immobilized organisms, or dead organisms, then no stress responses or alarms will be registered. Corresponding results were generated from *C. tentans* in this study. No higher frequencies associated with ventilitory behaviour were registered. Long durations of 'rest' were detected by the MFB, registered as an absence of amplitudes. Theoretically, stationary *C. tentans* larvae ventilitory behaviours would be detectable by the MFB, therefore differentiating between live motionless larvae and stressed immobile/dead ones. However no higher frequencies, as ventilation described by Gerhardt, were registered by the MFB while the larva was in rest. In such cases how would the MBF register a dead larva? How can any differentiation be possible without the registering of all behaviours used for stress identification? At this time the MBF appears to be unable to complete this task.

This study, in conjunction with previous works by Fleet, advocates that additional study of the MFB is required with strong attention paid to the variations in how the MFB registers behavioural changes along with methods of statistical analysis. The MFB must demonstrate successful reproducibility before it can be implemented into the field as a reliable component of an early-warning biomonitoring for drinking water.

## 4.0 SUMMARY

The following study concluded with the success of visually observing deviations in behavioural responses from both organisms upon induced stress from the four contaminants and/or positive control. The observations on benthic organism's *C. tentans* and *L. variegatus* have further demonstrated their suitability for behavioural biomonitoring. The lapse photography experiments illustrated that foraging and swimming behaviours are continual activities elicited from both *C. tenants* and *L. variegatus* with possible applications in non-visual biomonitoring systems. Burrow construction and colony formation proved to be unreliable parameters for non-visual biomonitoring systems. These two behaviours are reliant on the locomotion behaviours of the two organisms, and success or failure to construct burrows or form a colony can be ascertained by monitoring their foraging and swimming behaviours. As burrow construction and colony formation is dependent on foraging/swimming success, so is foraging/swimming dependent on correct body positioning. Therefore the monitoring of body positioning is also not needed. It is the opinion of this paper that future behavioural bioassays need focus on the foraging and swimming behaviour of *C. tentans* and *L. variegatus* for the identification of stress.

Work with the MFB concluded with mixed success. Under exposure to 100 μg/L of TBT, the MFB successfully identified the presence of the toxicant through the changes in locomotion behaviours of *L. variegatus* and *C. tentans*. Whereas visual observations from the time lapse photography failed to identify such changes in locomotion. In this case the MFB was more successful than visual observations. In the case of Cu, while there was no visual stress behaviours observed during the time lapse photography, L. variegatus did show stress in swimming behaviour under 500ug/L. However the MFB failed to register such stress in swimming behaviour for L. variegatus. In this case, visual observation was more successful than

the MFB. This calls into question if the results from the TBT treatment are due to TBT-induced stress on the organism's behaviour, or simply the large variability and plasticity of behaviour. To answer this multiple tests would need to be conducted with the MFB accompanied with large sample sizes.

The ability of the MBF to detect changes in locomotion where the naked eye fails corresponds to the sensitivity of the technology. However its inability to detect changes where the naked eye did calls into questions its accuracy. If a stress response was visible surely it would have been detectable from the MFB. In addition to this, its failure to register stress behaviour with the positive control tests calls into question the accuracy of the technology.

While the automated system proved effective in generating quantified numerical values from behaviour, the data rendered requires multiple categorizing and various statistical analyses before significances can be obtained. The MFB software does not complete this data manipulation and therefore the sensitivity and consistency of the alarm system must be questioned.

It is the recommendation of this study that the MFB is not ready to be included into an early-warning biomonitoring system as it stands to date. Further work on the MFB with both organisms is required. Attention needs be placed on data and signal analyses to correct for the problems of high variances and reproducibility; at which point the integration of the MFB into an early-warning biomonitoring for drinking water can be re-evaluated. However, until such time, the MFB remains too variable for present use.

# Appendix A

## MFB Data: BAND 1 readings

### *C. tentans*

| Chironomids | | | | | REF | | | | | Cu | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Channel | 1 | 3 | 9 | 10 | 11 | 4 | 5 | 6 | 7 | 8 |
| date | time | | | | | | | | | | | |
| 25.11.11 | 10:25:21 | | 34 | 42 | 7 | 31 | 0 | 42 | 35 | 31 | 41 | 32 |
| 25.11.11 | 10:35:21 | | 39 | 34 | 17 | 33 | 0 | 40 | 15 | 29 | 42 | 45 |
| 25.11.11 | 10:45:21 | | 39 | 34 | 4 | 35 | 0 | 51 | 42 | 24 | 35 | 40 |
| 25.11.11 | 10:55:21 | | 45 | 37 | 25 | 36 | 0 | 44 | 0 | 21 | 37 | 32 |
| 25.11.11 | 11:05:21 | | 33 | 33 | 0 | 42 | 0 | 34 | 2 | 10 | 8 | 31 |
| 25.11.11 | 11:15:21 | | 33 | 28 | 15 | 34 | 0 | 40 | 17 | 24 | 9 | 23 |
| 25.11.11 | 11:25:21 | | 11 | 5 | 26 | 48 | 0 | 35 | 9 | 3 | 5 | 37 |
| 25.11.11 | 11:35:21 | | 20 | 36 | 7 | 5 | 0 | 35 | 19 | 0 | 12 | 31 |
| 25.11.11 | 11:45:21 | | 7 | 10 | 27 | 22 | 0 | 41 | 17 | 23 | 30 | 34 |
| 25.11.11 | 11:55:21 | | 19 | 18 | 0 | 47 | 6 | 24 | 9 | 9 | 9 | 24 |
| 25.11.11 | 12:05:21 | | 24 | 11 | 0 | 28 | 11 | 36 | 36 | 35 | 28 | 27 |
| 25.11.11 | 12:15:21 | | 14 | 12 | 0 | 0 | 8 | 38 | 34 | 37 | 27 | 33 |
| 25.11.11 | 12:25:21 | | 32 | 36 | 0 | 3 | 0 | 19 | 28 | 37 | 27 | 32 |
| 25.11.11 | 12:35:21 | | 36 | 16 | 2 | 27 | 0 | 33 | 33 | 34 | 37 | 27 |
| 25.11.11 | 12:45:21 | | 22 | 30 | 5 | 34 | 0 | 47 | 9 | 43 | 29 | 46 |
| 25.11.11 | 12:55:21 | | 0 | 10 | 0 | 0 | 0 | 38 | 36 | 34 | 19 | 37 |
| 25.11.11 | 13:05:21 | | 0 | 42 | 5 | 3 | 0 | 31 | 29 | 32 | 23 | 35 |
| 25.11.11 | 13:15:21 | | 30 | 26 | 0 | 0 | 11 | 38 | 30 | 32 | 29 | 32 |
| 25.11.11 | 13:25:21 | | 36 | 16 | 0 | 0 | 0 | 34 | 21 | 33 | 37 | 20 |
| 25.11.11 | 13:35:21 | | 15 | 2 | 0 | 0 | 4 | 37 | 25 | 36 | 21 | 37 |
| 25.11.11 | 13:45:21 | | 3 | 0 | 0 | 6 | 3 | 40 | 3 | 31 | 5 | 31 |
| 25.11.11 | 13:55:21 | | 0 | 31 | 0 | 3 | 2 | 33 | 24 | 31 | 29 | 37 |
| 25.11.11 | 14:05:21 | | 25 | 20 | 0 | 6 | 13 | 39 | 28 | 30 | 29 | 32 |
| 25.11.11 | 14:15:21 | | 31 | 8 | 0 | 3 | 0 | 37 | 31 | 31 | 17 | 14 |

| | | | | | REF | | | | | TBT | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Channel | 1 | 3 | 9 | 10 | 11 | 4 | 5 | 6 | 7 | 8 |
| 27.11.11 | 15:00:50 | | 8 | 23 | 31 | 33 | 37 | 17 | 15 | 18 | 38 | 46 |
| 27.11.11 | 15:10:50 | | 33 | 36 | 34 | 29 | 38 | 35 | 38 | 0 | 14 | 13 |
| 27.11.11 | 15:20:50 | | 32 | 17 | 37 | 0 | 10 | 34 | 6 | 35 | 12 | 39 |
| 27.11.11 | 15:30:50 | | 32 | 41 | 37 | 0 | 36 | 2 | 0 | 27 | 36 | 30 |
| 27.11.11 | 15:40:50 | | 28 | 38 | 31 | 7 | 31 | 31 | 0 | 29 | 32 | 36 |
| 27.11.11 | 15:50:50 | | 34 | 29 | 32 | 44 | 37 | 36 | 18 | 28 | 34 | 33 |
| 27.11.11 | 16:00:50 | | 33 | 34 | 34 | 49 | 34 | 32 | 11 | 30 | 18 | 31 |
| 27.11.11 | 16:10:50 | | 32 | 38 | 33 | 40 | 33 | 30 | 21 | 15 | 3 | 31 |
| 27.11.11 | 16:20:50 | | 40 | 33 | 38 | 31 | 33 | 27 | 13 | 0 | 19 | 10 |
| 27.11.11 | 16:30:50 | | 26 | 30 | 32 | 37 | 34 | 28 | 13 | 7 | 33 | 18 |
| 27.11.11 | 16:40:50 | | 31 | 45 | 29 | 32 | 32 | 14 | 18 | 3 | 21 | 41 |
| 27.11.11 | 16:50:50 | | 6 | 33 | 32 | 32 | 34 | 31 | 9 | 0 | 43 | 26 |
| 27.11.11 | 17:00:50 | | 4 | 37 | 44 | 38 | 31 | 22 | 3 | 0 | 12 | 23 |
| 27.11.11 | 17:10:50 | | 31 | 36 | 37 | 32 | 40 | 32 | 2 | 0 | 3 | 18 |
| 27.11.11 | 17:20:50 | | 4 | 38 | 34 | 36 | 31 | 26 | 20 | 7 | 20 | 0 |
| 27.11.11 | 17:30:50 | | 33 | 34 | 38 | 39 | 35 | 25 | 0 | 0 | 7 | 29 |
| 27.11.11 | 17:40:50 | | 31 | 36 | 37 | 44 | 34 | 12 | 2 | 17 | 3 | 5 |
| 27.11.11 | 17:50:50 | | 30 | 40 | 34 | 38 | 33 | 35 | 23 | 4 | 21 | 20 |
| 27.11.11 | 18:00:50 | | 26 | 38 | 47 | 39 | 36 | 30 | 9 | 0 | 26 | 10 |
| 27.11.11 | 18:10:50 | | 15 | 40 | 27 | 9 | 36 | 26 | 7 | 0 | 6 | 11 |
| 27.11.11 | 18:20:50 | | 22 | 33 | 35 | 38 | 33 | 0 | 18 | 0 | 18 | 35 |
| 27.11.11 | 18:30:50 | | 32 | 33 | 33 | 39 | 32 | 18 | 9 | 11 | 6 | 7 |
| 27.11.11 | 18:40:50 | | 35 | 32 | 37 | 38 | 42 | 30 | 13 | 0 | 0 | 31 |
| 27.11.11 | 18:50:50 | | 39 | 35 | 33 | 31 | 33 | 11 | 0 | 0 | 27 | 7 |

| | | | | | REF | | | | | 10% EtOH | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Channel | 1 | 3 | 9 | 10 | 11 | 4 | 5 | 6 | 7 | 8 |
| 29.11.11 | 10:56:25 | | 23 | 31 | 37 | 22 | 28 | 17 | 16 | 23 | 21 | 19 |
| 29.11.11 | 11:06:25 | | 25 | 38 | 29 | 34 | 31 | 18 | 19 | 34 | 39 | 38 |
| 29.11.11 | 11:16:26 | | 34 | 34 | 44 | 27 | 35 | 29 | 24 | 34 | 30 | 47 |
| 29.11.11 | 11:26:26 | | 20 | 30 | 33 | 39 | 31 | 16 | 28 | 33 | 24 | 34 |
| 29.11.11 | 11:36:26 | | 42 | 30 | 33 | 33 | 34 | 17 | 28 | 33 | 32 | 24 |
| 29.11.11 | 11:46:26 | | 42 | 28 | 34 | 28 | 37 | 14 | 36 | 33 | 29 | 38 |
| 29.11.11 | 11:56:26 | | 42 | 38 | 32 | 27 | 34 | 16 | 33 | 35 | 33 | 30 |
| 29.11.11 | 12:06:26 | | 49 | 34 | 25 | 28 | 38 | 14 | 5 | 23 | 32 | 28 |
| 29.11.11 | 12:16:27 | | 45 | 34 | 38 | 27 | 39 | 41 | 17 | 30 | 29 | 15 |
| 29.11.11 | 12:26:27 | | 38 | 32 | 29 | 21 | 33 | 15 | 9 | 19 | 17 | 0 |
| 29.11.11 | 12:36:27 | | 34 | 34 | 27 | 15 | 36 | 11 | 25 | 28 | 28 | 5 |
| 29.11.11 | 12:46:27 | | 34 | 33 | 19 | 23 | 32 | 17 | 0 | 30 | 8 | 37 |
| 29.11.11 | 12:56:27 | | 32 | 35 | 19 | 26 | 38 | 13 | 35 | 32 | 7 | 23 |
| 29.11.11 | 13:06:27 | | 30 | 33 | 28 | 4 | 37 | 24 | 9 | 25 | 38 | 19 |
| 29.11.11 | 13:16:27 | | 33 | 7 | 32 | 21 | 38 | 30 | 9 | 33 | 22 | 5 |
| 29.11.11 | 13:26:27 | | 37 | 39 | 36 | 17 | 31 | 23 | 19 | 21 | 17 | 2 |
| 29.11.11 | 13:36:28 | | 34 | 39 | 3 | 14 | 23 | 26 | 28 | 25 | 4 | 0 |
| 29.11.11 | 13:46:28 | | 41 | 28 | 30 | 0 | 6 | 13 | 22 | 7 | 28 | 5 |
| 29.11.11 | 13:56:28 | | 32 | 38 | 28 | 0 | 35 | 33 | 0 | 6 | 26 | 0 |
| 29.11.11 | 14:06:28 | | 28 | 36 | 30 | 0 | 30 | 28 | 31 | 31 | 6 | 0 |
| 29.11.11 | 14:16:28 | | 30 | 20 | 34 | 6 | 19 | 22 | 18 | 13 | 22 | 16 |
| 29.11.11 | 14:26:28 | | 32 | 34 | 21 | 29 | 12 | 21 | 24 | 27 | 30 | 2 |
| 29.11.11 | 14:36:28 | | 28 | 35 | 10 | 2 | 37 | 31 | 19 | 17 | 10 | 6 |
| 29.11.11 | 14:46:28 | | 41 | 35 | 22 | 0 | 19 | 43 | 18 | 16 | 15 | 3 |

*L. variegatus*

| Limbriculus | | | | | REF | | | | | Cu | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Channel | 1 | 3 | 9 | 10 | 11 | 4 | 5 | 6 | 7 | 8 |
| date | time | | | | | | | | | | | |
| 24.11.11 | 10:40:10 | | 48 | 38 | 48 | 40 | 0 | 42 | 41 | 43 | *43* | 39 |
| 24.11.11 | 10:50:10 | | 44 | 39 | 49 | 50 | 0 | 41 | 39 | 45 | *52* | 44 |
| 24.11.11 | 11:00:10 | | 44 | 39 | 45 | 41 | 6 | 42 | 40 | 42 | *0* | 43 |
| 24.11.11 | 11:10:10 | | 43 | 51 | 42 | 38 | 8 | 49 | 40 | 42 | *0* | 52 |
| 24.11.11 | 11:20:10 | | 45 | 41 | 44 | 38 | 0 | 42 | 43 | 44 | *0* | 40 |
| 24.11.11 | 11:30:10 | | 41 | 40 | 46 | 44 | 0 | 40 | 39 | 43 | *0* | 39 |
| 24.11.11 | 11:40:10 | | 48 | 37 | 44 | 42 | 0 | 45 | 52 | 40 | *0* | 33 |
| 24.11.11 | 11:50:10 | | 47 | 45 | 44 | 39 | 0 | 49 | 49 | 41 | *0* | 45 |
| 24.11.11 | 12:00:10 | | 53 | 38 | 47 | 40 | 0 | 40 | 45 | 40 | *6* | 45 |
| 24.11.11 | 12:10:10 | | 43 | 38 | 39 | 41 | 0 | 42 | 40 | 47 | *13* | 48 |
| 24.11.11 | 12:20:10 | | 50 | 40 | 39 | 33 | 0 | 38 | 41 | 39 | *19* | 45 |
| 24.11.11 | 12:30:10 | | 47 | 46 | 44 | 32 | 0 | 40 | 45 | 47 | *3* | 44 |
| 24.11.11 | 12:40:10 | | 49 | 40 | 36 | 31 | 0 | 46 | 22 | 45 | *32* | 35 |
| 24.11.11 | 12:50:10 | | 40 | 46 | 43 | 22 | 0 | 40 | 13 | 43 | *36* | 37 |
| 24.11.11 | 13:00:10 | | 45 | 37 | 40 | 42 | 0 | 52 | 28 | 35 | *13* | 42 |
| 24.11.11 | 13:10:10 | | 39 | 46 | 15 | 38 | 0 | 49 | 25 | 34 | *2* | 40 |
| 24.11.11 | 13:20:10 | | 41 | 36 | 25 | 35 | 0 | 49 | 44 | 40 | *21* | 42 |
| 24.11.11 | 13:30:10 | | 41 | 49 | 42 | 32 | 0 | 45 | 41 | 39 | *19* | 36 |
| 24.11.11 | 13:40:10 | | 36 | 33 | 7 | 42 | 0 | 40 | 38 | 35 | *11* | 48 |
| 24.11.11 | 13:50:10 | | 26 | 35 | 36 | 41 | 0 | 40 | 34 | 37 | *12* | 47 |
| 24.11.11 | 14:00:10 | | 37 | 41 | 42 | 40 | 0 | 42 | 40 | 47 | *13* | 45 |
| 24.11.11 | 14:10:10 | | 39 | 41 | 44 | 41 | 0 | 40 | 34 | 41 | *9* | 39 |
| 24.11.11 | 14:20:10 | | 27 | 50 | 47 | 42 | 0 | 47 | 32 | 37 | *6* | 45 |
| 24.11.11 | 14:30:10 | | 31 | 36 | 39 | 40 | 0 | 39 | 28 | 35 | *24* | 48 |

| | | | | | REF | | | | | TBT | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Channel | 1 | 3 | 9 | 10 | *11* | 4 | 5 | 6 | 7 | 8 |
| date | time | | | | | | | | | | | |
| 26.11.11 | 15:00:13 | | 37 | 48 | 45 | 47 | *0* | 42 | 39 | 35 | 40 | 45 |
| 26.11.11 | 15:10:13 | | 39 | 46 | 43 | 39 | *0* | 50 | 40 | 40 | 45 | 44 |
| 26.11.11 | 15:20:13 | | 42 | 35 | 38 | 49 | *0* | 47 | 41 | 45 | 45 | 47 |
| 26.11.11 | 15:30:13 | | 37 | 40 | 36 | 47 | *0* | 42 | 45 | 44 | 37 | 42 |
| 26.11.11 | 15:40:13 | | 47 | 37 | 37 | 40 | *0* | 40 | 46 | 40 | 41 | 42 |
| 26.11.11 | 15:50:13 | | 42 | 38 | 40 | 40 | *0* | 39 | 38 | 34 | 40 | 42 |
| 26.11.11 | 16:00:13 | | 42 | 39 | 37 | 46 | *0* | 40 | 37 | 38 | 42 | 39 |
| 26.11.11 | 16:10:13 | | 50 | 37 | 43 | 38 | *0* | 50 | 42 | 43 | 41 | 38 |
| 26.11.11 | 16:20:13 | | 45 | 37 | 35 | 36 | *0* | 39 | 40 | 50 | 37 | 42 |
| 26.11.11 | 16:30:13 | | 50 | 38 | 37 | 40 | *0* | 37 | 36 | 51 | 41 | 41 |
| 26.11.11 | 16:40:13 | | 44 | 37 | 44 | 41 | *0* | 51 | 38 | 38 | 40 | 43 |
| 26.11.11 | 16:50:13 | | 50 | 48 | 35 | 45 | *0* | 53 | 39 | 38 | 36 | 38 |
| 26.11.11 | 17:00:13 | | 46 | 36 | 42 | 45 | *0* | 41 | 40 | 38 | 43 | 37 |
| 26.11.11 | 17:10:13 | | 36 | 39 | 36 | 37 | *0* | 39 | 39 | 30 | 43 | 41 |
| 26.11.11 | 17:20:13 | | 48 | 41 | 40 | 40 | *0* | 44 | 41 | 56 | 38 | 41 |
| 26.11.11 | 17:30:13 | | 29 | 39 | 34 | 33 | *0* | 39 | 39 | 39 | 42 | 41 |
| 26.11.11 | 17:40:13 | | 40 | 40 | 46 | 37 | *0* | 39 | 43 | 40 | 37 | 28 |
| 26.11.11 | 17:50:13 | | 32 | 43 | 45 | 41 | *0* | 38 | 39 | 32 | 38 | 40 |
| 26.11.11 | 18:00:13 | | 45 | 39 | 45 | 36 | *0* | 41 | 35 | 37 | 33 | 38 |
| 26.11.11 | 18:10:13 | | 46 | 42 | 40 | 37 | *0* | 38 | 41 | 40 | 38 | 26 |
| 26.11.11 | 18:20:13 | | 34 | 42 | 40 | 45 | *0* | 13 | 30 | 41 | 38 | 40 |
| 26.11.11 | 18:30:13 | | 44 | 45 | 47 | 44 | *0* | 40 | 39 | 37 | 34 | 30 |
| 26.11.11 | 18:40:13 | | 43 | 41 | 48 | 39 | *0* | 46 | 38 | 37 | 36 | 37 |
| 26.11.11 | 18:50:13 | | 43 | 41 | 36 | 48 | *0* | 41 | 44 | 34 | 29 | 40 |

| | | | | | REF | | | | | EtOH | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Channel | 1 | *3* | 9 | 10 | 11 | 4 | 5 | 6 | 7 | 8 |
| date | time | | | | | | | | | | | |
| 28.11.11 | 16:30:07 | | 33 | *36* | 50 | 51 | 9 | 43 | 36 | 38 | 42 | 39 |
| 28.11.11 | 16:40:07 | | 40 | *40* | 45 | 44 | 0 | 49 | 38 | 40 | 38 | 49 |
| 28.11.11 | 16:50:07 | | 49 | *32* | 36 | 38 | 0 | 46 | 30 | 29 | 43 | 49 |
| 28.11.11 | 17:00:07 | | 37 | *34* | 36 | 39 | 5 | 40 | 31 | 40 | 44 | 48 |
| 28.11.11 | 17:10:07 | | 42 | *42* | 36 | 46 | 5 | 33 | 33 | 45 | 38 | 45 |
| 28.11.11 | 17:20:07 | | 38 | *35* | 36 | 38 | 0 | 37 | 21 | 43 | 40 | 45 |
| 28.11.11 | 17:30:07 | | 40 | *10* | 48 | 48 | 0 | 45 | 11 | 38 | 38 | 36 |
| 28.11.11 | 17:40:07 | | 50 | *2* | 42 | 39 | 36 | 47 | 26 | 37 | 42 | 35 |
| 28.11.11 | 17:50:07 | | 38 | *0* | 35 | 38 | 0 | 41 | 24 | 48 | 49 | 46 |
| 28.11.11 | 18:00:07 | | 47 | *0* | 49 | 39 | 0 | 35 | 41 | 33 | 36 | 41 |
| 28.11.11 | 18:10:07 | | 40 | *5* | 38 | 38 | 0 | 48 | 38 | 41 | 38 | 47 |
| 28.11.11 | 18:20:07 | | 47 | *6* | 36 | 39 | 0 | 35 | 36 | 37 | 38 | 37 |
| 28.11.11 | 18:30:07 | | 46 | *0* | 42 | 41 | 8 | 45 | 45 | 45 | 36 | 45 |
| 28.11.11 | 18:40:07 | | 50 | *0* | 44 | 48 | 0 | 48 | 39 | 40 | 47 | 37 |
| 28.11.11 | 18:50:07 | | 42 | *0* | 46 | 49 | 11 | 39 | 29 | 39 | 37 | 49 |
| 28.11.11 | 19:00:07 | | 46 | *0* | 48 | 37 | 4 | 45 | 29 | 40 | 47 | 35 |
| 28.11.11 | 19:10:07 | | 50 | *0* | 48 | 38 | 0 | 43 | 33 | 45 | 36 | 47 |
| 28.11.11 | 19:20:07 | | 48 | *6* | 37 | 48 | 30 | 36 | 44 | 37 | 44 | 49 |
| 28.11.11 | 19:30:07 | | 45 | *2* | 43 | 42 | 0 | 44 | 44 | 39 | 44 | 40 |
| 28.11.11 | 19:40:07 | | 47 | *5* | 44 | 45 | 33 | 42 | 45 | 46 | 36 | 40 |
| 28.11.11 | 19:50:07 | | 49 | *0* | 49 | 37 | 0 | 39 | 38 | 41 | 36 | 46 |
| 28.11.11 | 20:00:07 | | 42 | *0* | 41 | 49 | 0 | 38 | 38 | 44 | 48 | 38 |
| 28.11.11 | 20:10:07 | | 44 | *2* | 47 | 50 | 0 | 44 | 44 | 39 | 45 | 38 |
| 28.11.11 | 20:20:07 | | 35 | *0* | 50 | 43 | 4 | 45 | 39 | 38 | 47 | 44 |

Bin sortings

*C. tentans*

| | Reference Channles | | | | | Cu Channels | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Bin* | *1* | *3* | *9* | *10* | *11* | *4* | *5* | *6* | *7* | *8* |
| 10 | 5 | 6 | 19 | 12 | 21 | 0 | 6 | 4 | 5 | 0 |
| 20 | 5 | 6 | 2 | 0 | 3 | 1 | 4 | 0 | 3 | 2 |
| 30 | 4 | 3 | 3 | 3 | 0 | 1 | 7 | 6 | 10 | 4 |
| 40 | 9 | 7 | 0 | 6 | 0 | 17 | 6 | 13 | 4 | 16 |
| More | 1 | 2 | 0 | 3 | 0 | 5 | 1 | 1 | 2 | 2 |

| | Reference channels | | | | | TBT channels | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Bin* | *1* | *3* | *9* | *10* | *11* | *4* | *5* | *6* | *7* | *8* |
| 10 | 4 | 0 | 0 | 4 | 1 | 2 | 11 | 15 | 7 | 6 |
| 20 | 1 | 1 | 0 | 0 | 0 | 5 | 9 | 4 | 7 | 5 |
| 30 | 5 | 3 | 2 | 1 | 0 | 9 | 2 | 4 | 4 | 4 |
| 40 | 14 | 18 | 20 | 16 | 22 | 8 | 1 | 1 | 5 | 7 |
| More | 0 | 2 | 2 | 3 | 1 | 0 | 0 | 0 | 1 | 2 |

| | Refernce channels | | | | | 10% EtOH Channels | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Bin* | *1* | *3* | *9* | *10* | *11* | *4* | *5* | *6* | *7* | *8* |
| 10 | 0 | 1 | 2 | 7 | 1 | 0 | 6 | 2 | 5 | 11 |
| 20 | 1 | 1 | 2 | 3 | 3 | 12 | 7 | 4 | 3 | 4 |
| 30 | 6 | 4 | 10 | 11 | 3 | 8 | 7 | 9 | 11 | 4 |
| 40 | 10 | 18 | 9 | 3 | 17 | 2 | 4 | 9 | 5 | 4 |
| More | 7 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 1 |

*L. variegatus*

| Reference channles | | | | | | Cu channels | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Bin* | *1* | *3* | *9* | *10* | *11* | *4* | *5* | *6* | *7* | *8* |
| 10 | 0 | 0 | 1 | 0 | 24 | 0 | 0 | 0 | 11 | 0 |
| 20 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 7 | 0 |
| 30 | 2 | 0 | 1 | 1 | 0 | 0 | 4 | 0 | 2 | 0 |
| 40 | 6 | 14 | 6 | 13 | 0 | 9 | 10 | 11 | 2 | 9 |
| 50 | 15 | 9 | 15 | 10 | 0 | 14 | 8 | 13 | 1 | 14 |
| More | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 |

| Reference channels | | | | | | TBT channels | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Bin* | *1* | *3* | *9* | *10* | *11* | *4* | *5* | *6* | *7* | *8* |
| 10 | 0 | 0 | 0 | 0 | 24 | 0 | 0 | 0 | 0 | 0 |
| 20 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| 30 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 3 |
| 40 | 7 | 14 | 14 | 13 | 0 | 11 | 15 | 16 | 14 | 9 |
| 50 | 16 | 10 | 10 | 11 | 0 | 9 | 8 | 5 | 9 | 12 |
| More | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 |

| Reference channels | | | | | | 10 % EtOH channels | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Bin* | *1* | *3* | *9* | *10* | *11* | *4* | *5* | *6* | *7* | *8* |
| 10 | 0 | 18 | 0 | 0 | 20 | 0 | 0 | 0 | 0 | 0 |
| 20 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 | 0 | 1 | 0 | 6 | 1 | 0 | 0 |
| 40 | 8 | 5 | 8 | 11 | 2 | 9 | 11 | 14 | 12 | 10 |
| 50 | 16 | 1 | 16 | 12 | 0 | 15 | 6 | 9 | 12 | 14 |
| More | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |

APPENDIX B

Time-Lapse Photography Statistics

*C.tentans* Non-parametirc Wilcoxon Signed Rank Tests

Chironomid foraging and body positioning 10 % EtOH

**Notes**

| | | |
|---|---|---|
| Output Created | | 16-Jan-2012 11:25:41 |
| Comments | | |
| Input | Active Dataset | DataSet0 |
| | Filter | <none> |
| | Weight | <none> |
| | Split File | <none> |
| | N of Rows in Working Data File | 240 |
| Missing Value Handling | Definition of Missing | User-defined missing values are treated as missing. |
| | Cases Used | Statistics for each test are based on all cases with valid data for the variable(s) used in that test. |
| Syntax | | NPAR TESTS /WILCOXON=VAR00001 WITH VAR00002 (PAIRED) /STATISTICS DESCRIPTIVES QUARTILES /MISSING ANALYSIS. |
| Resources | Processor Time | 00 00:00:00.016 |
| | Elapsed Time | 00 00:00:00.018 |
| | Number of Cases Allowed[a] | 112347 |

a. Based on availability of workspace memory.

**Descriptive Statistics**

| | N | Mean | Std. Deviation | Minimum | Maximum | Percentiles | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | 25th | 50th (Median) | 75th |
| VAR00001 | 240 | 1.0000 | .00000 | 1.00 | 1.00 | 1.0000 | 1.0000 | 1.0000 |
| VAR00002 | 240 | 2.9875 | .14410 | 1.00 | 3.00 | 3.0000 | 3.0000 | 3.0000 |

## Wilcoxon Signed Ranks Test

**Ranks**

| | | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| VAR00002 - VAR00001 | Negative Ranks | 0[a] | .00 | .00 |
| | Positive Ranks | 239[b] | 120.00 | 28680.00 |
| | Ties | 1[c] | | |
| | Total | 240 | | |

a. VAR00002 < VAR00001

b. VAR00002 > VAR00001

c. VAR00002 = VAR00001

**Test Statistics[b]**

| | VAR00002 - VAR00001 |
|---|---|
| Z | -15.428[a] |
| Asymp. Sig. (2-tailed) | .000 |

a. Based on negative ranks.

b. Wilcoxon Signed Ranks Test

*L. variegatus* Non-parametirc WIlcoxon Signed Rank Tests

*Lumbriculus* swimming & body positioning: 400ug/L Cu

**Notes**

| | | |
|---|---|---|
| Output Created | | 16-Jan-2012 11:30:05 |
| Comments | | |
| Input | Active Dataset | DataSet0 |
| | Filter | <none> |
| | Weight | <none> |
| | Split File | <none> |
| | N of Rows in Working Data File | 240 |
| Missing Value Handling | Definition of Missing | User-defined missing values are treated as missing. |
| | Cases Used | Statistics for each test are based on all cases with valid data for the variable(s) used in that test. |
| Syntax | | NPAR TESTS /WILCOXON=VAR00001 WITH VAR00002 (PAIRED) /STATISTICS DESCRIPTIVES QUARTILES /MISSING ANALYSIS. |
| Resources | Processor Time | 00 00:00:00.016 |
| | Elapsed Time | 00 00:00:00.125 |
| | Number of Cases Allowed[a] | 112347 |

a. Based on availability of workspace memory.

**Descriptive Statistics**

| | N | Mean | Std. Deviation | Minimum | Maximum | Percentiles | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | 25th | 50th (Median) | 75th |
| VAR00001 | 240 | 1.0000 | .00000 | 1.00 | 1.00 | 1.0000 | 1.0000 | 1.0000 |
| VAR00002 | 240 | 1.2667 | .44314 | 1.00 | 2.00 | 1.0000 | 1.0000 | 2.0000 |

## Wilcoxon Signed Ranks Test

**Ranks**

| | | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| VAR00002 - VAR00001 | Negative Ranks | 0[a] | .00 | .00 |
| | Positive Ranks | 64[b] | 32.50 | 2080.00 |
| | Ties | 176[c] | | |
| | Total | 240 | | |

a. VAR00002 < VAR00001

b. VAR00002 > VAR00001

c. VAR00002 = VAR00001

**Test Statistics[b]**

| | VAR00002 - VAR00001 |
|---|---|
| Z | -8.000[a] |
| Asymp. Sig. (2-tailed) | .000 |

a. Based on negative ranks.

b. Wilcoxon Signed Ranks Test

*Lumbriculus* swimming and body positioning: 500ug/L Cu

**Notes**

| | | |
|---|---|---|
| Output Created | | 16-Jan-2012 11:32:44 |
| Comments | | |
| Input | Active Dataset | DataSet0 |
| | Filter | <none> |
| | Weight | <none> |
| | Split File | <none> |
| | N of Rows in Working Data File | 240 |
| Missing Value Handling | Definition of Missing | User-defined missing values are treated as missing. |
| | Cases Used | Statistics for each test are based on all cases with valid data for the variable(s) used in that test. |
| Syntax | | NPAR TESTS /WILCOXON=VAR00001 WITH VAR00002 (PAIRED) /STATISTICS DESCRIPTIVES QUARTILES /MISSING ANALYSIS. |
| Resources | Processor Time | 00 00:00:00.000 |
| | Elapsed Time | 00 00:00:00.008 |
| | Number of Cases Allowed[a] | 112347 |

**Descriptive Statistics**

| | N | Mean | Std. Deviation | Minimum | Maximum | Percentiles | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | 25th | 50th (Median) | 75th |
| VAR00001 | 240 | 1.0000 | .00000 | 1.00 | 1.00 | 1.0000 | 1.0000 | 1.0000 |
| VAR00002 | 240 | 1.4625 | .49963 | 1.00 | 2.00 | 1.0000 | 1.0000 | 2.0000 |

## Wilcoxon Signed Ranks Test

**Ranks**

| | | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| VAR00002 - VAR00001 | Negative Ranks | 0[a] | .00 | .00 |
| | Positive Ranks | 111[b] | 56.00 | 6216.00 |
| | Ties | 129[c] | | |
| | Total | 240 | | |

**Ranks**

| | | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| VAR00002 - VAR00001 | Negative Ranks | 0[a] | .00 | .00 |
| | Positive Ranks | 111[b] | 56.00 | 6216.00 |
| | Ties | 129[c] | | |
| | Total | 240 | | |

a. VAR00002 < VAR00001

b. VAR00002 > VAR00001

c. VAR00002 = VAR00001

**Test Statistics[b]**

| | VAR00002 - VAR00001 |
|---|---|
| Z | -10.536[a] |
| Asymp. Sig. (2-tailed) | .000 |

a. Based on negative ranks.

b. Wilcoxon Signed Ranks Test

*Lumbriculus* swimming and body positioning: 5000ug/L Cu

**Notes**

| | | |
|---|---|---|
| Output Created | | 16-Jan-2012 11:39:47 |
| Comments | | |
| Input | Active Dataset | DataSet0 |
| | Filter | <none> |
| | Weight | <none> |
| | Split File | <none> |
| | N of Rows in Working Data File | 240 |
| Missing Value Handling | Definition of Missing | User-defined missing values are treated as missing. |
| | Cases Used | Statistics for each test are based on all cases with valid data for the variable(s) used in that test. |
| Syntax | | NPAR TESTS /WILCOXON=VAR00001 WITH VAR00002 (PAIRED) /STATISTICS DESCRIPTIVES QUARTILES /MISSING ANALYSIS. |
| Resources | Processor Time | 00 00:00:00.000 |
| | Elapsed Time | 00 00:00:00.000 |
| | Number of Cases Allowed[a] | 112347 |

**Descriptive Statistics**

| | N | Mean | Std. Deviation | Minimum | Maximum | Percentiles | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | 25th | 50th (Median) | 75th |
| VAR00001 | 240 | 1.0000 | .00000 | 1.00 | 1.00 | 1.0000 | 1.0000 | 1.0000 |
| VAR00002 | 240 | 2.4625 | .85732 | 1.00 | 3.00 | 2.0000 | 3.0000 | 3.0000 |

## Wilcoxon Signed Ranks Test

**Ranks**

| | | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| VAR00002 - VAR00001 | Negative Ranks | 0[a] | .00 | .00 |
| | Positive Ranks | 182[b] | 91.50 | 16653.00 |
| | Ties | 58[c] | | |
| | Total | 240 | | |

**Ranks**

| | | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| VAR00002 - VAR00001 | Negative Ranks | 0[a] | .00 | .00 |
| | Positive Ranks | 182[b] | 91.50 | 16653.00 |
| | Ties | 58[c] | | |
| | Total | 240 | | |

a. VAR00002 < VAR00001

b. VAR00002 > VAR00001

c. VAR00002 = VAR00001

**Test Statistics[b]**

| | VAR00002 - VAR00001 |
|---|---|
| Z | -13.069[a] |
| Asymp. Sig. (2-tailed) | .000 |

a. Based on negative ranks.

b. Wilcoxon Signed Ranks Test

*Lumbriculus* swimming and body positioning: 1% EtOH

**Notes**

| | | |
|---|---|---|
| Output Created | | 16-Jan-2012 11:41:03 |
| Comments | | |
| Input | Active Dataset | DataSet0 |
| | Filter | <none> |
| | Weight | <none> |
| | Split File | <none> |
| | N of Rows in Working Data File | 240 |
| Missing Value Handling | Definition of Missing | User-defined missing values are treated as missing. |
| | Cases Used | Statistics for each test are based on all cases with valid data for the variable(s) used in that test. |
| Syntax | | NPAR TESTS /WILCOXON=VAR00001 WITH VAR00002 (PAIRED) /STATISTICS DESCRIPTIVES QUARTILES /MISSING ANALYSIS. |
| Resources | Processor Time | 00 00:00:00.000 |
| | Elapsed Time | 00 00:00:00.000 |
| | Number of Cases Allowed[a] | 112347 |

**Descriptive Statistics**

| | N | Mean | Std. Deviation | Minimum | Maximum | Percentiles | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | 25th | 50th (Median) | 75th |
| VAR00001 | 240 | 1.0000 | .00000 | 1.00 | 1.00 | 1.0000 | 1.0000 | 1.0000 |
| VAR00002 | 240 | 1.0458 | .20956 | 1.00 | 2.00 | 1.0000 | 1.0000 | 1.0000 |

## Wilcoxon Signed Ranks Test

**Ranks**

| | | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| VAR00002 - VAR00001 | Negative Ranks | 0[a] | .00 | .00 |
| | Positive Ranks | 11[b] | 6.00 | 66.00 |
| | Ties | 229[c] | | |
| | Total | 240 | | |

**Ranks**

| | | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| VAR00002 - VAR00001 | Negative Ranks | 0[a] | .00 | .00 |
| | Positive Ranks | 11[b] | 6.00 | 66.00 |
| | Ties | 229[c] | | |
| | Total | 240 | | |

a. VAR00002 < VAR00001

b. VAR00002 > VAR00001

c. VAR00002 = VAR00001

**Test Statistics[b]**

| | VAR00002 - VAR00001 |
|---|---|
| Z | -3.317[a] |
| Asymp. Sig. (2-tailed) | .001 |

a. Based on negative ranks.

b. Wilcoxon Signed Ranks Test

*Lumbriculus* swimming and body positioning: 10% EtOH

**Notes**

| | | |
|---|---|---|
| Output Created | | 16-Jan-2012 11:41:42 |
| Comments | | |
| Input | Active Dataset | DataSet0 |
| | Filter | <none> |
| | Weight | <none> |
| | Split File | <none> |
| | N of Rows in Working Data File | 240 |
| Missing Value Handling | Definition of Missing | User-defined missing values are treated as missing. |
| | Cases Used | Statistics for each test are based on all cases with valid data for the variable(s) used in that test. |
| Syntax | | NPAR TESTS /WILCOXON=VAR00001 WITH VAR00002 (PAIRED) /STATISTICS DESCRIPTIVES QUARTILES /MISSING ANALYSIS. |
| Resources | Processor Time | 00 00:00:00.000 |
| | Elapsed Time | 00 00:00:00.032 |
| | Number of Cases Allowed[a] | 112347 |

**Descriptive Statistics**

| | N | Mean | Std. Deviation | Minimum | Maximum | Percentiles | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | 25th | 50th (Median) | 75th |
| VAR00001 | 240 | 1.0000 | .00000 | 1.00 | 1.00 | 1.0000 | 1.0000 | 1.0000 |
| VAR00002 | 240 | 1.9667 | .17988 | 1.00 | 2.00 | 2.0000 | 2.0000 | 2.0000 |

## Wilcoxon Signed Ranks Test

**Ranks**

| | | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| VAR00002 - VAR00001 | Negative Ranks | 0[a] | .00 | .00 |
| | Positive Ranks | 232[b] | 116.50 | 27028.00 |
| | Ties | 8[c] | | |
| | Total | 240 | | |

**Ranks**

| | | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| VAR00002 - VAR00001 | Negative Ranks | 0[a] | .00 | .00 |
| | Positive Ranks | 232[b] | 116.50 | 27028.00 |
| | Ties | 8[c] | | |
| | Total | 240 | | |

a. VAR00002 < VAR00001

b. VAR00002 > VAR00001

c. VAR00002 = VAR00001

**Test Statistics[b]**

| | VAR00002 - VAR00001 |
|---|---|
| Z | -15.232[a] |
| Asymp. Sig. (2-tailed) | .000 |

a. Based on negative ranks.

b. Wilcoxon Signed Ranks Test

*C. tentans* and *Lumbriculus* time-lapse photography
All replicates that had identical behaviour to reference

**Notes**

| | | |
|---|---|---|
| Output Created | | 16-Jan-2012 11:43:47 |
| Comments | | |
| Input | Active Dataset | DataSet0 |
| | Filter | <none> |
| | Weight | <none> |
| | Split File | <none> |
| | N of Rows in Working Data File | 240 |
| Missing Value Handling | Definition of Missing | User-defined missing values are treated as missing. |
| | Cases Used | Statistics for each test are based on all cases with valid data for the variable(s) used in that test. |
| Syntax | | NPAR TESTS /WILCOXON=VAR00001 WITH VAR00002 (PAIRED) /STATISTICS DESCRIPTIVES QUARTILES /MISSING ANALYSIS. |
| Resources | Processor Time | 00 00:00:00.000 |
| | Elapsed Time | 00 00:00:00.000 |
| | Number of Cases Allowed[a] | 112347 |

**Descriptive Statistics**

| | N | Mean | Std. Deviation | Minimum | Maximum | Percentiles | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | 25th | 50th (Median) | 75th |
| VAR00001 | 240 | 1.0000 | .00000 | 1.00 | 1.00 | 1.0000 | 1.0000 | 1.0000 |
| VAR00002 | 240 | 1.0000 | .00000 | 1.00 | 1.00 | 1.0000 | 1.0000 | 1.0000 |

## Wilcoxon Signed Ranks Test

**Ranks**

| | | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| VAR00002 - VAR00001 | Negative Ranks | 0[a] | .00 | .00 |
| | Positive Ranks | 0[b] | .00 | .00 |
| | Ties | 240[c] | | |
| | Total | 240 | | |

**Ranks**

| | | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| VAR00002 - VAR00001 | Negative Ranks | 0[a] | .00 | .00 |
| | Positive Ranks | 0[b] | .00 | .00 |
| | Ties | 240[c] | | |
| | Total | 240 | | |

a. VAR00002 < VAR00001

b. VAR00002 > VAR00001

c. VAR00002 = VAR00001

**Test Statistics[b]**

| | VAR00002 - VAR00001 |
|---|---|
| Z | .000[a] |
| Asymp. Sig. (2-tailed) | 1.000 |

a. The sum of negative ranks equals the sum of positive ranks.

b. Wilcoxon Signed Ranks Test

Independent T-tests on burrow time of *C. tentans*

TBT 1.0µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 3.502 | .135 | -.815 | 4 | .461 |
| | Equal variances not assumed | | | -.815 | 2.683 | .481 |

TBT: 10ug/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 10.381 | .032 | .341 | 4 | .751 |
| | Equal variances not assumed | | | .341 | 2.112 | .764 |

TBT: 100ug/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 9.732 | .036 | -1.142 | 4 | .317 |
| | Equal variances not assumed | | | -1.142 | 2.093 | .367 |

Atrazine: 5µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 4.795 | .094 | 1.476 | 4 | .214 |
| | Equal variances not assumed | | | 1.476 | 2.641 | .248 |

Atrazine: 50µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | .949 | .385 | .078 | 4 | .941 |
| | Equal variances not assumed | | | .078 | 3.583 | .942 |

Atrazine: 500µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | .048 | .838 | -.343 | 4 | .749 |
| | Equal variances not assumed | | | -.343 | 3.973 | .749 |

Copper: 100µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 1.442 | .296 | .605 | 4 | .578 |
| | Equal variances not assumed | | | .605 | 2.875 | .590 |

Copper: 400µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 6.206 | .067 | 1.425 | 4 | .227 |
| | Equal variances not assumed | | | 1.425 | 2.003 | .290 |

Copper: 500µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | .242 | .648 | .299 | 4 | .780 |
| | Equal variances not assumed | | | .299 | 3.917 | .780 |

Copper: 5000µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 5.439 | .080 | -1.087 | 4 | .338 |
| | Equal variances not assumed | | | -1.087 | 2.319 | .377 |

Cipro: 1.0µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 8.673 | .042 | -1.600 | 4 | .185 |
| | Equal variances not assumed | | | -1.600 | 2.220 | .239 |

Cipro: 10µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | .000 | 1.000 | -2.885 | 4 | .045 |
| | Equal variances not assumed | | | -2.885 | 3.987 | .045 |

Cipro: 100µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 13.559 | .021 | -1.134 | 4 | .320 |
| | Equal variances not assumed | | | -1.134 | 2.009 | .374 |

EtOH 0.1%

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | . | . | -49.667 | 2 | .000 |
| | Equal variances not assumed | | | -49.667 | 1.000 | .013 |

EtOH 1.0%  and 10%

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | . | . | -49.667 | 2 | .000 |
| | Equal variances not assumed | | | -49.667 | 1.000 | .013 |

*Lumbriculus variegatus* clumping time

TBT: 1µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | .099 | .768 | -2.022 | 4 | .113 |
| | Equal variances not assumed | | | -2.022 | 3.885 | .115 |

TBT: 10µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 10.878 | .030 | -3.893 | 4 | .018 |
| | Equal variances not assumed | | | -3.893 | 2.064 | .057 |

TBT: 10µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 2.427 | .194 | -1.200 | 4 | .296 |
| | Equal variances not assumed | | | -1.200 | 2.302 | .339 |

Atrazine 5µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | .541 | .503 | -.954 | 4 | .394 |
| | Equal variances not assumed | | | -.954 | 3.747 | .398 |

Atrazine 50µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | .075 | .798 | -1.028 | 4 | .362 |
| | Equal variances not assumed | | | -1.028 | 3.767 | .365 |

Atrazine 500µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | .008 | .933 | .699 | 4 | .523 |
| | Equal variances not assumed | | | .699 | 3.988 | .523 |

Copper 100µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 6.779 | .060 | -3.138 | 4 | .035 |
| | Equal variances not assumed | | | -3.138 | 2.396 | .070 |

Copper 200µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 2.207 | .212 | -4.230 | 4 | .013 |
| | Equal variances not assumed | | | -4.230 | 3.183 | .021 |

Copper 300µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 8.029 | .047 | -1.681 | 4 | .168 |
| | Equal variances not assumed | | | -1.681 | 2.283 | .219 |

Copper 400µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 7.105 | .056 | -1.029 | 4 | .361 |
| | Equal variances not assumed | | | -1.029 | 2.355 | .397 |

Copper 500µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 3.818 | .122 | -.043 | 4 | .968 |
| | Equal variances not assumed | | | -.043 | 2.810 | .969 |

Copper 5000µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 10.543 | .031 | -5.206 | 4 | .006 |
| | Equal variances not assumed | | | -5.206 | 2.000 | .035 |

Cipro 1µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | .425 | .550 | -8.115 | 4 | .001 |
| | Equal variances not assumed | | | -8.115 | 3.836 | .001 |

Cipro 10µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 3.847 | .121 | -4.067 | 4 | .015 |
| | Equal variances not assumed | | | -4.067 | 2.713 | .032 |

Cipro 100µg/L

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | 11.960 | .026 | -.865 | 4 | .436 |
| | Equal variances not assumed | | | -.865 | 2.066 | .476 |

EtOH 0.1%, 1.0%, and 10%

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | |
|---|---|---|---|---|---|---|
| | | F | Sig. | t | df | Sig. (2-tailed) |
| Time | Equal variances assumed | . | . | -26.765 | 2 | .001 |
| | Equal variances not assumed | | | -26.765 | 1.000 | .024 |

APPENDIX C

MFB statistics

Discriminant Analysis

*C. tenans* TBT

**Wilks' Lambda**

| Test of Function(s) | Wilks' Lambda | Chi-square | df | Sig. |
|---|---|---|---|---|
| 1 | .060 | 15.503 | 5 | .008 |

*C. tenans* Copper

**Wilks' Lambda**

| Test of Function(s) | Wilks' Lambda | Chi-square | df | Sig. |
|---|---|---|---|---|
| 1 | .296 | 7.308 | 4 | .120 |

*C. tenans* EtOH

**Wilks' Lambda**

| Test of Function(s) | Wilks' Lambda | Chi-square | df | Sig. |
|---|---|---|---|---|
| 1 | .434 | 5.011 | 4 | .286 |

*L. variegatus* TBT

**Wilks' Lambda**

| Test of Function(s) | Wilks' Lambda | Chi-square | df | Sig. |
|---|---|---|---|---|
| 1 | .090 | 13.268 | 5 | .021 |

*L. variegatus* Copper

**Wilks' Lambda**

| Test of Function(s) | Wilks' Lambda | Chi-square | df | Sig. |
|---|---|---|---|---|
| 1 | .733 | 1.705 | 5 | .888 |

*L. variegatus* EtOH

**Wilks' Lambda**

| Test of Function(s) | Wilks' Lambda | Chi-square | df | Sig. |
|---|---|---|---|---|
| 1 | .259 | 7.426 | 5 | .191 |

Factor Analysis

*C. tenans* TBT

Extraction Method: Principal Component Analysis.

**Component Matrix[a]**

| | Component | |
|---|---|---|
| | 1 | 2 |
| VAR00002 | .812 | -.490 |
| VAR00003 | .884 | -.123 |
| VAR00004 | .521 | .796 |
| VAR00005 | -.966 | .170 |
| VAR00006 | -.736 | -.347 |

**Rotated Component Matrix[a]**

| | Component | |
|---|---|---|
| | 1 | 2 |
| VAR00002 | .948 | -.014 |
| VAR00003 | .826 | .338 |
| VAR00004 | .049 | .950 |
| VAR00005 | -.920 | -.340 |
| VAR00006 | -.461 | -.671 |

**Component Transformation Matrix**

| Component | 1 | 2 |
|---|---|---|
| 1 | .864 | .504 |
| 2 | -.504 | .864 |

## *C. tenans* Copper

Extraction Method: Principal Component Analysis.

**Component Matrix[a]**

| | Component | |
|---|---|---|
| | 1 | 2 |
| VAR00002 | -.906 | -.399 |
| VAR00003 | -.327 | .603 |
| VAR00004 | .130 | .829 |
| VAR00005 | .938 | -.056 |
| VAR00006 | .826 | -.265 |

**Rotated Component Matrix[a]**

| | Component | |
|---|---|---|
| | 1 | 2 |
| VAR00002 | -.927 | -.347 |
| VAR00003 | -.293 | .620 |
| VAR00004 | .177 | .821 |
| VAR00005 | .934 | -.109 |
| VAR00006 | .810 | -.311 |

**Component Transformation Matrix**

| Component | 1 | 2 |
|---|---|---|
| 1 | .998 | -.057 |
| 2 | .057 | .998 |

*C. tenans* EtOH

Extraction Method: Principal Component Analysis.

**Component Matrix[a]**

| | Component | |
|---|---|---|
| | 1 | 2 |
| VAR00002 | .593 | -.622 |
| VAR00003 | .590 | .443 |
| VAR00004 | .644 | .181 |
| VAR00005 | -.941 | -.238 |
| VAR00006 | -.317 | .733 |

**Rotated Component Matrix[a]**

| | Component | |
|---|---|---|
| | 1 | 2 |
| VAR00002 | .237 | -.826 |
| VAR00003 | .728 | .119 |
| VAR00004 | .655 | -.139 |
| VAR00005 | -.943 | .226 |

| | | |
|---|---|---|
| VAR00006 | .059 | .797 |

**Component Transformation Matrix**

| Component | 1 | 2 |
|---|---|---|
| 1 | .886 | -.464 |
| 2 | .464 | .886 |

*L. variegatus* TBT

Extraction Method: Principal Component Analysis.

**Component Matrix[a]**

| | Component | |
|---|---|---|
| | 1 | 2 |
| VAR00002 | -.996 | -.068 |
| VAR00003 | .066 | .844 |
| VAR00004 | .342 | -.463 |
| VAR00005 | .787 | .198 |
| VAR00006 | .770 | -.302 |
| VAR00007 | .126 | .885 |

**Rotated Component Matrix[a]**

| | Component | |
|---|---|---|
| | 1 | 2 |
| VAR00002 | -.995 | -.078 |
| VAR00003 | .058 | .845 |
| VAR00004 | .347 | -.460 |
| VAR00005 | .785 | .206 |
| VAR00006 | .773 | -.294 |
| VAR00007 | .117 | .886 |

**Component Transformation Matrix**

| Component | 1 | 2 |
|---|---|---|
| 1 | 1.000 | .010 |
| 2 | -.010 | 1.000 |

*L. variegatus* Copper

Extraction Method: Principal Component Analysis.

**Component Matrix[a]**

| | Component | |
|---|---|---|
| | 1 | 2 |
| VAR00002 | -.900 | -.377 |
| VAR00003 | -.660 | .535 |
| VAR00004 | -.173 | .775 |
| VAR00005 | .853 | .084 |
| VAR00006 | .887 | .002 |
| VAR00007 | .094 | .781 |

**Rotated Component Matrix[a]**

| | Component | |
|---|---|---|
| | 1 | 2 |
| VAR00002 | -.942 | -.255 |
| VAR00003 | -.584 | .617 |
| VAR00004 | -.069 | .791 |
| VAR00005 | .857 | -.029 |
| VAR00006 | .880 | -.115 |

| | | |
|---|---|---|
| VAR00007 | .196 | .762 |

**Component Transformation Matrix**

| Component | 1 | 2 |
|---|---|---|
| 1 | .991 | -.132 |
| 2 | .132 | .991 |

*L. variegatus* EtOH

Extraction Method: Principal Component Analysis.

**Component Matrix[a]**

| | Component | |
|---|---|---|
| | 1 | 2 |
| VAR00002 | .874 | -.459 |
| VAR00003 | .781 | .533 |
| VAR00004 | .386 | .906 |
| VAR00005 | -.747 | .530 |
| VAR00006 | -.901 | -.011 |
| VAR00007 | -.295 | -.072 |

**Rotated Component Matrix[a]**

| | Component | |
|---|---|---|
| | 1 | 2 |
| VAR00002 | -.982 | .104 |
| VAR00003 | -.353 | .877 |
| VAR00004 | .182 | .968 |

| | | |
|---|---|---|
| VAR00005 | .915 | .025 |
| VAR00006 | .743 | -.510 |
| VAR00007 | .206 | -.224 |

**Component Transformation Matrix**

| Component | 1 | 2 |
|---|---|---|
| 1 | -.831 | .556 |
| 2 | .556 | .831 |

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