# Evaluation of the Multispecies Freshwater Biomonitor to determine behavioural effects of Tributyltin and Atrazine on *Daphnia magna* and *Hyalella azteca*

By

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# Abstract

Early-warning biomonitoring systems (EWBS), when rigorously tested and assessed for scientific veracity, can provide rapid identification and continuous monitoring of changes in water quality in order to prevent consumption of contaminated water. The Multispecies Freshwater Biomonitor (MFB) is supposedly just such a system, recording the behaviour of aquatic organisms using an electric field and detecting changes in the movements of biota which may be caused by external stressors such as aquatic contaminants. In this study, the MFB was used in an extensive suite of experiments in an effort to detect behavioural changes of *Daphnia magna* and *Hyalella azteca* when exposed to tributyltin (TBT), a biocide used primarily on the hulls of ships, and atrazine, a pesticide used extensively on corn in southern Ontario. The applicability of the MFB to be used as a monitor of drinking water quality as well as the usefulness of the organisms in this automated system was determined. While responses in behaviour were seen with the human eye, it was determined that neither contaminant brought about behavioural changes in either organism that were detectable by the MFB, even at the highest tested concentrations. While extensive literature indicated that this system was indeed useful for field applications, this study concluded that the MFB is not yet ready for use in the field to detect contaminants entering a water system when using *D. magna* or *H. azteca* as test species. Future research is required for examination of other species' ability to detect aquatic contaminants and whether the MFB is able to detect such responses.

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## CONTENTS

# 1 Introduction

## 1.1 Purpose

The purpose of this study is to determine the applicability of the Multispecies Freshwater Biomonitor (MFB) in monitoring drinking water supplies using *Daphnia magna* and *Hyalella azteca* as test organisms. The method of pollutant detection is based on aquatic organisms' behavioural responses to contaminant exposure. Such biological monitoring allows for sensitive, environmentally-relevant, cost-effective and rapid detection of aquatic contaminants to occur. In this study, the ability of the MFB automated system to detect behavioural changes of the aquatic organisms when they are exposed to various pollutants identified as a concern to the Niagara and Great Lakes regions in southern Ontario was determined. The contaminants investigated in this study are tributyltin, an antifouling agent used on the hulls of domestic and international ships, and atrazine, a pesticide widely used through the agricultural landscape of the area.

This study is part of a larger NSERC project that aims to develop a more holistic, real-time multi-organism early-warning biomonitoring technology that aims to be fully implemented in a water treatment facility in the Welland Canal within the next 5 years. This technology will build upon current early-warning biomonitoring systems to provide a new and more effective way to detect pollutants in the water systems and prevent consequent consumption of contaminated water.

With the growing population, increased levels of human, agricultural and industrial wastes are being created and finding their way into freshwater systems through direct and indirect means (Maal-Bared *et al.*., 2008). Methods for detecting large influxes of pollutants into drinking water supplies which pose a threat to human and ecosystem health need to be implemented in order to strengthen water treatment systems already in place. Use of proper monitoring can also identify polluters upstream in order for them to be held accountable for their actions (Mikol *et al.*., 2007).

Traditional monitoring of water systems includes spot sampling followed by chemical, and possibly microbial, analysis in a laboratory (Roig *et al.*., 2007). This method does not, however, allow for continuous monitoring of water systems and turnover times for laboratory results are not rapid enough to prevent consumption of contaminated water. Additionally, chemical analysis does not give environmentally-

relevant results. If a contaminant's presence is identified in the water at a given concentration, chemical analysis cannot determine whether said concentration will have an effect and, if so, the extent. Drinking water sources must be secured with appropriate monitors and alarms in place, followed by proper treatment and back-up systems, so that contamination can be detected and dealt with efficiently (McQuiggie, 2002).

Early-warning biomonitoring systems (EWBS) detect contaminants in freshwater based on monitoring behavioural responses of organisms. Any changes in behaviour relative to a reference lets operators know a contaminant is present. Several systems have been developed in Europe with the aim to have eventual widespread implementation in water treatment plants throughout the world. However, although there is much potential for use of these systems, limitations have been noted and their application to date has been limited.

The system under development will be used as a "miner's canary" to rapidly alert operators of water treatment plants of stressors in incoming water. Chemical testing is not sufficient for such applications and monitoring of water quality through organism behavioural reactions will offer a continuous and environmentally-relevant detection of pollutants. Application of this system could be done with subsequent follow-up to identify culprits with chemical analysis, to offer a first-line of rapid detection followed by specific identification of contaminants present. As stated, this study assesses the applicability of the MFB automated system as a component of the 'miner's canary' early warning system. Additional biomonitors are being evaluated by other members of the team conjunctly for the purpose of multiple systems being applied in the final EWBS to be used in the field. This will allow for the reactions of multiple species to be monitored simultaneously, offering a more holistic assessment of water quality given the relative differences in sensitivities of different organisms to different stressors.

The initial implementation location of the developed EWBS is to be in the DeCew Falls Water Treatment Plant located in the Niagara Region of southern Ontario. This water treatment plant is the largest in the Regional Municipality of Niagara serving St. Catherines, Thorold, Niagara-on-the-Lake, and Jordan with up to 227 million litres of water per day (DeCew Falls WTP Annual Summary, 2008). The DeCew Falls plant takes its drinking water from Lake Erie via the Welland Canal which is a high-density shipping

area with many domestic and international vessels passing through it everyday during the open water season. Tributyltin (TBT) is a highly toxic biocide that is added to antifouling paints used on the hulls of many domestic and internationally-registered ships. Although TBT use is banned in Canada for vessels less than 25 metres and restricted in many other countries, numerous countries still allow its employment on large vessels. Because of the shipping density, the Welland Canal is at risk for increased levels of TBT to be present which would therefore enter the DeCew Falls plant. The second contaminant of concern analysed in this study is atrazine. Atrazine is an herbicide that is widely and heavily applied to agricultural lands such as those surrounding the Welland Canal. Atrazine enters the water system through runoff from the fields, most notably in the spring after pesticide application. TBT and atrazine can readily enter Welland Canal water and pass on to the DeCew Falls water treatment plant. The analysis of the effect which these pollutants have on two aquatic organisms, *D. magna* and *H. azteca*, will be presented in this study.

Implementation and use of a EWBS in the treatment plant would allow for the presence of these contaminants, among many others, to be identified in order for more intensive and better targeted treatment methods to be applied. The primary objective of the system's development is for application in water treatment plants; however, this system could also be used to monitor effluent from facilities into larger water systems to ensure good quality. Additionally, EWBS offer a more extensive monitoring for a significantly lower cost than traditional methods. This would allow for application of the system in marginalized communities throughout the world including First Nation Reserves across Canada.

## 1.2 Test Organisms: The Use of *Daphnia magna* and *Hyalella azteca*

### *1.2.1 Daphnia magna*

*Daphnia magna* (Figure 1.1) are relatively small (0.5 – 5 mm in length) crustaceans that are common in freshwater systems such as lakes, rivers, ponds and other surface waters throughout Canada and the United States, including the Great Lakes ecosystem (Dodson & Hanazato, 1995; Ryan & Dodson, 1998). *D. magna* are an important addition to the aquatic food web as they act as the link between primary producers, such as phytoplankton, and secondary consumers such as fish (Dodson & Hanazato, 1995; Dodson *et al.*., 1995; Fischer *et al.*., 2006). They are widely used in ecotoxicological testing for a variety of reasons including the ease by which they are cultured in the lab, their rapid response to a range of contaminants and the array of behavioural characteristics they elicit when exposed to contaminants (Giesy & Hoke, 1989; Ren *et al.*, 2009). Low culturing cost and short life cycles, which allow for rapid reproduction and maturation, have resulted in widespread studies on *D. magna* behavioural and physiological responses of both short and long-term duration (e.g. Barber *et al.*, 1990; Goodrich & Lech, 1990; Arner & Koivisto, 1993; Gerhardt & Svensson, 1994; Schmidt *et al.*, 2006; Barber *et al.*, 1990; Ren *et al.*, 2007 Watson *et al.*, 2007).

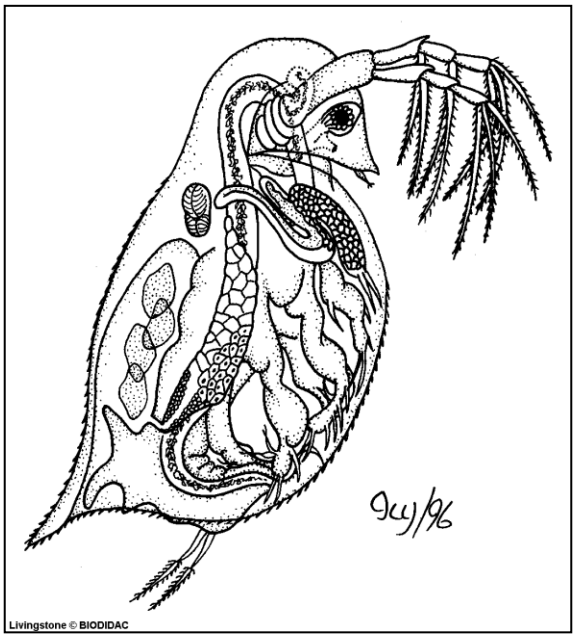



**Figure 1.1:** *Daphnia magna* (BIODIDAC, 1996)

*D. magna* are highly sensitive to a large range of chemicals at low concentrations that can be found contaminating aquatic systems and are therefore used as model organisms for predicting impacts of such contaminants on the environment (Kieu *et al.*, 2001; Kiss *et al.*, 2003; Schmidt *et al.*, 2005; Ren *et al.*, 2009). *D. magna* have two sets of antennae with the smaller set located along the front used for breathing and acquiring food, and the larger secondary set located on the head used for propulsion (Untersteiner *et al.*, 2003). The body of *D. magna* is covered by a clear carapace which allows for

observation and monitoring of the inner organism, such as the presence of neonates and heart rate. It has been suggested that *D. magna* are highly sensitive to contaminant exposure as antennae and the overall body's surface area are continuously exposed to any contaminants which are dissolved or suspended in the aquatic environment (Green *et al.*, 2003). Because of this, *D. magna* are likely to show behavioural and physiological changes when exposed to extremely low concentrations of a pollutant, adding to their usefulness as a test organism (Green *et al.*, 2003).

#### *1.2.1.1 Behaviour and Behavioural Bioassays*

*Daphnia magna* are permanently swimming organisms which move constantly throughout the water column in search of food, phytoplankton and algae (Dodson *et al.*, 1995; Fischer *et al.*, 2006). Changes in their movement patterns caused by contaminant exposure or other stressors can have detrimental impact on their survival (Schmidt *et al.*, 2005). Normal swimming patterns of *D. magna* include strong, smooth strokes with their secondary antennae propelling them in a straight direction. Their style of swimming is a distinct saltatory or jumping style, earning them the name "water fleas" (Dodson & Hanazato, 1995). It is through this movement that *D. magna* are able to find food sources which are located throughout the water column as well as to group with other daphnids for safety purposes (Ryan & Dodson, 1998, Christensen *et al.*, 2005). It is also important for the organisms to have the ability to swim in controlled, straight lines for efficient avoidance of predators (Ryan & Dodson, 1998). Predator escape responses of *D. magna* are quick jerky swimming from side to side and may include some spinning behaviour (Dodson *et al.*, 1995). Changes in swimming behaviour also occur when the organisms are exposed to stressors such as an aquatic pollutant. Observing swimming behavioural changes offers a useful endpoint in ecotoxicological research (Baillieul & Scheunders, 1998; Ren *et al.*, 2008; Marshall, 2009). Specific behaviours and behavioural changes of *D. magna* have been classified and quantified in accordance with sensitivity and usefulness in ecotoxicological bioassays. These behaviours include ability to 1) swim through the water column (swimming height), 2) swimming style, 3) immobilization, 4) secondary antennae use, and 5) spinning movements (Marshall, 2009).

As *D. magna* are organisms which constantly swim throughout the water column, searching for food sources and avoiding predators, one of the most responsive indicators

of stress has been identified as the inability for individual organisms to do so (Ryan & Dodson, 1998; Green *et al.*, 2003; Marshall, 2009). If the organisms are no longer able to move in a controlled manner through the water column, they become susceptible to predators and are no longer able to obtain sufficient nutrients. The ability to swim up and down through the water column is closely related to diurnal patterns of the organisms. During the day, under more intense light conditions, *D. magna* avoid the surface of the water and gather near the bottom of the water-body (Cushing, 1951; Martins *et al.*, 2007). This is associated with avoidance of predators such as fish (Cushing, 1951). In the evening and through the night, *D. magna* swim towards the surface of the water column to graze on phytoplankton under the cover of lower light levels (Cushing, 1951; Ryan & Dodson, 1998).

Controlled movement throughout the water column has been identified as one of the most sensitive behavioural traits to be monitored when using *D. magna* as a test species (Marshall, 2009). Movement through the water column is the first behavioural trait that was affected when *D. magna* were exposed to several aquatic contaminants, including TBT and atrazine (Marshall, 2009). The inability of *D. magna* to move upwards and downwards through the water has been shown to occur within hours of contaminant exposure at very low concentrations (Kieu *et al.*, 2001; Michels *et al.*, 2001; Martins *et al.*, 2007; Marshall, 2009).

Specific swimming style has also been identified in *D. magna* as a highly-sensitive behavioural indicator of organism stress (Marshall, 2009). Altered swimming style can arise from either direct physical impairment caused by toxicant exposure, or from avoidance attempts made by the organism (Green *et al.*, 2003). Exposure to low concentrations of certain aquatic contaminants have been shown to cause rapid alteration of swimming style in *D. magna* within hours of exposure, and are therefore deemed a good indicator of water quality and organism stress (Marshall, 2009).

As previously stated, normal swimming patterns include strong, controlled thrusts from the secondary antennae which results in the distinct saltatory moving style of *D. magna* (Dodson & Hanazato, 1995). When *D. magna* are under stressful conditions, their swimming style can change to the use of jerky, short strokes and they may use the bottom of the test vessel for propulsion upwards (Marshall, 2009). Swimming style is highly

important to *D. magna* as it influences both their ability to gather food and to avoid predation (Ryan & Dodson, 1998, Ren *et al.*, 2007). It has been shown that jerky movements can attract the attention of certain predators, such as the bluegill sunfish which consumes larger quantities of *D. magna* showing erratic swimming style (Ryan & Dodson, 1998).

Immobilization can occur in *D. magna* for several reasons. If exposure to a toxicant affects organism metabolism and other internal functions, energy usually used for swimming can be diverted to maintenance of organism wellbeing, resulting in decreased locomotive activity and eventual immobility (Untersteiner *et al.*, 2003; Schmidt *et al.*, 2005). A toxicant may also directly affect the coordination and muscle activity of *D. magna* required for movement resulting in immobility (Untersteiner *et al.*, 2003; Schmidt *et al.*, 2005). Immobilization is associated with complete exhaustion of an organism, when adaptation to or avoidance of a contaminant is no longer possible (Ren *et al.*, 2008). Immobilization is a good indicator of organism stress as it has been shown to occur rapidly when exposed to low concentrations of various aquatic contaminants (Marshall, 2009).

Other specific behavioural parameters have been identified as indicators of organism stress, and consequently water quality, including secondary antennae usage, spinning movements, and body orientation (Marshall, 2009). Although these changes are able to determine when *D. magna* are under stress, they have been shown not to be as sensitive or rapid as indicators compared with ability to move through the water column, swimming style and immobilization (Marshall, 2009). These latter three responses were thus used extensively in the current study.

Application of *D. magna* in biomonitoring systems is extensive and the variety of behavioural responses they elicit make them ideal organisms for application in behavioural analysis (Dodson *et al.*, 1995; Bailleul & Scheunders, 1998; Lechelt *et al.*, 2000; Green *et al.*, 2003; Gerhardt *et al.*, 2006a; Gerhardt *et al.*, 2006b; Martins *et al.*, 2007; Ren *et al.*, 2007; Watson *et al.*, 2007; Ren *et al.*, 2008). Several of these studies are detailed below, in context with the biomonitoring systems used in each.

#### *1.2.1.2 Rationale for Use of Daphnia magna in the miner's canary*

*Daphnia magna* are of ecological significance to Canadian waters, including the Niagara and Welland Canal regions, and they have been shown to elicit rapid responses to contaminant exposure. The ability of *D. magna* to respond to the presence of pollutants with a variety of traits makes it a good test organism for use in biomonitoring procedures. They have specifically been shown to elicit distinct and rapid responses to contaminants identified as concern to the Welland Canal region, including tributyltin and atrazine (Marshall, 2009), and therefore their applicability as a test species in the miner's canary early-warning biomonitoring system under development is to be assessed. Their behavioural responses have been classified, recently and thoroughly, through visual analysis, and some work has been done with automated technology (to be described in following sections). An extensive literature review has revealed their relevance to the ecological stability of the Welland Canal ecosystem. These factors make *D. magna* an ideal candidate for the addition to the miner's canary system.

### *1.2.2 Hyalella azteca*

*Hyalella azteca* (Figure 1.2) is a freshwater benthic amphipod ubiquitous to North and South America, and present throughout the Great Lakes region (Blockwell *et al.*, 1998; Wang *et al.*, 2004). In their natural habitat, *H. azteca* are omnivorous detrivores that feed on algae, leaf litter, small isopods, bacteria and aquatic plants among other detritus, greatly influencing the recycling of nutrients in the water ecosystem (Blockwell *et al.*, 1998; Wang *et al.*, 2004). *H. azteca* can grow up to 5mm in length and a variety of ages and sizes have been used in toxicity testing (Collyard *et al.*, 1994). Relatively simple culturing and quick maturation makes *H. azteca* a useful test organism (Collyard *et al.*, 1994; Borgmann *et al.*, 1996; Wang *et al.*, 2004).

**Figure 1.2:** *Hyalella azteca* image (DEC, New York)

*H. azteca* is widely used in sediment toxicology testing due to their close and regular contact with the sediment. *H. azteca* are most commonly found on solid substrates, either in the sediment or among rocks or algal mats, generally within the top 1 to 2 cm of sediment (Collyard *et al.*, 1994; Borgmann *et al.*, 1996; Hatch & Burton, 1999; Wang *et al.*, 2004).

#### *1.2.2.1 Behaviour and Behavioural Bioassays*

*H. azteca* is highly sensitive to aquatic contaminants and can respond with a variety of behavioural traits to toxicant exposure (Wang *et al.*, 2004; Marshall, 2009). It is a good biological indicator of sediment quality as it is in contact with the sediment through burrowing and feeding. Several studies have shown that the majority of contaminant exposure to *H. azteca* comes not from the sediment itself, but rather from food sources and from the water column (Suedel & Rogers, 1996; Wang *et al.*, 2004; Moore *et al.*, 2006). Indication of water quality is shown as the organism is able to swim freely though the lower portion of the water column, therefore having contact with contaminants found in the water column, and will burrow more readily in the sediment to escape from contaminants that have hydrophilic tendencies (Borgmann *et al.*, 1996; Hatch & Burton, 1999). Behavioural responses of *H. azteca* include immobilization, erratic swimming and avoidance, such as hiding in sediment and under leaves, as well as clumping of individual organisms together.

One of the most sensitive behavioural parameters to be viewed under stress conditions is immobilization of *H. azteca* (Marshall, 2009). Stress conditions also affect other behaviours which can be monitored including substrate crawling and body length (Marshall, 2009). Burrowing into the sediment has been identified as a behavioural parameter that may be assessed as it represents predator avoidance and foraging for food (Hatch & Burton, 1999; Wang *et al.*, 2004). Burrowing gives several potential factors that can be measured including changes in burrowing behaviour, the amount of time spent burrowing and the percentage of a population that is burrowing (Wang *et al.*, 2004). However, there is some question as to whether this burrowing behaviour is altered under stress conditions and whether or not the time spent on burrowing is affected by contaminant exposure (Wang *et al.*, 2004). Burrowing into the sediment has been shown not to be as a sensitive an indicator as immobility, substrate crawling or body length of

the organism (Marshall, 2009). It is these latter responses that were assessed in the current study.

*H. azteca* are considered to be immobilized if there in no movement of any body parts occurring, including not only swimming and walking movements but also burrowing, body contractions and leg movements while the organism is lying on its side (Marshall, 2009). Mobility is a highly important ability for *H. azteca*, as with *Daphnia magna*, being required for foraging, predator avoidance, and mating (Untersteiner *et al.*, 2003; Schmidt *et al.*, 2005). It is also likely that *H. azteca* lose mobility completely in the case of total depletion of energy reserves. This energy loss may be caused by the organism's avoidance or attempt to acclimatize to the contaminant. Immobility may also be caused by the direct influence of the contaminant on inner functions of the organism. *H. azteca* have been shown to lose mobility completely within hours of exposure to low concentrations of aquatic contaminants. Marshall (2009) showed that after one hour of exposure to a solution of 5µg/L of atrazine, significant immobility was noted in *H. azteca*. Immobility is a good indicator of *H. azteca* stress and has been shown to be the most sensitive and reliable response elicited in the organism (Marshall, 2009).

Substrate crawling is important to *H. azteca* for foraging purposes as much of their nutrition comes from algae growing on the sediment/water interface and other detritus that has settled on this surface (Wang *et al.*, 2004). If the crawling ability of the organism is impaired for long periods of time, lack of nutrition may affect survival (Wang *et al.*, 2004). Extent of substrate crawling has been identified as a reliable indicator of organism stress and its monitoring has been suggested to have possible use in automated biomonitoring technology (Marshall, 2009).

Body length has also been identified as a sensitive and rapid response parameter for measuring *H. azteca* health and presence of a contaminant (Marshall, 2009). Under normal conditions, *Hyalella* bodies are fully extended and elongated during swimming, walking and resting activities. Shortened body lengths are observed under certain stress conditions and may be interpreted as an avoidance behaviour, in an attempt to reduce surface area exposed to a contaminant. Although this parameter has been shown to be reliable for certain contaminants, such as tributyltin, it does not always give dependable

results (Marshall, 2009). It should therefore be used in conjunction with analyses of other behavioural patterns and not in isolation.

#### *1.2.2.2 Rationale for Use of Hyalella azteca in the miner's canary*

Sediment toxicity tests with benthic dwelling species are ecologically-relevant as various routes of exposure can be integrated. Therefore, by having multiple species which live in different portions of the water system, a complete and thorough analysis of water quality can be achieved. *Hyalella azteca* have been frequently used in aquatic testing and are known to be highly sensitive to a variety of aquatic contaminants found in the Great Lakes watershed. *H. azteca* are naturally found in the Great Lakes and Niagara regions and their addition to the miner's canary may give ecologically significant results for the system implemented in the Welland Canal water treatment plant.

## 1.3 Bioassay Contaminants

### *1.3.1 Tributyltin*

Tributyltin (TBT) (Figure 1.3) is a tri-substituted organo-tin that is highly toxic to organisms at concentrations of nanograms per litre, and is very persistent in the aquatic environment (Alzieu *et al.*, 1989; Alzieu, 1998; Horry *et al.*, 2004). There are several forms of TBT including oxides, chlorides, fluorides and acetate (Alzieu, 1998). Each compound has a slightly differing solubility value, ranging from 1-10 mg/L for TBT oxide and below 20 mg/L for other species (Alzieu,1998). TBT is a hydrophobic substance with an octanol-water partitioning coefficient ranging from 3.21 to 3.85 (Alzieu, 1998).

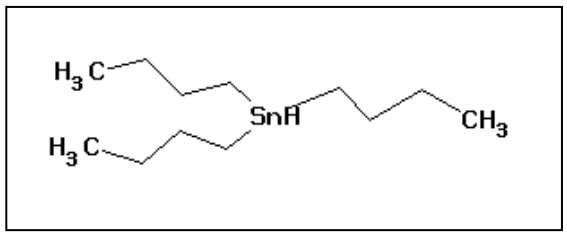



**Figure 1.3:** Tributyltin (merck-chemicals.com)

TBT enters the aquatic environment primarily as an antifouling agent used on the hulls of ships (Weis & Cole, 1989; Schmidt *et al.*, 2006). As the speed and efficiency of ships is impaired by the attachment of barnacles and other sea life to their hulls, keeping the hull surfaces clean is important. In the 1970s, paints which contained organic tin-

based compounds, primarily TBT, came into use. These paints needed only to be reapplied every 6 or 7 years, as opposed to the annual application required for previously used copper-based paints, and the use of organic tin-based paints increased dramatically (Weis & Cole, 1989). It was during the early 1980s that the toxic effects of TBT on the aquatic environment and non-target species began to be noticed. It was suggested by several studies conducted then that TBT could cause toxic harm to a variety of species at concentrations as low as 1 µg/L (Smith, 1981; Thain, 1983; Waldock & Thain, 1983). In 1984, Alzieu and Heral published a study of their observations of TBT toxicity on the oyster *Crassostrea gigas*. Near mooring areas in France, a large number of malformed oysters were found. In areas with many mooring ships, the shells of the oysters were unusually thick causing stunted growth and inability to be sold on the market (Alzieu & Heral, 1984). High levels of tin were found in the oysters suggesting that it was the organo-tins which caused the malformations. It was determined that malformations of oyster shells could occur at concentrations as low as 150 ng/L (Waldock & Thain, 1983). The decrease in the number of oyster offspring in the harbours was also linked to organo-tin exposure (Alzieu & Heral, 1984).

TBT is not only used as an additive to antifouling paint, but can also enter the aquatic ecosystem through other industrial uses. Such uses include slime control in paper mills, disinfection of circulating industrial cooling water and the preservation of wood (Antizar-Ladislao, 2008).

TBT acts as a toxin on invertebrates likely through impairment of muscle function (Alzieu, 1998). By preventing the breakdown of ATP to ADP, TBT causes the muscles to become deficient in energy and therefore unable to perform movement activities. Overall, after exposure to TBT, invertebrates experience a shutting down of many biological functions throughout the body rather than localized effects on a single organ system (Schmidt *et al.*, 2005). In addition to malformation and movement inhibition, TBT is also a potent endocrine disruptor. Anatomical malformations were first found in female snails (*Nucella lapillus*) by Gibbs and Bryan (1986). In areas with high levels of boating activity, imposex (a single organism containing non-functioning gonads of both genders) was noted and considered responsible for the population decline of the snails (Bryan *et*

*al.*, 1986). Such malformations were produced in the laboratory with very low concentrations of 20 ng/L (Bryan *et al.*, 1986).

Because of TBT toxicity, many countries, including Canada, regulated its antifouling uses during the 1980s or early 1990s (Chau *et al.*, 1997; Maguire, 2000). Bans on TBT use began in France with restrictions placed on vessels less than 25 meters in length and with mean leaching rates of more than 4µg/cm$^2$/day (Chau *et al.*, 1997; Alzieu, 1998). Other countries soon followed suit including the United Kingdom (1987), the United States (1988), Australia (1989), and the Netherlands, Hong Kong and Japan (1992) (Chau *et al.*, 1997). Reduced environmental concentrations have been noted in some areas owing to the regulations as well as the development of slow-release TBT-containing antifouling paints. However, this reduction has not been seen throughout the world as use of TBT on large vessels continues to be legal in some countries. In addition, persistence in sediment allows release from sediment to be a source for TBT in the water column (Chau *et al.*, 1997; Maguire & Batchelor, 2005).

Measurable presence of TBT in Canadian waters is largely confined to harbours, marinas and shipping channels where higher levels of boating and shipping activities can be found (Lee *et al.*, 2004; Maguire & Batchelor, 2004). Canada first regulated the use of TBT in 1989 with a prohibition of use of antifouling paints on vessels less than 25m in length. A leaching rate restriction for use on larger vessels was also implemented at that time (Agriculture Canada, 1989). In 1994, a survey of Canadian water showed that TBT concentrations had reduced slightly in freshwater, but not in marine water or in sediments (Chau *et al.*, 1997). Significant concentrations of TBT have been found in waters (Grinwis *et al.*, 1998); however, higher levels are located in sediments (Weis & Cole, 1989; Maguire & Batchelor, 2005). Concentrations of TBT up to 5.76 µg/L have been measured in Canadian freshwater, 1.5 µg/L in marine water in France, and 7.2 µg/L in harbours in the Netherlands (Grinwis *et al.*, 1998; Schmidt *et al.*, 2005). Levels as high as 61.8 µg/L have been measured in industrial effluent near Bremen, Germany (Schulte-Oehlmann *et al.*, 2006). TBT has a half-life of more than one year in water and up to 15 years in sediment (Maguire, 2000). Benthic invertebrates may be particularly susceptible to TBT toxic effects as they are constantly burrowing in and ingesting sediment, and therefore, are continuously exposed to higher concentrations than found in the water

column (Bartlett *et al.*, 2007). Water quality guidelines have been set at 9.6 ng/L of water by Environment Canada. This level was selected through determining the lowest chronic exposure effect found in the literature and applying a safety factor of 10 (Chau *et al.*, 1997). A complete ban of the presence of TBT in antifouling paints used in Canada was slated to come into effect in January 2008 (Pest Management Regulatory Agency, 2002); however, an overall assessment of TBT concentrations has yet to be conducted and toxic concentrations may still be present in areas with high international ship traffic.

Current methods of TBT detection rely on gas chromatography/mass spectroscopy or inductively-coupled plasma mass spectroscopy (Horry *et al.*, 2004). These processes give highly accurate results but are expensive and time-consuming. Behavioural effects of TBT have been shown in *D. magna* with rapid decreases in swimming activity and changes in preferred swimming depth noted at 7.1µg/L (Schmidt *et al.*, 2006). The application of automated behavioural monitoring could allow for early detection of TBT in Canadian freshwater systems at relevant concentrations.

### *1.3.2 Atrazine*

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) (Figure 1.4) is one of the most widely-applied herbicides in North America (McElroy *et al.*, *2007*). It is a chloro-N-dialkyl substituted triazine herbicide with a low water solubility of approximately 33 mg/L at 25 degrees Celsius (Health Canada, 1993; USEPA, 2002). Atrazine has an octanol-water partition coefficient of 2.82 and a hydrolysis half-life of over 1000 days. Such chemical properties make atrazine a persistent contaminant in aquatic environments (USEPA, 2003).

Atrazine is a herbicide that, along with other triazine herbicides such as cyanazine, propazine and simazine, is the most heavily-used class of pesticides in the world (Gammon *et al.*, 2005). These herbicides are Photosystem II inhibitors and are widely applied to control broadleaf and grass weeds (Solomon *et al.*, 1996; Gammon *et al.*, 2005).

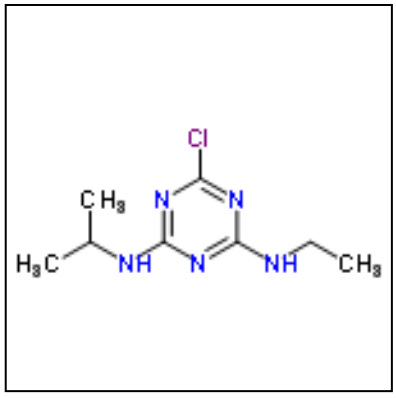



**Figure 1.4:** Atrazine (Chemspider.com)

Atrazine was developed and patented in the late 1950s in Switzerland and was registered for use in the United States in 1959. Since that time, atrazine has been used extensively

around the world. In North America, atrazine is primarily applied to corn fields but is also used on sugarcane, vegetables, grain fields and other crops (Solomon *et al.*, 1996; Anderson & Zhu, 2004). It was in 1994 that the USEPA first began to take note of possible ecological effects which atrazine could produce.

Drinking of atrazine-contaminated water has been linked to a number of health issues in humans in both acute and chronic exposures (Health Canada, 1993). Nausea and dizziness have been reported after immediate consumption of contaminated water (Health Canada, 1993) and chronic atrazine exposures have been associated with an increased risk of ovarian cancer, malignant tumours in uteruses and breasts and non-Hodgkin's lymphoma (Donna *et al.*, 1984; Hoar *et al.*, 1988; Health Canada, 1993; Gammon *et al.*, 2005). Atrazine acts on the pituitary-gonadal system and therefore influences regulation of several hormones which in turn increases the risk of reproductive system tumours (Health Canada, 1993). A 2003 Canadian study demonstrated an increased risk of prostrate cancer in farmers and suggested herbicide use as a culprit (Mills & Yang, 2003); however, triazines were not directly implicated. Health Canada has classified atrazine as a Group 3 Carcinogen (possibly carcinogenic to humans) as no conclusive findings about atrazine's role have been determined (Health Canada, 1993). In 1999, the International Agency for Research on Cancer (IARC) followed, classifying atrazine as a possible human carcinogen (IARC, 1999). In order to reduce the risks associated with atrazine intake, the World Health Organization (WHO) and Health Canada have recommended a maximum of 0.5 mg of atrazine per kilogram of body weight per day.

Although there is not conclusive evidence as to how atrazine toxically affects a non-target organism, changes in movement behaviour have been seen in a number of aquatic organisms (Wan *et al.*, 2006). It has been suggested that atrazine suppresses the enzyme acetylcholinesterase, thereby allowing the accumulation of acetylcholine. This build-up will cause constant stimulation of nerve and muscle fibres in the affected organism leading eventually to paralysis, followed by death (Forget *et al.*, 2003). Atrazine exposure may also lead to affected ATP availability (Liu *et al.*, 2005) and ion regulation (Waring and Moore, 2004). Another possible mode of action is through oxidative stress. Increased levels of harmful reactive oxygen species have been noted in organisms after atrazine exposure. With increased levels, damage to lipids, proteins and

eventually DNA will occur leading to possible cancers and tumours developing in the organism (Sanchez *et al.*, 2008, Song *et al.*, 2009).

With between 70,000 and 90,000 tonnes applied each year, atrazine is the most heavily-applied herbicide in North America (Graymore *et al.*, 2001). Atrazine can then enter the water system easily through runoff from agricultural fields and leaching (DeNoyelles *et al.*, 1982). Contamination of well and surface waters has been detected in many provinces including Prince Edward Island, Nova Scotia, Quebec, Ontario, Saskatchewan and British Columbia (Health Canada, 1993). In Canada, concentrations of up to 81 µg/L have been detected in drinking water after application of the herbicide in the spring, which greatly exceeds the stipulated levels (Graymore *et al.*, 2001). Health Canada has set drinking water levels of atrazine to 5 µg/L (Health Canada, 1993) while 2 µg/L has been set by the Canadian Water Quality Guidelines to prevent harm to aquatic life (Canadian Water Quality Guidelines, 2008). In the United States, drinking water levels of atrazine are not to exceed 3 µg/L (USEPA, 2003).

The high levels of atrazine found in Canadian and North American drinking waters, which greatly exceed stipulated levels (most notably in the spring), make it a contaminant of concern requiring seasonal, if not continuous, monitoring. Present methods of atrazine detection include gas chromatography followed by flame ionization, electron capture or mass spectroscopy (Health Canada, 1993). In order to increase the speed of obtaining results and to reduce the cost of chemical testing, the application of an automated biomonitoring system is suggested. Atrazine can be readily removed from contaminated water through ozone oxidation, UV radiation and granular activated carbon among other methods (Jiang *et al.*, 2006). It is important to detect large concentrations of atrazine entering a drinking water treatment plant in order for proper methods to be used for targeted removal.

### *1.3.3 Dimethyl sulfoxide*

Dimethyl sulfoxide (DMSO) is an organic solvent that is used often in biological testing (e.g. Bowman *et al.*, 1981; Ura *et al.*, 2002; Hallare *et al.*, 2006; Marshall, 2009) and was used in the current study as a carrier to dissolve the hydrophobic TBT and atrazine and disperse them in the water column. It is required as many organic pollutants and pesticides have low water solubilities and must be dissolved in an organic solvent

prior to being placed into the test water (Bowman *et al.*, 1981). DMSO ensures that the compound of interest is evenly distributed throughout the test water. DMSO is not only used as a solvent for ecotoxicology, but also in human and veterinary therapeutics due to its relatively low toxicity (Barbosa *et al.*, 2003).

Although some concern has been raised regarding the toxic effect and subsequent alteration of results that may be caused by use of DMSO in toxicological studies, DMSO has been shown to have a safe working concentration of 0.1% v/v (Martins *et al.*, 2007; Hutchison *et al.*, 2006; Ren *et al.*, 2008; Ren *et al.*, 2009). It has also been shown to be less toxic than other organic solvents such as methanol, ethanol, acetone and acetonitrile (Bowman *et al.*, 1981).

## 1.4 Early-Warning Biomonitoring Systems (EWBS)

### *1.4.1 Background and Rationale for EWBS use*

The traditional approaches to ensuring water security that include periodic monitoring of known chemical constituents and the use of a variety of indicator organisms for pathogen detection are no longer adequate. These analyses are off-line, indirect procedures requiring more than 24 hours to either culture microorganisms or extract and analyse chemicals. Consequently, evidence that drinking water safety is being compromised can come too late. Cost, effort and expertise prohibit continuous monitoring at present. Furthermore, the diversity of potential contaminants makes it difficult to anticipate which pollutants are necessary to monitor, and difficult to predict effects of simultaneous exposure to different contaminants.

Periodic monitoring followed by laboratory analysis is a useful and cost-effective method of monitoring waters that already meet good quality standards, that are not dynamic (i.e. water quality can change rapidly through time) and where only surveillance monitoring is necessary. However, in bodies of water which are failing to meet the standards, or which are dynamic such as rivers or shipping channels, spot sampling practises are inadequate and continuous monitoring is required (Roig *et al.*, 2007). Periodic sampling is also insufficient for use in complex systems (e.g. tidal waters) or systems that are subject to temporal fluctuations in pollution levels (e.g. seasonal use of

pesticides or weather patterns) as they do not provide a representative picture of water quality (Roig *et al.*, 2007). Applying frequent and/or widespread sampling methods results in high costs for labour and transport, the need for a large number of analyses, and delayed result time. Therefore, alternative methods must be used (Roig *et al.*, 2007).

Early-warning biomonitoring systems (EWBS) allow for *in situ*, automated biomonitoring of aquatic systems to detect pollution, pursue polluters and provide warnings of changes in water quality in order to protect sensitive sites such as drinking water-intake points, through monitoring organism behavioural changes (Day & Scott, 1990; Johnstone *et al.*, 2006; Barata *et al.*, 2007; Roig *et al.*, 2007; Ha & Choi, 2008; Kristoff *et al.*, 2008).

The use of EWBS was validated in November of 1986 when a Sandoz chemical storage building in Basel, Switzerland caught fire. The result was a release of approximately 40 tonnes of insecticides and 400 kg of atrazine into the Rhine River. This discharge caused significant damage to a large portion of the River's ecological community, as well as drinking water production in an already polluted river. However, the presence of the toxicants was detected and reported shortly after the incident by the Dynamic *Daphnia* test, an automated biomonitoring system located 500 kilometres downstream of the facility that electronically measured the altered swimming behaviour of *Daphnia magna* and registered an alarm.

Subsequently, implementation of EWBS has occurred along several rivers throughout Europe employing the use of a variety of species. They are predominantly located along large rivers and rivers that cross country borders, such as the Rhine. Such systems currently include, but are not limited to, the Dynamic *Daphnia* test (De Zwart *et al.*, 1995), the bbe *Daphnia*Toximeter (Lechelt *et al.*, 2000), the Mosselmonitor, and the Dreissena-monitor (Borcherding & Volpers, 1994). Luminescent bacteria have also been employed for water biomonitoring using the BioLum Luminous Bacteria Test (Kuster *et al.*, 2004). In North America, development and implementation of EWBS has so far been limited to the United States Military (Van Der Schalie *et al.*, 2001). The Intelligent Aquatic BioMonitoring System is a portable system that detects changes in the movement of bluegills (*Lepomis macrochirus*) using electrodes suspended above and below each fish in a chamber. Currently, these commercially-available devices are typically based on

single-organism response(s). Since organisms respond to different chemical stressors in different ways, these devices may over-predict, or more dangerously, under-predict risks posed by a certain contaminant, suite of contaminants or pathogens.

Desired parameters of EWBS include full automation to minimize operator involvement, real-time detection of contaminants in the water system, and alarm signal generation that can be sent remotely (Bode & Nusch, 1999; Lechelt *et al.*, 2000). They must be sensitive to a variety of contaminants and produce rapid responses from organism exposure to low concentrations. Reliable alarm interpretation is highly important to minimize generation of false alarms. Finally, in order to have widespread use, the costs, maintenance efforts, and training requirements must all be minimal. To ensure this, the implemented monitors should be able to work unmanned for a minimum of five to seven consecutive days (Lechelt *et al.*, 2000).

The future of EWBS lies in the development of widely-applicable systems. They must be capable of adapting to a variety of situations and provide fast, reliable and accurate responses. The choice of organisms to be used as component biological indicators is highly important as each provides a different response to different toxicants. In order to have a comprehensive system able to detect a myriad of possible toxicants, it is necessary to include multiple species and be able to interpret the changes in their behaviour consistently.

### *1.4.2 Daphnia EWBS: Development over the years*

*Daphnia magna* are known to be sensitive to the presence of a large number of pollutants and can respond with a variety of behavioural traits, including inability to move throughout the water column, swimming style and immobilization (Dodson & Hanazato, 1995; Green *et al.*, 2003; Christensen *et al.*, 2005; Marshall, 2009). A range of early-warning biomonitoring systems have been developed to detect changes in their swimming behaviours (Knie, 1978; Kerren, 1991; Bailleul & Scheunders, 1998; Lechelt *et al.*, 2000; Michels *et al.*, 2000; Green *et al.*, 2003). The Dynamic *Daphnia* Test, a monitor developed by Knie (1978), uses the swimming activity of *Daphnia magna* to assess stress. Stress resulting from pollution exposure is measured as the organisms swim through multiple infrared (IR) light beams. A baseline level is established for organism

activity. If the activity of the organisms increase, corresponding to higher stress levels, they will cross a higher number of IR beams. An alarm is generated once a certain threshold is passed. The disadvantage of the Dynamic *Daphnia* Test stems from the lack of definition of number of IR beams crossed by a given number of organisms. If many organisms cross a small number of beams, the generated measurement could be the same as several organisms crossing a larger number of beams (Michels *et al.*, 2000). This system is not specific with the types of organism behaviour which represents stress to *Daphnia* and can therefore may not give any indication of the type of stressor which has entered the water system. This system is not available on the market, but is still operated at water quality monitoring sites (Jeon *et al.*, 2008).

More recently, the bbe *Daphnia* Toximeter was developed by bbe Moldaenke (1997). This system uses an alarm analysis based on swimming velocity where a camera frame grabber digitizes the images and enters them into a computer program. From here, a trajectory analysis is performed for each daphnid in the chamber (Bailleul & Scheunders, 1998; Lechelt *et al.*, 2000). The traced movement of the center of each organism is provided on a black and white vector image where the displacement of objects can be described through a simple geometrical model. Graphical and tabular outputs are available. The instrument consists of 2 simultaneously observable channels each containing media and up to 25 organisms can be tracked simultaneously (Bailleul & Scheunders, 1998). Parameters associated with swimming are monitored including average velocity, fractal dimension (measure of turning and circling by daphnids) of the organisms, a V-class index (compares velocity ranges of organisms under various treatments), average height in the water column, distance between organisms, and the number of organisms moving (Lechelt *et al.*, 2000; Green *et al.*, 2003). The bbe Moldaenke *Daphnia* Toximeter has been shown to rapidly and effectively detect changes in behaviours of *D. magna* when exposed to certain contaminants (Lechelt *et al.*, 2000; Green *et al.*, 2003; Watson *et al.*, 2007).

Several limitations, however, are in place with the application of the bbe Moldaenke *Daphnia* Toximeter. Although this system allows for continual detection of hazardous compounds, the individual *Daphnia* cannot be recognized and the average of swimming velocities is used. A delayed trigger of the alarm may result if the activity of a

highly-activated organism, induced by exposure to toxicants, is compensated for by that of slowly moving organisms. This system is also limited to the use of a single species, *D. magna*. As discussed ealier, since organisms respond to different chemical stressors in different ways, these devices may be too simplified. This system is also based on image analysis which requires clarity of the sample water and a sufficient light source for the organisms to be located. If the water sample must be filtered before entering the Toximeter, is it highly possible for substances to be removed and the contamination of the water to be under-predicted. Addition of an external light source may also alter the natural diurnal behaviours of the organisms and cause an altered response to water contamination to be given.

### *1.4.3 Automated Grid Counter*

Another optical EWBS has been developed in Korea by Jeon *et al.* (2008). The Automated Grid Counter device (Figure 1.5) is a multichannel biological monitoring system for individual analysis of *D. magna*. The focus of this team was to produce a precise and efficient monitor that was simple and cheap to implement (Jeon *et al.*, 2008). By using individual animals, a more sensitive response to pollutants may be given, as well as obtaining more detailed information on the behaviour of the organism. Therefore, this system employs multiple channels for individual organism analysis (Jeon *et al.*, 2008). The system consists of 6 channels, each with an individual organism whose movements are recorded by a web-camera (Figure 1.5).

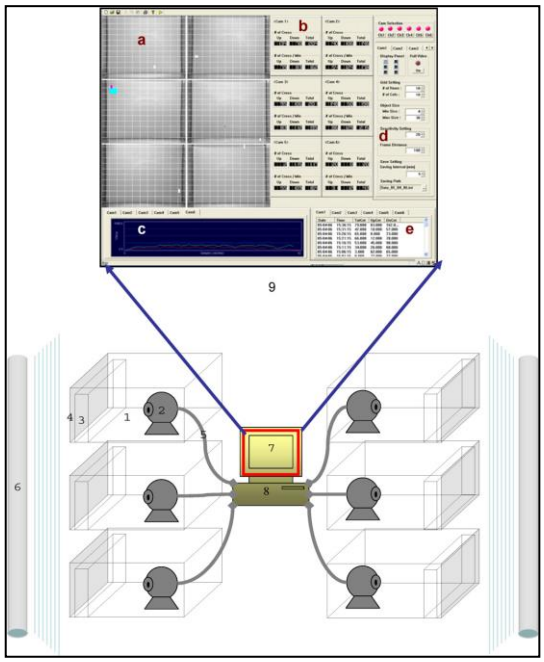



**Figure 1.5**: Automated Grid Counter (Jeon *et al.*, 2008)

The information gathered is sent to the computer unit where the image signals from all 6 channels can be processed in real-time. For the data to be analysed, the image

must first be binarized. The territory of the moving object must then be established on the pixel array and the reflection of the *Daphnia* is described within the grid (Figure1.6). The center of the object can be monitored on the capture window which is set up with a virtual image of horizontal and vertical lines. The Grid Counter (GC) registers one event when the center of the *D. magna* crosses a grid line. A large number of events represent *Daphnia* swimming activity. If this activity passes a certain threshold, the alarm is activated. The sensitivity of the GC can be controlled by the size of the objects being monitored as well as the number of horizontal and vertical lines within the grid. By defining the exact size of the objects, errors caused by other objects (such as water bubbles) can be avoided (Jeon *et al.*, 2008).

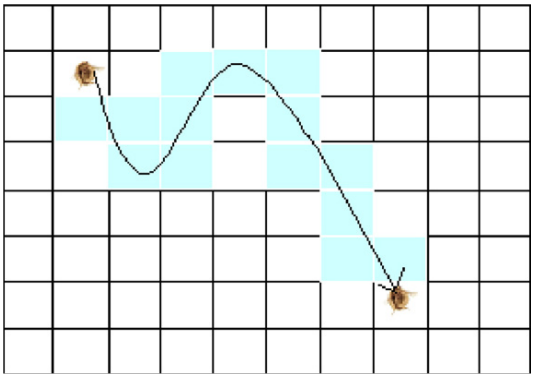


**Figure 1.6:** Grid Counter (Jeon *et* al, 2008)

Because of the simplicity of this system, the amount of data collected does not need to be processed by a high-performance computer. With the smaller amount of data being processed, a larger number of channels with individual organisms can be simultaneously observed. The materials used in the apparatus are also simple and relatively inexpensive, especially in comparison to other EWBS. This is apparent with the use of web-cams as opposed to high-tech and tailored video cameras used in other systems (Jeon *et al.*, 2008).

Although the Automated Grid Counter is a seemingly robust system, it presents the same limitations as the bbe Moldaenke *Daphnia*Toximeter. This system is also based on visual image analysis. In the Grid Counter, the issue of water clarity and sufficient light source may be a larger issue as the quality of the cameras are not as high. The use of web-cameras, although cheaper, will give a lower resolution and reduce clarity of the image. High filtration may be required, thereby removing possible water toxicity, and adequate lighting can result in altered organism behaviour and skewed results.

## 1.5 The Multispecies Freshwater Biomonitor

According to the myriad of published literature (Gerhardt & Svensson, 1994; Gerhardt, 1995; Gerhardt, 1998; Gerhardt & Palmer, 1998; Gerhardt & Schmidt, 2002; Gerhardt *et al.*, 2002a; Gerhardt *et al.*, 2002b; Gerhardt *et al.*, 2003; Gerhardt *et al.*, 2004; de Bisthoven *et al.*, 2004; Gerhardt *et al.*, 2005a; Gerhardt *et al.*, 2005b, de Bisthoven *et al.*, 2006; Gerhardt *et al.* 2006; Kirkpatrick *et al.*, 2006a; Kirkpatrick *et al.*, 2006b; Gerhardt, 2007a; Gerhardt, 2007b; Gerhardt *et al.*, 2007; Macedo-Sousa *et al.*, 2007; Sardo *et al.*, 2007; Ren *et al.*, 2007; Kienle & Gerhardt, 2008; Kienle *et al.*, 2008; Macedo-Sousa *et al.*, 2008; Ren *et al.*, 2008; Gerhardt, 2009; Kienle *et al.*, 2009; Holmstrup *et al.*, 2009; Peeters *et al.*, 2009; Ren *et al.*, 2009; Langer-Jaesrich *et al.*, 2010; Sardo & Soares, 2010), the Multispecies Freshwater Biomonitor (MFB) is a fully-automatic, online, real-time biomonitor designed to record behavioural patterns of aquatic vertebrates and invertebrates, size permitting (Figure 1.7).

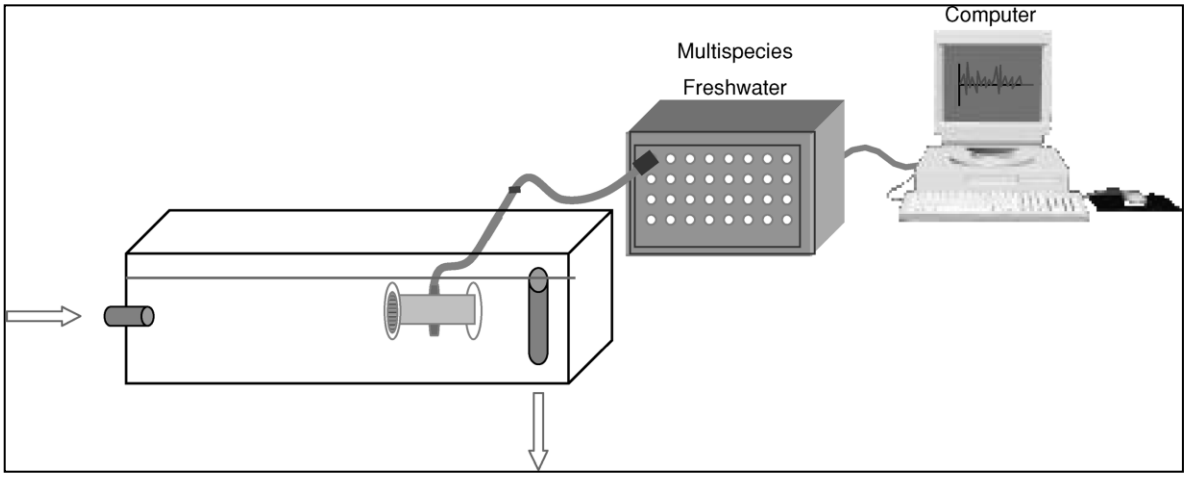



**Figure 1.7:** Schematic diagram of the Multispecies Freshwater Biomonitor (Gerhardt, 2007a)

It is a non-optical EWBS that uses an electrical field within individual chambers to monitor the behaviour and behavioural changes or aquatic organisms. The system can have a high number of replicates, anywhere from 8 to 96 channels, each containing an individual animal and can be employed simultaneously. This set-up also can allow for monitoring multiple species at the same time. The MFB has several advantages over other *in situ* online biomonitors as it requires no filtration or pre-treatment of the water samples. Additionally, since it is a non-optical system, measurement of benthic

organisms within their appropriate substrate is possible and organisms can be monitored under night or turbid conditions.

### *1.5.1 Apparatus Set-up, Signal Generation and Alarm System*

The MFB system is made up of the test chambers, with the individual organisms placed inside, connected to the MFB impedance recording instrument (Figure 1.8). The total space required for the apparatus is approximately 1 $m^2$ (Gerhardt, 2007).

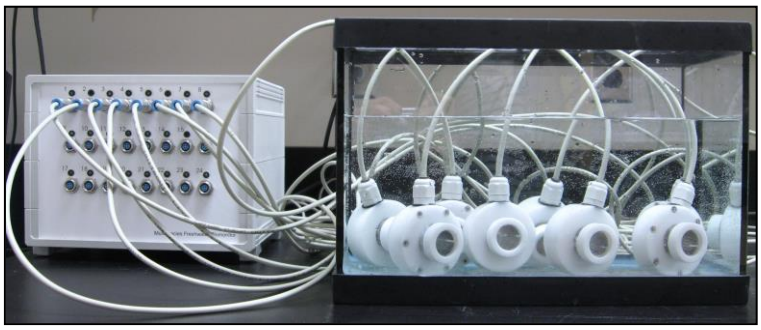


**Figure 1.8:** Laboratory Set-up of the Multispecies Freshwater Biomonitor chambers and Impedance Recording Instrument

Inside each chamber are two set of stainless steel plate electrodes, located at the walls of each chamber. The first pair of electrodes creates a high frequency signal of alternating current of up to 50 kHz resulting in an electric field being produced inside each chamber. The organisms are placed inside the chambers and are able to swim and behave freely within the electrical field. Any movement is registered as an impedance signal within the field and is picked up by the second pair of electrodes, which are non-current carrying. The impedance signals are then sent to the recording device and continue onto a computer for data analysis. Interference between the chambers is avoided by sending the signals to the MFB-impedance recorder in synchronicity (Gerhardt *et al.*, 2006).

Signals generated within the chambers by the organism will change in amplitude in correlation with the size of their movements. The amplitude of the signal is also dependant on both the size of the animal relative to the size of the chamber, as well as the degree of activity of the organism. The amplitudes (in volts) of organism movements are measured for the duration of 250 seconds, with one series of measurements being taken every ten minutes. The amplitude values from the real-time reading are given in volts against time, in seconds (Figure 1.9).

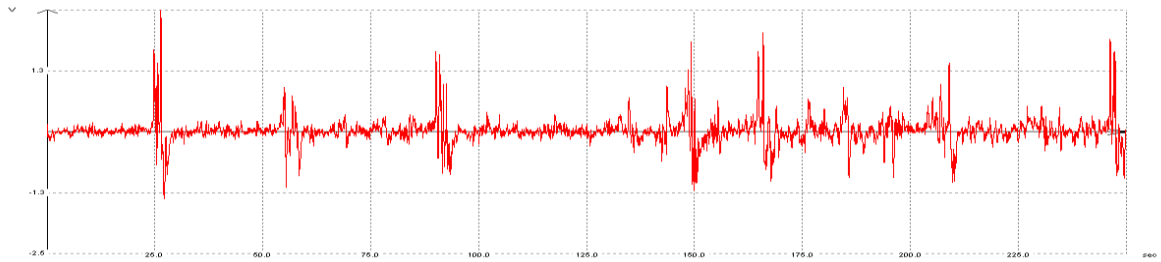


**Figure 1.9:** Movement Pattern *Hyalella azteca*

These values are then transformed by the MFB software using discrete Fast Fourier Transformation. This transformation converts the amplitude values into frequencies in hertz (Figure 1.10) (Gerhardt *et al.*, 2006). The lower amplitude voltages correspond with high frequency hertz values. The different frequencies range from 0.0 to 10.0 Hz and are analyzed in intervals of 0.5 Hz. Within this range, the low frequencies (from 0.5 to 2.5 Hz) are attributed to locomotion and other slow activities. The higher frequencies (from 3.0 to 8.0 Hz) are caused by ventilation (respiratory patterns of the organism) and faster movements.

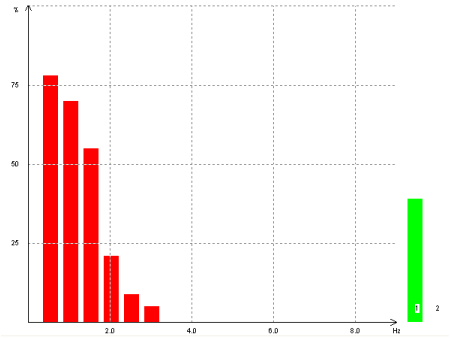


**Figure 1.10:** Fast Fourier Histogram of *Hyalella azteca*

Prognosis values are calculated from the moving average of the preceding 5 values. The alarm is generated when the values of the frequency of behavioural signal differs by 10% from the prognosis value. There is also a mortality alarm that is activated when the number of chambers with inactive organisms exceeds a defined number. This parameter can be set depending on the degree of specificity desired (Gerhardt *et al.*, 2006).

### *1.5.2 Organisms and Toxicants analysed thus far with the MFB*

To date, the MFB has been used in multiple experiments, analysing the viability of different indicator organisms and their responses to a variety of chemicals and toxicants. The MFB does not detect specific movements of an organism, such as antennae

or movement of other appendage. Rather, it groups the behaviours into ranges of frequencies obtained by the transformation of signal amplitudes into frequencies. As mentioned, the lower range of frequencies (from 0.5 to 2.5 Hz) corresponds to locomotion of the organism. The locomotive activities are described as the "overall behavioural strength" elicited by an organism (Ren *et al.*, 2007). Behavioural strength is defined as the "measure of intensity of behavioural parameters representing motility" including swimming velocity, behavioural frequency and movement extent (Ren *et al.*, 2007; Ren *et al.*, 2008). It is from this range that most results in the following studies come. The ventilation frequencies (from 3.0 to 8.0 Hz) are generally dwarfed by locomotion frequencies, as the amplitude is very small (Kirkpatrick *et al.*, 2006b). In several studies, measurable ventilation frequencies were not generated at all (Kirkpatrick *et al.*, 2006a). Many studies focused on the locomotive activities of the organisms only, rather than including ventilation in monitoring practises, as the values were not generated reliably if at all (Gerhardt & Palmer, 1998; de Bisthoven *et al.*, 2006; Kirkpatrick *et al.*, 2006b; Kienle *et al.*, 2008; Sardo & Soares, 2010).

*Gammarus pulex* is a freshwater amphipod which feeds on detritus and is commonly found in European streams (Gerhardt, 1998). It has been used in MFB application since the first experiments and publications (Gerhardt & Svensson, 1994; Gerhardt, 1995; Gerhardt *et al.*, 1998). Its locomotive activites to a range of chemicals and toxicants has been analysed and contaminants include pharmaceuticals, such as fluoxetine, ibuprofen and carbamazepine (De Lange *et al.*, 2009), metals such as Cd, Cu, Ni, Pb and Zn (Gerhardt *et al.*, 1998; Gerhardt *et al.*, 2007), and acidic conditions (Gerhardt *et al.*, 2003).

It should be noted that no work has previously been done using *Hyalella azteca* in the MFB. *H. azteca* is used in this study because of its ecological importance in the Great Lakes (Geisy & Hoke, 1989) and the ease with which it is cultured in the laboratory (Nebeker & Miller, 1988). Previous work has been conducted to determine *H. azteca* response to both tributyltin (TBT) and atrazine using visual analysis (Marshall, 2009). This study demonstrated that *H. azteca* was indeed a good indicator organism to be used for detection of these aquatic contaminants. The present study aimed to build on Marshall's body of work through application of *H. azteca* in an automated system. It also

aims to build on the work conducted with the MFB by determining if *H. azteca* is an appropriate organism for use in this automated technology.

The effect of three organophosphorus pesticides on the locomotive activity of *Daphnia magna* was analysed using the MFB (Ren *et al.*, 2007). It was found that low concentrations of dipterex, malathion and parathion caused significant decreases in the behavioural strength of exposed *D. magna* over a 24-hour period (Ren *et al.*, 2007). The effect of several other pesticides on *D. magna* locomotive activity was also assessed in a similar study (Ren *et al.*, 2009). It was found that exposure to various concentrations of deltamethrin, chlorothalonil and nitrofen caused significant decreases in the overall behavioural strength of adult *D. magna* after 48 hours of exposure (Ren *et al.*, 2009). In both instances, behavioural changes were time- and concentration- dependant, with higher concentrations causing stronger alterations on locomotive activity over shorter exposure times (Ren *et al.*, 2007; Ren *et al.*, 2009).

From these studies, it was shown that *D. magna* are able to elicit marked changes in locomotive activity over longer time periods of 24 to 48 hours. In the present study, the aim was to use *D. magna* in the MFB over a shorter time frame. As the miner's canary system requires detection of aquatic contaminants rapidly, within hours depending on the location of the system in regards to the water treatment plant intake pipe, the applicability of *D. magna* in the first and second hours of exposure to environmentally-relevant concentrations of TBT and atrazine is to be assessed.

### *1.5.3 In situ application of the MFB*

The MFB has yet to be implemented and included for permanent use in monitoring stations; however, some preliminary short-term work has been conducted to determine usability in field situations (Gerhardt *et al.*, 2003; Gerhardt *et al.*, 2007). In one study, the MFB was used to detect behavioural responses of *Gammarus pulex* and *Daphnia magna* in a drinking water processing plant in the Rhine River in Germany (Gerhardt *et al.*, 2003). *In situ* analysis was conducted over a time period of one month using unfiltered surface water. Additionally, multiple (5) *D. magna* were placed within each chamber rather than individual organisms per chamber. It was determined that use of multiple organisms within each chamber reduced the variability as there was higher probability of having unresponsive organisms. During the first 20 days, both species

demonstrated a stable baseline for locomotion. It was determined that *G. pulex* and *D. magna* showed stable locomotive behaviour, although better survival was noted in *G. pulex*. It was concluded that *G. pulex* survived better and for longer periods than *D. magna*, who has a shorter life cycle which may also pose issues with reproduction as presence of *Daphnia* neonates may alter the generated results (Gerhardt *et al.*, 2003).

The second *in situ* analysis was slightly more in depth, with three locations used to assess viability of the MFB outside of the laboratory. The rivers selected were the Meuse (Netherlands), the Aller (Germany), and the Rhine (France) and the test organism was *G. pulex* (Gerhardt *et al.*, 2007). In the Aller River, in situ monitoring with the MFB was conducted for two weeks with no maintenance or upkeep duties conducted on the system for the duration of the test. For that study, the Aller River represented a clean water system with no industrial effluent or other detrimental uses being present. After the two weeks, all seven *G. pulex* organisms survived with no alterations in their locomotive patterns being detected. Chemical analysis did not detect any contaminants or threats, validating the organism response results (Gerhardt *et al.*, 2007).

The experimental set-ups in the Meuse and Rhine Rivers were conducted for 10 days and six weeks, respectively. These set-ups did not only monitor the natural water, but spikes of trace metals (Cd, Cu, Ni, Pb and Zn) were added as well as polycyclic aromatic hydrocarbons, polychlorinated biphenyls and a range of polar and hydrophobic pesticides (Gerhardt *et al.*, 2007). *G. pulex* reacted very rapidly to the trace metal pulses as behavioural warnings were detected within a few hours at sublethal levels. Mortality alarms occurred towards the end of the exposure and only after repeated pulses and stress. The response of *G. pulex* to pulse exposure of organic pollutants occurred more slowly than for the trace metals pulses; however, behavioural warnings and mortality alarms were also recorded by the MFB (Gerhardt *et al.*, 2007).

Although the MFB has not yet been permanently implemented into use for contaminant detection entering a drinking water treatment plant or from the effluent of a facility in a water system, the results from the above studies show potential for this application. *G. pulex* demonstrated slightly more consistent responses than *D. magna* and were able to survive for longer periods of time. However, the use of singular *D. magna* has been shown to identify contaminants readily (Ren *et al.*, 2007; Ren *et al.*, 2009). In

addition, *D. magna* has been used throughout ecotoxicological studies and its inclusion in the final miner's canary system will allow for comparison to previous work (Marshall, 2009).

## 1.6 Issues to be considered with EWBS

The use of automated biomonitoring systems for source water protection offers several advantages over traditional chemical spot sampling methods. First, EWBS respond rapidly to a large variety of contaminants (Mikol *et al.*, 2007). Organism response will occur if the concentration and exposure time exceed a given threshold, regardless of the contaminant. This allows for the presence of any possible pollutant or cocktail of contaminants to be recognized and does not rely on certain tests to be conducted (Diamond *et al.*, 1988). Secondly, as automated monitors provide continuous information regarding water quality without constant maintenance or supervision, the number of personnel required for source water evaluation can be reduced. Alerts to potential problems can be sent to one operator from multiple biomonitors for further action to be taken (Bode & Nusch, 1999). Thirdly, EWBS are applicable in multiple situations for different purposes such as detection of pollutants entering a water treatment plant or monitoring industrial facility effluents. Source water and distribution systems can have increased security by using biomonitors to detect any spills coming into the facility. Detection of incoming spills will allow for further treatment to be administered as needed. Effluent monitoring is also possible through application of biomonitors (Shedd *et al.*, 2001) as is watershed protection (USEPA, 2001). Identification of polluters and origination points of spills is important for liability and protection of the environment.

Biomonitors are able to detect the presence of aquatic contaminants based on organism behavioural changes. They are also even able to identify the class to which a pollutant may belong as different species can react with different behaviours when exposed to different contaminants. It is then up to modelling systems of the biomonitors as well as chemical analysis to determine what an exact culprit may be. Although a large range of contaminants can be detected using biomonitors, it is difficult if not impossible to specifically identify which contaminant, or mixture of contaminants, has entered the water system (Mikol *et al.*, 2007). Further chemical analysis is required for accurate identification. The use of multiple organisms, however, can facilitate the identification of

the class or family to which the given toxicant belongs permitting better targeted chemical analysis. Databases detailing the behavioural responses of organisms to different contaminants must be compiled for comparisons to be made. Once the class to which the contaminant belongs has been determined, chemical analysis can be conducted for identification of the specific contaminant.

The cost of the equipment is also to be considered. The cost of commercially-available biomonitors generally ranges from approximately $10,000 (US) to $100,000 (US) (Mikol *et al.*, 2007). Personnel costs are reduced as little maintenance and upkeep is required for the biomonitors and organisms, and data can be checked routinely and remotely.

Aside from the actual responses generated from the automated biomonitors, the usefulness of the system is based on sound decision-making so as to confirm occurrence of an event and to notify involved parties. This means that alarms are further verified through the use of chemical analysis (Gunatilaka & Diehl, 2000; Kramer & Foekema, 2000). A formal decision-making framework is required so response to a chemical contaminant event can occur efficiently, and a response plan must be in place before an event occurs which outlines follow-up procedures that are proportional to the severity of the event (Mikol *et al.*, 2007).

## 1.7 Summary

The use of organism behaviour as a means to assess water quality offers a scientifically-valid, environmentally-relevant, rapid, and cost-effective addition to traditional chemical analysis methods. Chemical analyses, although offering low detection limits and specific contaminant identification, are unable to provide rapid results and this could result in the human consumption of polluted drinking water. With the high number of possible contaminants and mixtures present in certain water systems, chemical testing is unfeasible as specific standards are required. Additionally, chemical analytical costs can be prohibitive. Conversely, behaviour of aquatic organisms can change with exposure to any contaminant or mixture of pollutants. Additionally, many aquatic organisms are highly sensitive to very low levels of pollutants as they are in full and constant contact with water and/or sediment which may be contaminated. By using organism responses as an additional layer of protection that complements chemical

assessment, environmentally-relevant analysis can be made and a true picture of water quality and potability can be obtained.

When designing a biological early-warning system, some of the most important factors to be taken in account include reliability, generation of minimal false alarms, and having a decision-making framework in place to deal with generated alarms. In order for a full and complete analysis of water quality to be done, EWBS can, and arguably should, be used in conjunction with chemical testing for specific identification of a contaminant or cocktail coming through a water system. Through use of multiple species of aquatic organisms which inhabit varying positions in the ecosystem and may have assorted behavioural responses to different contaminants, a fuller picture can be established and aide in the identification of the family to which a given contaminant belongs.

The Multispecies Freshwater Biomonitor is a system that is non-species specific and can theoretically analyze the behavioural responses of several species at once. It is a non-optical system, allowing for observations to be made through the full 24-hour day cycle as well as analysis of organisms which prefer to burrow and live in the sediment, or for full analysis under turbid conditions such as those occurring after a thunderstorm or ship passage. The MFB can be set up in a flow-through system which creates a continual monitoring device for water assessment and offers constant protection for human consumption of drinking water.

This study is part of a larger project which aims to develop a miners' canary that can be used to readily detect quality of drinking water to prevent human consumption of contaminated water. The final product will be applicable in multiple locations along water systems used for monitoring of drinking water intake and discharge from facilities along the water side. By placing an EWBS adjacent to a drinking water intake pipe, incoming plumes of contaminants can easily be detected. This will then allow for the intake pipe to be closed and prevent any water for being brought into the WTP until after the plume has passed. Identification of the incoming contaminants will also allow for proper measures, such as increased treatment or longer residence times, to be taken to ensure complete removal of the contaminant from the water system.

In addition to monitoring water flowing into intake pipes, EWBS can also be placed downstream or at the exit point of a facility that discharges its effluent into the

aquatic environment. By placement of the system in such a location, continuous monitoring of the facility will ensure early detection of pollutant discharge. Detection can prevent contamination of drinking water and increase the chance of liability attaching to the companies at fault for the spill as well as give auditable assurance that the given company is in compliance and not liable for the spill from another company's facility.

## 1.8 Objectives

In vulnerable and remote communities, both in Canada and around the world, there is a need for a scientifically-rigorous, affordable and rapid system to determine water quality. The use of an early-warning biomonitoring system can offer this with minimal resources required for allocation to maintenance of the system and to operator training. In this study, the applicability of the MFB as a component of a more holistic EWBS was evaluated for use in the Niagara and Great Lakes region. The organisms used were *D. magna* and *H. azteca*, and while they have been deemed as highly sensitive test species with varying behavioural responses in past studies, they have been further studied in the current research to assess their efficacy in MFB technologies. If the MFB is able to detect the effect of TBT and atrazine on the organisms' behaviour, then the MFB and the organisms studied could be candidates for inclusion in the final early-warning biomonitoring system under development.

# 2 Materials and Method

## 2.1 Overview

It should be emphasized at this juncture that a thorough ecological knowledge of the behavioural responses of the daphnids and amphipods is critical before these behaviours can be assessed in automated systems such as the Multispecies Freshwater Biomonitor. Thus, long before the actual MFB arrived in the laboratory, extensive experimentation was conducted on *Daphnia magna* and *Hyalella azteca* to assess visually both normal behaviours and stressed responses from the organisms when exposed to contaminants such as ethanol. The responses that were examined will be detailed further in this section.

Additionally, it should be emphasized that while *D. magna* had been used in MFB technologies in Europe, this is the first time that *H. azteca* was used as a test organism with MFB.

## 2.2 Washing Procedure

### *2.2.1 Glassware, aquaria and other reusable pieces of lab equipment*

Washing procedures for all glassware, aquaria and other reusable equipment followed the "Ryerson University" protocol (McCarthy *et al.*. – personal communication), which was developed from the Environment Canada method (1996). Equipment was thoroughly washed before use to ensure no traces of contaminants were transferred from experiment to experiment.

All glassware was first rinsed with acetone three times to remove organic compounds, followed by one rinse with dechlorinated municipal drinking water. It was then left to soak in a solution of Extran®MN 01 Powder soap for 15 minutes to remove organic contaminants. After such time, the glassware was manually scrubbed while wearing Nitrile gloves to remove any soap residue and rinsed with dechlorinated municipal drinking water. Finally, the glassware was dipped in 10% (v/v) HCl to remove heavy metals and rinsed three times with distilled water. Equipment was placed in an inverted position and allowed to air dry.

### *2.2.2 Multispecies Freshwater Biomonitor*

In order to ensure no contamination of successive experiments by previous ones, the MFB chambers (Figure 2.1) were washed thoroughly following each use.

**Figure 2.1:** MFB Chambers in individual vessels

Washing protocols could not follow the "Ryerson University" procedure as contact with 10% (v/v) HCl would cause the oxidation of the metal chamber anodes. Acetone could also not be used as the chambers contained acrylic plastic tubing that is soluble in acetone. Thus, to clean the equipment, the MFB chambers were soaked in a solution of Extran®MN 01 Powder soap for 20 minutes to remove organic contaminants. They were then scrubbed to remove any residue and rinsed three times with distilled water. The chambers were air dried prior to use. Nitex screens were used at either end of the chamber cylinders (Figure 2.2) to prevent organism escape. Screens were replaced on the chambers for each series of bioassays using a different contaminant to prevent transfer of adsorbed chemicals.

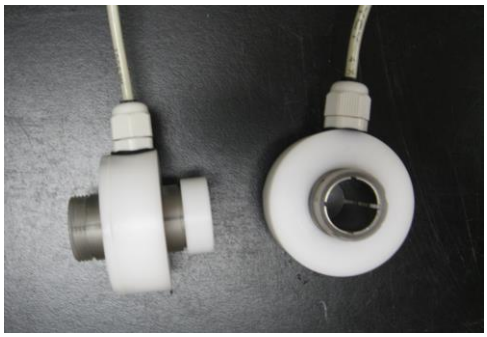


**Figure 2.2:** MFB Chamber without Nitex mesh

## 2.3 Culturing of Bioassay Organism

### *2.3.1 Daphnia magna*

Culturing procedures for rearing *Daphnia magna* were adapted from the Environment Canada (1996) and the USEPA (2002) protocols with additional modifications developed in the Ryerson Laboratories (McCarthy *et al.*). Three cultures of *D. magna* were established in the laboratory to be used for behavioural analysis. Cultures were begun in 9L Teflon-sealed glass aquaria by addition of 7 L of dechlorinated

municipal drinking water to each vessel. Culture water was aerated for a minimum of 48 hours before use to ensure no less than 80% oxygen saturation (DO) of the water. Before addition of initial organism populations, 5mL of Nutrafin fish food flakes and 20mL of *Pseudokirchneriella subcapitata* algae ($1.0 \times 10^5$ cells/mL) were added ("Ryerson University" Protocol; Environment Canada, 2000). The aquaria were allowed to equilibrate for 24 hours after which 40 adult organisms were added to each. The tanks were continuously aerated with an aquarium aerator and covered with plexiglass sheets to discourage contamination by atmospheric dust.

Temperature, DO and pH were checked in the culture water before addition of organisms and the levels were maintained throughout culturing and experimental period within a range of $20 \pm 2^{o}C$ for temperature, 80% air saturation for DO, and 6.0 – 8.5 for pH (Environment Canada, 2000). Aquaria were exposed to indirect and diffused natural daylight supplemented with fluorescent light (intensity of 5 – 10 $\mu E\ m^{-2}\ s^{-1}$) with spring/summer photoperiod of 16 ± 1 hour light: 8 ± 1 hour dark cycle. This light: dark cycle was maintained throughout the experimental period as well as culturing period in order to prevent behavioural changes caused by differing diurnal rhythms (Environment Canada, 2000).

Organisms were fed thrice weekly with an appropriate mixture (see above for dose amounts). To remove waste, 70% of culture water was replenished with fresh dechlorinated municipal drinking water weekly. In order to prevent over-crowding and accumulation of waste and to reduce stress on the organisms, the population of *D. magna* was thinned weekly or as needed to twenty or fewer animals per litre (Environment Canada, 2000). Thinning was conducted by transferring adult daphnids by gentle pipetting using a sterile pipette with a 5 mm opening and minimal carry over of "old" water. Water used in bioassays was from the same source (dechlorinated municipal drinking water) and had the same physical conditions as the culture water in order to lessen stress of the organisms and consequently cause altered behavioural results.

### *2.3.2 Hyalella azteca*

Culturing procedures for rearing *Hyalella azteca* were adapted from the Environment Canada (1996) and the USEPA (2002) protocols with additional modifications developed in the Ryerson Laboratory (McCarthy *et al.*). Culturing followed

the same procedures as for *Daphnia magna* with the following modifications. To each aquarium, sterile cotton gauze was added for use as substrate. Substrate is added to *H. azteca* culture tanks in order to mimic natural habitat of the benthic organisms, such as leaves and other detritus found at the bottom of lakes and rivers (Environment Canada, 1997). Temperature, DO and pH were checked in the culture water before addition of organisms and the levels were maintained throughout culturing and experimental period within a range of 23 ± 2ºC for temperature, 80-100% air saturation for DO, and 7.4-8.5 for pH (Environment Canada, 1997). Organisms were fed twice weekly with an appropriate amount of food (see above for dose amounts). Sterile gauze was replaced weekly. Thirty to fifty percent of culture water was replaced weekly. Dechlorinated municipal drinking water was continuously aerated and available for replenishment of the aquaria (Environment Canada, 1997). Water used in bioassays was from the same source and had the same physical conditions as the culture water in order to lessen stress of the organisms and consequently cause altered behavioural results.

## 2.4 Dilutions

An organic solvent was required as tributyltin and atrazine are hydrophobic contaminants. Dimethyl sulfoxide (DMSO) was used as a carrier for the following dilutions in order to ensure even distribution of the contaminants and to prevent adsorption to any surfaces in the test vessels.

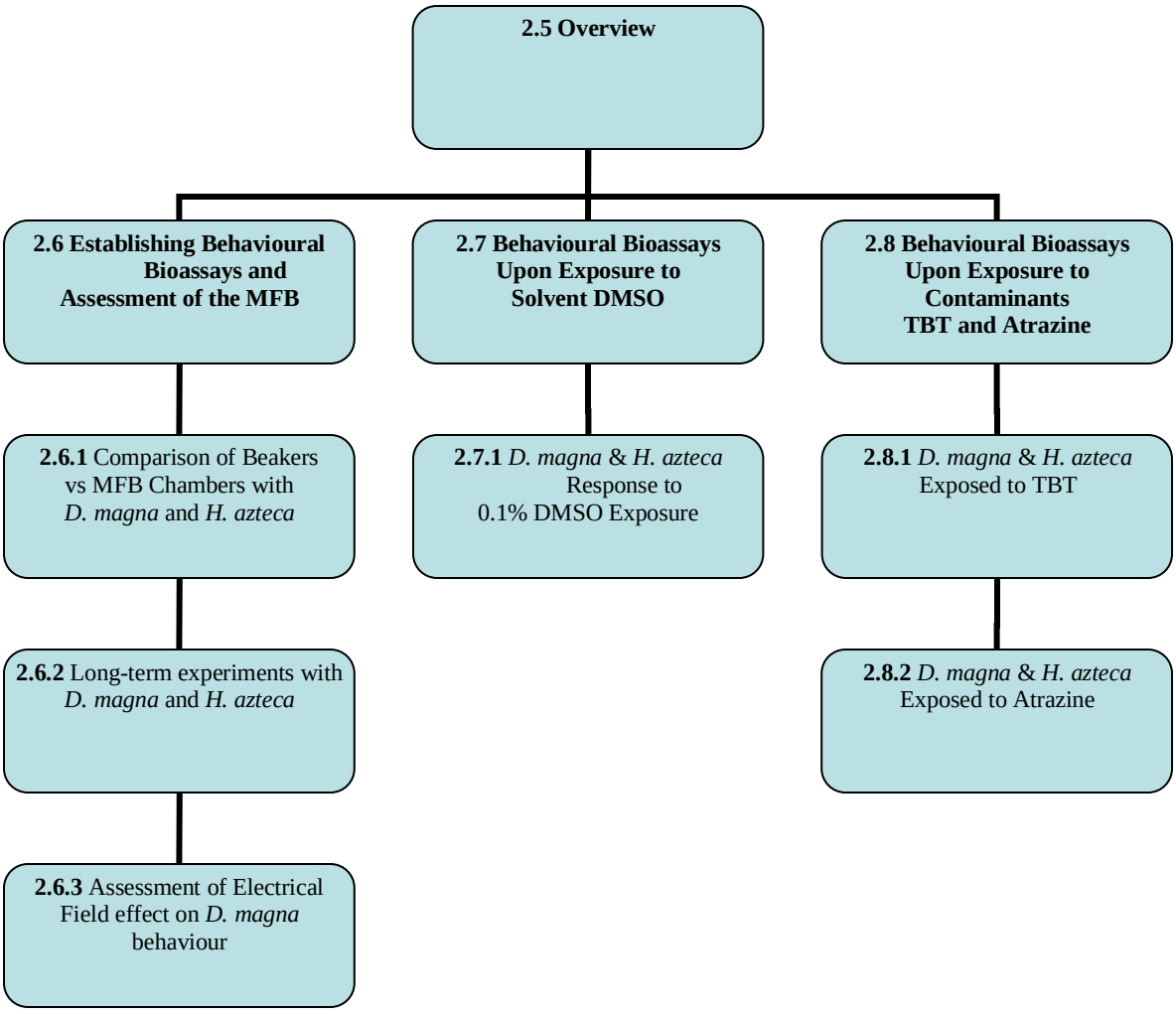
An initial stock solution of 100 mg/L of tributyltin (TBT) and 500 mg/L of atrazine were made in DMSO. From these, subsequent solutions of desired concentrations were made by diluting appropriate volumes of the stock solution in DMSO (Appendix A). These working stocks were then used in experiments by adding 100 µL of working stock per 100 mL of water, creating a final concentration of 0.1% DMSO (v/v) for all test vessels across all contaminant test concentrations. For each bioassay series, a Reference of dechlorinated municipal tap water and a 0.1% DMSO Reference were included to ensure no adverse behaviour was caused by DMSO presence. The use of 0.1% DMSO as an organic carrier has been shown to have no effect on survival or behaviour of *D. magna* and *H. azteca* (Martins *et al.,* 2007; Hutchison *et al.*, 2006; Ren *et al.*, 2008; Ren *et al.,* 2009). It has also been shown to be less toxic than

other organic solvents such as methanol, ethanol, acetone and acetonitrile (Bowman *et al.*, 1981).

## 2.5 Overview

The experiments conducted through this study are outlined in Figure 2.3. Initial behavioural bioassays were conducted to learn the normal swimming and behavioural patterns of *Daphnia magna* and *Hyalella azteca* as well as the responses and behavioural patterns of the organisms under various stress conditions. This was followed by a comparison of normal and stress behaviours of both the organisms in open test vessels versus normal and stress behaviours of both the organisms when placed within the Multispecies Freshwater Biomonitor chambers. This was done to assess whether *D. magna* or *H. azteca* elicited comparable responses when confined to the smaller volume of the MFB chambers. Assessment of the MFB also consisted of long-term experiments in which the organisms remained inside the MFB chambers for one week. Finally, an assessment of whether the electrical field generated across the MFB chambers had an effect on the behaviour of the organisms was conducted.

**Figure 2.3:** Overview of Experiments Conducted




The second phase of experiments assessed whether in fact the use of 0.1% dimethylsulfoxide had an influence on the organism. This was conducted in open test vessels and with the MFB chambers for both *D. magna* and *H. azteca*. The third and final phase of experiments was the exposure of both organisms to the contaminants of concern: tributyltin and atrazine. These bioassays were conducted with the MFB chamber as only and did not include open test vessels as previous work demonstrated the respective behaviours of *D. magna* and *H. azteca* in response to the contaminants (Marshall, 2009).

## 2.6 Establishing Behavioural Bioassays

### *2.6.1 Daphnia magna and Hyalella azteca Comparison of open test vessels and MFB Chambers*

Extensive experimentation was conducted to establish if any variability in organism behaviour could be detected inside or outside of the MFB chambers. Thus, bioassays were initially established in the laboratory before the arrival of the MFB and behaviours assessed when organisms were exposed to conventional stressors such as ethanol and salts. Square beakers with a total volume of 200mL were used as bioassay vessels. Reference and 0.1% DMSO Reference treatments were analysed for behavioural responses. Upon arrival of the MFB, these experiments were repeated, using both beakers and MFB chambers.

In each experiment, singular organisms were placed either in the open beaker or within an MFB chamber. *D. magna* used were 24 hours old. *H. azteca* had been previously sieved through Nitex screen with pores sizes between 400 and 750 microns and allowed 24 hours recovery.

Each Reference had 12 replicates, (6 for beakers and 6 for chambers) for a total of 24 vessels. The test vessels were placed in the randomly-arranged beakers and chambers and acclimatized for two hours. Changes in swimming behaviour were made through video analysis and personal observations throughout the 6- hour duration of the experiment.

Behavioural patterns were classified based on previous research done by Marshall (2009). Through Marshall's research, behavioural characteristics of *D. magna* and *H. azteca* were quantified and qualitatively ranked. It was established that the most sensitive characteristics of *D. magna* for assessment of stress were 1) swimming height in the water column, 2) swimming style, and 3) immobilization. For *H. azteca* the most sensitive characteristics were established to be 1) immobilization, 2) substrate crawling, and 3) body length. These characteristics were used to evaluate changes in individual behaviour over time. Details of how characteristics of *D. magna* and *H. azteca* behaviour were classified can be found in Table 2.1 and 2.2.

**Table 2.1:** Measurements of Behavioural patterns Analysed to Determine Stress of *Daphnia magna* (Environment Canada, 2000; Marshall, 2009)

| **Swimming height** | **Swimming style** | **Immobilization** |
|---|---|---|
| - Test vessels were separated into 3 equal sections: top, middle and bottom<br><br>- Organisms were observed for 2 minutes during each hour of observation<br><br>- If the organisms were unable move outside of one section, their ability to move through the water column was considered impaired | - Natural swim style of *D. magna* is continuous movement made by strong smooth strokes with 2º antennae<br><br>- Under stress swimming style changes to short, jerky strokes and organisms may require bottom of test vessel for propulsion | - No movement of any kind including locomotive behaviours or antennae<br><br>- Mortality was determined through observation of the heart through carapace<br><br>- If observation of heart cannot be made, the solution is gently agitated and ability during the next 15 min was observed |

**Table 2.2:** Measurements of Behavioural patterns Analysed to Determine Stress of *Hyalella azteca* (Environment Canada, 1997; Marshall, 2009)

| **Immobilization** | **Body Length** |
|---|---|
| - No movement of any kind including locomotive behaviours or antennae and leg movements<br><br>- If no movement, the solution is gently agitated and ability during the next 15min was observed<br><br>- Organisms which did not swim after stimulation were considered dead | - Under normal conditions, organism bodies are fully extended and elongated during swimming, walking and resting activities<br><br>- Shortened bodies are observed under stress conditions in an attempt to reduce surface area of organism |

### *2.6.2 Long term survival of Daphnia magna and Hyalella azteca*

It was crucial to conduct long-term experiments to determine if *Daphnia magna* and *Hyalella azteca* would be able to survive for a week within the MFB chambers. Literature had suggested (Gerhardt *et al.*, 2003) that *D. magna* would be fine in a long-term MFB bioassay but this was needed to be ascertained. Additionally, and as mentioned earlier, this was the first time *Hyalella* had been used in MFB experiments. Four aquaria, with conditions similar to the culture aquaria, were set up for each species (Figure 2.4). Five MFB chambers were placed in each aquarium and every chamber contained an individual organism. The aquaria were left undisturbed for 7 days. *D. magna* and *H. azteca* were fed in accordance to the culturing protocol listed previously and survivorship of the organisms was observed daily.

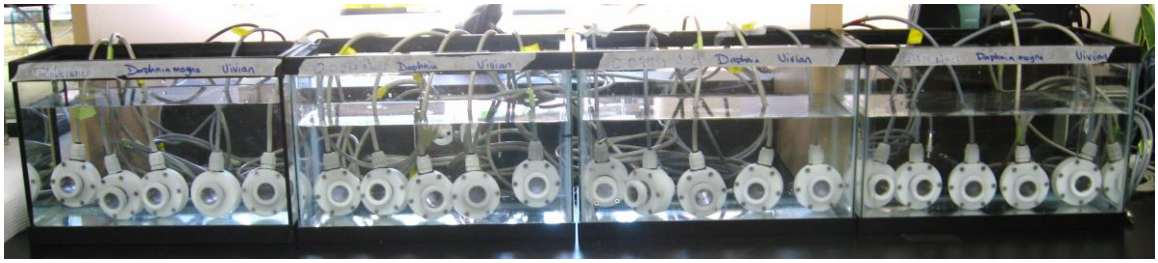


**Figure 2.4:** Long term experiments with *Daphnia magna* in MFB chambers

### *2.6.3 Assessment of MFB Electrical Field on Daphnia magna Behaviour*

Analysis of the effect of the electrical field created within the MFB chambers was done to determine if presence of an electrical field within the MFB chambers would affect the organisms located inside. Two chambers were attached using PVC tubing with an inner diameter of 2cm. The Nitex screen was removed from between the chambers to allow for free swimming of the organism between them. This chamber pair was placed in Reference water with an individual *Daphnia magna* inside each pair. One end of the chamber pair was pressed up against the aquarium glass to prevent escape of the *D. magna* and to allow for visual observations to be made. The other end was covered with Nitex screen to allow the free flow of water and prevent escape of the organism. Four chamber pairs were made.

The experiment was conducted by turning on the electric field in only one of the chambers in the pair for a period of 4 minutes. The location of the organism, whether in

the ‘on’ or ‘off’ chamber of a pair, was recorded once every minute for four minutes. The four-minute assessment was conducted four times over two days. During each assessment, the chambers were alternated between being turned ‘on’ and ‘off’.

## 2.7 Behavioural Bioassays upon Exposure to Solvent DMSO

### *2.7.1 Daphnia magna and Hyalella azteca in 0.1% DMSO*

As previously mentioned, DMSO is used as an organic solvent to ensure even distribution of the hydrophobic contaminants throughout the test water and prevent adsorption to the surfaces of the test vessels. The effect of DMSO has been examined by multiple researchers (Bowman *et al.*, 1981; Stratton *et al.*, 1985; Barbosa *et al.*, 2003; Marshall, 2009) but no work has been done using the MFB to evaluate this. Reactions of individual organisms both inside and outside of MFB chambers were measured using increasing concentrations of DMSO.

Individual *Daphnia magna* were placed in 400mL beakers with 200mL of test solution and observed for behavioural changes over 24hrs of exposure. The behaviours monitored throughout this experiment were the ability of *D. magna* to swim throughout the water column, the overall swimming activity of the organisms and immobility of any organisms. Concentrations of 0.1%, 1.0% and 10% DMSO were used, as well as Reference conditions. Each of the three DMSO concentrations and the Reference were set up for comparison in open vessels and inside the MFB chambers. Organisms were placed in the vessels and inside the chambers and acclimatized for two hours. There were three replicates of each treatment, for a total of 24 beakers. Test beakers were randomly arranged in order to control for variations that may be caused by temperature, lighting and other conditions in the laboratory. Changes in swimming behaviour were recorded based on visual observations throughout the duration of the experiment. Observations were made at 1hr, 2hrs, 3hrs and 24hrs of exposure to the test solutions to determine any effects caused by the DMSO. This experiment was repeated with *Hyalella azteca*.

After 24hrs of exposure, organisms were examined for mortality. Any organism not moving in either the beaker or inside the MFB chamber was gently prodded with a sterile transfer pipette to look for signs of life (Marshall, 2009). If no movement was

observed, the organism was considered dead. Mortality was not included in the study objectives, but deaths were recorded as important data.

## 2.8 Behavioural Bioassays upon Exposure to Contaminants Tributyltin (TBT) and Atrazine

### *2.8.1 Daphnia magna and Hyalella azteca exposure to Tributyltin*

The effect of three different concentrations of TBT on the behavioural activity of *Daphnia magna* and *Hyalella azteca* was determined using the MFB. Bioassay vessels used were 400mL beakers each with 200mL of test solution. A Reference of aerated dechlorinated municipal drinking water was used as well as 0.1% DMSO Reference to determine if any negative effects would arise from the organic solvent. The three concentrations of TBT tested were 1, 10 and 100 µg/L TBT in 0.1% DMSO. Four replicates of each treatment and reference were used. The MFB model utilized can hold a maximum of 24 chambers at one time. Two extra chambers were placed into Reference conditions with no organisms placed inside to be used as blanks for detection of any background disturbances. With the implementation of the blanks, a maximum of four chambers could be used per treatment and reference. In order to increase the number of replicates, the exact same experiment was repeated the following day. Therefore, each reference and treatment had a total of eight replicates, four from each day.

The vessels were randomized and data was analyzed for the 1st, 2nd, 6th and 12th hour of exposure. Organisms were placed in the chambers and given 2 hours for acclimatization before the data were collected. This experiment was repeated similarly for *Hyalella azteca*.

### *2.8.2 Daphnia magna and Hyalella azteca exposure to Atrazine*

The effect of three different concentrations of atrazine on the behavioural activity of *Daphnia magna* and *Hyalella azteca* was determined using the MFB. Bioassay vessels used were 400mL beakers each with 200mL of test solution. The three concentrations tested were 5, 50 and 500 µg/L atrazine in 0.1% DMSO. A Reference of aerated dechlorinated municipal drinking water was used as well as a 0.1% DMSO Reference to

determine if any negative effects would arise from the organic solvent. As with the TBT bioassay, a maximum of four replicates per reference and treatment could be used in a single test. The same conditions were repeated the following day in order to increase the number of replicates. Each reference and treatment had a total of eight replicates, four from each day. The vessels were randomised and data was analyzed for the 1st, 2nd, 6th and 12th hour of exposure. Organisms were placed in the chambers and given 2 hours for acclimatization before the data were collected.

Averages of the lowest frequency class (0.5Hz) were presented as mean over an hour, based on six measurements taken every hour. Organism behaviour was highly variable and multiple measurements of individual organisms were averaged to obtain each replicate value. All *D. magna* used in the following experiments were from the same brood and between 6 to 24 hours in age, as suggested by previous studies (Gerhardt *et al.*, 2003; Ren *et al.*, 2007). The *H. azteca* were sieved through Nitex mesh of 400 and 750 microns. At this size, the *H. azteca* are between 20 and 25 days of age (Othman & Pascoe, 2001).

## 2.9 The MFB Readouts

The final readouts generated by the MFB are presented as locomotive or ventilation activity in percentage. The activity values are derived from movements made by the individual organism over the period of continual monitoring. The monitoring periods last for four minutes and are taken once every ten minutes. During these periods, the MFB records the activities of the organism within the chamber and produces differing amplitude values that correspond to movements. The movements are grouped by the magnitude of impedance they produce, not by specific movement type such as swimming, antennae use or walking. The amplitudes are Fast Fourier Transformed by the MFB software to give different frequencies, measured in hertz. Each frequency, ranging from 0.5 to 8.5 Hz, corresponds to movements of a given magnitude made by the organism. The lower range of frequencies, from 0.5 to 2.5 Hz, represents movements of larger magnitude, predominantly locomotion. The upper range of frequencies, from 3.0 to 8.5 Hz, represents movements of smaller magnitude, such as those associated with ventilation. The 0.5Hz values for each monitoring period are the largest, with the

subsequent frequencies decreasing proportionally. Values for frequencies over 3.5Hz are rarely detected or presented by the MFB; therefore, the effects of the contaminants on organism ventilation were not analyzed. Locomotive activity, in percent, is the variable which is used to determine any changes in organism behaviour through a given experiment. Attempts were made to reduce the size of the standard deviations through several means. The number of replicates used for each test ranged from 7 to 9, as suggested by previous studies (Gerhardt *et al.*, 2003; Ren *et al.*, 2007). In addition, all tests were conducted beginning at the same time to prevent any diurnal activity changes of both *D. magna* and *H. azteca* from affecting their responses (Kirkpatrick *et al.*, 2006a). Other parameters were controlled for as much as possible as well. These included constant strength of the light source and covering the test vessels in order to block movement outside and in front of the chambers from influencing the organisms inside the chambers. The variable which was most difficult to control for was temperature, although paired tests were conducted at temperatures within 3 degrees of each other.

One of the most important variables was the size of the organisms. The amplitude of the locomotive activities is directly correlated to the relative size of the organisms within the chambers (Gerhardt, 1995). For example, a larger *D. magna* would register larger movement amplitude than even a slightly smaller *D. magna* performing the same movement in the same-sized chamber. If different sizes are used in the same test, very large standard deviations will occur, masking any treatment effects that may be caused by a contaminant.

As the goal is to determine early response behaviours of *D. magna* and *H. azteca* to TBT and atrazine to be used in an early-warning biomonitoring system, the $1^{st}$, $2^{nd}$ and $6^{th}$ hours of exposure were selected for analysis. In some cases, the $12^{th}$ hour of exposure is given to further assess reactions elicited by the organisms.

The values on the following graphs are the mean locomotive activity (%) of the organisms. The error bars represent standard deviation from the mean.

## 2.10 Statistical Analysis

For the purposes of the experiments in this study, only the lower hertz values were analyzed. Analysis of the data was done using Systat software version 12 (Systat Software, Inc, Chicago, IL). For experiments described in Section 2.8, as these tests were conducted over different days, a two-way ANOVA was used with treatment (i.e. contaminant concentration) as an independent variable and day (i.e. Day 1 or Day 2) as a blocking term. For other experiments, one-way ANOVA tests were used with treatment as the independent variable. For one-way ANOVA tests that determined significant differences among treatments, post-hoc pairwise comparisons (Tukey's HSD) were conducted to determine which treatment groups were significantly different from one another.

# 3 Results and Discussion

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## 3.1 Overview

Visual observations have been used for decades in traditional bioassays to classify and describe organism behavioural under both reference and stress conditions (e.g. Beattie & Pascoe, 1978; Flickinger *et al.*, 1982; Hill, 1989; Mackie, 1989; Bitton *et al.*, 1995). There is a human element that may result in error that is involved when performing visual observations, however, and developing automated systems to analyse organism behaviour has been undertaken throughout Europe, Asia and North America (e.g. Borcherding & Volpers, 1994; De Zwart *et al.*, 1995; Echelt *et al.*, 2000; Michels *et al.*, 2000; Tahedl & Hader, 2000; Van Der Schalie *et al.*, 2001; Gerhardt *et al.*, 2007; Jeon *et al.*, 2008). In the following sections, experimental results from both visual analysis and use of the MFB automated system are articulated and discussed. Additionally, the appropriateness of using *Daphnia magna* and *Hyalella azteca* as test organisms in the MFB is investigated in addition to whether the MFB itself is suitable for field use to monitor drinking water quality in southern Ontario. This study was the first to use *H. azteca* in MFB multi-organism systems.

In order to determine the suitability of the MFB, comparisons have been made between visual observations in beakers and automated readings in MFB chambers. The effect of the MFB chambers on the behaviour and survival of the organisms has also been investigated, and ultimately, the ability of the MFB to detect behavioural changes of the organisms when exposed to two contaminants was assessed.

Previous work has classified and quantified typical behavioural characteristics of *D. magna* and *H. azteca*, including the responses most likely to be observed under various stress conditions. This research was conducted by Marshall (2009) in a related project and is used as a foundation for the work presented in this study. Marshall determined which behaviours were exhibited most readily and reliably by *D. magna* and *H. azteca* for varying concentrations of tributyltin (TBT) and atrazine and the current study has built upon this information.

## 3.2 Establishing Behavioural Bioassays

In the present study, initial experiments using visual observations were conducted to analyse and observe the basic stress patterns of *Daphnia magna* and *Hyalella azteca* at

varying concentrations of TBT and atrazine. The influence of dimethyl sulfoxide (DMSO), an organic carrier, was also tested thoroughly to determine the appropriate concentration to be used so as not to generate a response from the organisms.

Additionally, before analysis of the behavioural effects of TBT and atrazine on *D. magna* and *H. azteca* in MFB could be conducted, assessment of the MFB and its chambers itself was required. This assessment focused primarily on whether placement of the organisms inside the MFB chambers would produce an altered behavioural reaction. Three different sets of experiments were thus conducted. Firstly, organism behaviour inside the MFB chambers was visually analysed and compared to organism behaviour when they were tested in regular test vessels ("beaker" experiments versus "chamber" experiments). This analysis was conducted for the first twelve hours of chamber placement to identify any short-term variability that may occur. Secondly, the assessment of the MFB equipment was conducted in a long-term bioassay. The survival of the organisms within the chambers was observed over 7 days to determine if it is possible to leave the equipment and the organisms unattended for longer durations, as they would be in a remote monitoring system. Thirdly, the possible effect of the electrical field that is created across the MFB chambers was tested on *D. magna* through analysis of attraction to or avoidance of chambers that were turned 'on' or 'off'.

### *3.2.1 Comparison of Beakers versus Chambers using Daphnia magna*

In order to determine if placement of *D. magna* inside the MFB chambers would cause an alteration of their normal behaviour, parallel bioassays were run with organisms in Reference conditions to analyse their swimming patterns both inside and out of the confines of the chambers. The methods used here to analyse *D. magna* behaviour were derived from the methods developed by Marshall (2009). Marshall determined that swimming height in the water column was the most sensitive response elicited by *D. magna* under stressful conditions. The second and third most sensitive parameters were swimming style and immobilization. It was determined that spinning, body orientation, and secondary antennae use were also characteristics that could demonstrate organism stress, although they are not as sensitive (Marshall, 2009). The first three parameters,

swimming height, swimming style and immobilization, were used in the analysis described here.

There was no change in swimming height in the water column for *D. magna* in reference conditions either inside or out of the MFB chambers from the first to the twelfth hour. There was also no change noted in the organisms in 0.1% DMSO solution after 12 hour relative to reference organisms.

There was no change in swimming style made by the *D. magna* or in the control they demonstrated over their movements comparing 0.1% DMSO with Reference. No impaired swimming was noted in the *D. magna* inside or out of the MFB chambers. Lastly, none of the organisms were immobile in either the Reference or DMSO conditions throughout the 12-hour duration of the experiment and survival was 100% in all test vessels inside and outside of MFB chambers.

Finally, as the chambers did not cause any visually detectable changes in swimming height, swimming style or mobility in *D. magna*, it was concluded that analysis of *D. magna* behaviour can be conducted using the MFB without the chambers affecting results or contributing to false alarms being produced.

### *3.2.2 Comparison of Beakers versus Chambers using Hyalella azteca*

As with the *D. magna*, before analysis of the effect of contaminants of concern could commence, comparative assessment of *Hyalella azteca's* behaviour inside and outside of the MFB chambers was required.

The most sensitive behavioural parameters for detecting stress in *H. azteca*, as defined by Marshall (2009), are immobilization, substrate crawling and body length. Marshall also analysed the usability and effectiveness of swimming events, burrowing, grouping and body orientations. It was established that these parameters were not as effective at describing organism stress levels when exposed to TBT or atrazine. In the following analysis of the possible influence of the MFB chambers on *H. azteca* behaviour, immobilization and body length were used as parameters to assess organism stress. Substrate crawling was not used to analyse overall stress of the organisms, although it was deemed an accurate measurement parameter by Marshall (2009). This parameter was not assessed as no additional substrate was placed in either the MFB

chambers or the test vessels. The chambers themselves contained grooves between the anodes which give some shelter to the *H. azteca* while still allowing them to be visually monitored at all times. Introduction of an external substrate, such as gauze (Marshall, 2009) or leaves (Gerhardt, 1998), may prevent visual analysis from being possible. Comparison between substrate crawling in the chambers versus in the test vessels was not attempted as any preference or repulsion of the organisms to the different surface materials (glass, stainless steel, acrylic etc.) was unknown.

As defined by Marshall (2009), no movement of any *H. azteca* body parts could occur in order to classify the organism as immobile. This included locomotion, such as swimming and substrate crawling, as well as movements associated with burrowing, any contractions of the body, and any movement of the legs if the organisms were lying on their side in the sediment. Using this description, none of the organisms were considered immobile throughout the 12-hour duration of the experiment in either the chambers or in the 0.1% DMSO solution.

Body length was also not affected by placement of the organisms in the chambers. Throughout the 12-hour duration of the experiment, the individuals maintained elongated and fully extended bodies.

As explained in Section 3.2.1, mobility is highly important to the survival and biological functions of organisms such as predator avoidance, mating and foraging. Immobilization likely occurs when *H. azteca* are under different levels of stress as they attempt to avoid or adapt to a contaminant. It was also suggested by Marshall (2009) that because of differing response times for different concentrations of TBT and atrazine, it may be possible for the immobilization parameter to be used to differentiate between the two classes of contaminants. Body length could be explained as a stress response by reducing the amount of body surface area exposed to the contaminant or predator. It was shown to have a rapid response time to various TBT and atrazine concentrations, although the responses caused by the different contaminants were not significantly different. Body length therefore cannot be used to differentiate between these two contaminants (Marshall, 2009).

As the chambers did not cause any visually detectable changes in mobility or body length in *H. azteca*, it can be concluded that analysis of their behaviour can be

conducted using the MFB without the chambers affecting results or contributing to false alarms.

### *3.2.3 Daphnia magna and Hyalella azteca Long-Term in the MFB*

In order for the MFB to be used in the field as a remote water monitor, it is essential that it be able to be left unattended for extended periods. A time of five to seven days has been noted to be the realistic time a biomonitor can be left in the field without being attended (Lechelt *et al.*, 2000). To determine if placement in the MFB chambers may cause adverse effects to *Daphnia magna* and *Hyalella azteca* over longer periods of time, the organisms were placed inside the chambers without interference for seven days. After 7 days in the chambers, 100% of both *D. magna* and *H. azteca* survived. It should also be noted that after 5 days in the chambers, neonates were present in one of the *D. magna* chambers.

This test was not done to assess behavioural changes of the organisms, but rather whether or not survival would be an issue for the organisms within a confined space for longer durations. The issue of confined space is more prevalent when assessing the applicability of *D. magna* as a test organism with the MFB. *D. magna* are phototactic by nature (Martins *et al.*, 2007) as their food source, algae, is located relative to light sources. Organisms were being fed invertebrate food as well as algae in this experiment; however, it is possible that being restricted in their swimming patterns might have caused extra stress to the organisms and limited their ability to survive and give useful responses. They are also continuously swimming through the water under normal conditions and restriction of swimming space, especially as the organism grows in size, may act as a detriment.

From initial conclusions, *H. azteca* appears to be an adequate species to be used for longer testing periods in the MFB. Previous experiments have been conducted with other freshwater amphipods in the MFB such as *Gammarus pulex* (Gerhardt ,1995; Gerhardt *et al.*, 1998), *C. pseudogracilis* (Kirkpatrick *et al.*, 2006a) and *Corophium volutator* (Kirkpatrick *et al.*, 2006b). Most notably, long-term experimentation in the field has been conducted using *G. pulex* (Gerhardt *et al.*, 2006). It was concluded that *G. pulex* survived better and had more stable locomotive behaviour during long-term

monitoring than *D. magna* over the duration of the test and that clearer, more accurate responses were elicited from the amphipod (Gerhardt *et a*l, 2003). This is not to say that *D. magna* is necessarily an inappropriate species for MFB use, or that an untested amphipod (*H. azteca*) is necessarily an appropriate species. Previous studies have shown that *D. magna* behaviour is a sensitive indicator of sublethal stress and can be used in the MFB efficiently (Ren *et al.*, 2007; Gerhardt *et al.*, 2005, Gerhardt *et al.*, 2006).

There is possible issue, however, with the reproduction and growth rate of *D. magna*. Young neonates are able to freely move through the mesh of the MFB chambers and escape to the outer test solution. If any of the neonates remain within the chamber, they may cause increased impedances within the electrical field of the chamber. This in turn may cause false alarms and/or altered responses to be picked up by the equipment. Thus, in order to prevent the birth of neonates during the duration of the experiment or in field application, younger organisms must be initially placed within the chambers. A second issue arises from the fairly rapid growth rate of *D. magna* within the first 7 to 10 days of life. The signal picked up by the MFB of the organism's movement is directly correlated to the size of the organism relative to the size of the chamber (Gerhardt, 1995). As the organisms grow within the chamber, the relative signals produced will also increase. Since *D. magna* grow so rapidly, different signals will be generated over the course of a week and false alarms may be produced. *D. magna* have been used successfully in MFB bioassays in short-term, acute toxicity tests of 24 and 48 hours (Gerhardt *et al.*, 2005; Ren *et al.*, 2007). There have been no assessments of individual *D. magna* applicability in the long-term use of the MFB. In one study, Gerhardt *et al.* (2003) assessed the use of five *D. magna* placed simultaneously into a single MFB chamber. They concluded that although *D. magna* were able to detect changes in the water quality, the use of multiple organisms in a single chamber increased variability, thereby reducing sensitivity. It was suggested that the amphipod *Gammarus pulex* was more appropriate for long-term (3 – 4 weeks) assessment using the MFB than *D. magna* because of longer life cycles. The life cycle of daphnids is short and during the one month duration of the *in situ* test, many daphnids died due to normal ageing. When *D. magna* are used in biomonitors, they must be replaced more often (every 1 – 2 weeks) when compared to *G. pulex* (every 3 – 4weeks) (Gerhardt *et al.*, 2003). To optimize survival and sensitivity of

*D. magna* in the MFB, Gerhardt *et al.* (2003) concluded that daphnids should be placed individually in the chambers.

The experiment conducted in the present study suggests that *H. azteca* do not appear to have issues with survival in the MFB chambers for long periods. While studies have shown that *D. magna* are fully applicable in short-term biomonitoring, further analysis should be conducted in order to determine if they are ideal organisms to be used in long-term biomonitoring using the MFB. From this study, *D. magna* appears to be applicable for long-term testing for up to 1 week. Thorough analysis must be conducted to determine the effects of different contaminants on *D. magna* at different ages as daily differences in locomotive activity levels have been noted (Matthias & Puzicha, 1990).

### *3.2.4 Influence of MFB Electrical Field on Daphnia magna*

When an experiment is conducted with the MFB, a pair of electrodes inside each selected chamber creates an electric field within each chamber through which the test organism swims (see Sec. 2.9 for detailed explanation of MFB measurements). It is the impedance created by organism movement within this field which generates the MFB data values. The parameters of the MFB can be set to select which chambers are to be used during each test. This experiment was designed to determine if the generated electrical field inside the chamber would affect the behaviour of the organisms swimming through it. To do so, two chambers were attached and the Nitex mesh was removed to allow free movement of the organism between them. The parameters were set to select one chamber of each pair to be turned "on" while the other remained "off". The location of each *D. magna* was recorded every sixty seconds for the duration of the test. Chi-squared analysis was applied to determine if the *D. magna* had a preference for either the 'on' or 'off' chamber. No significant attraction to or avoidance of chambers that were turned 'on' or 'off' were observed at any time period (Fig 3.1; Table 3.1).

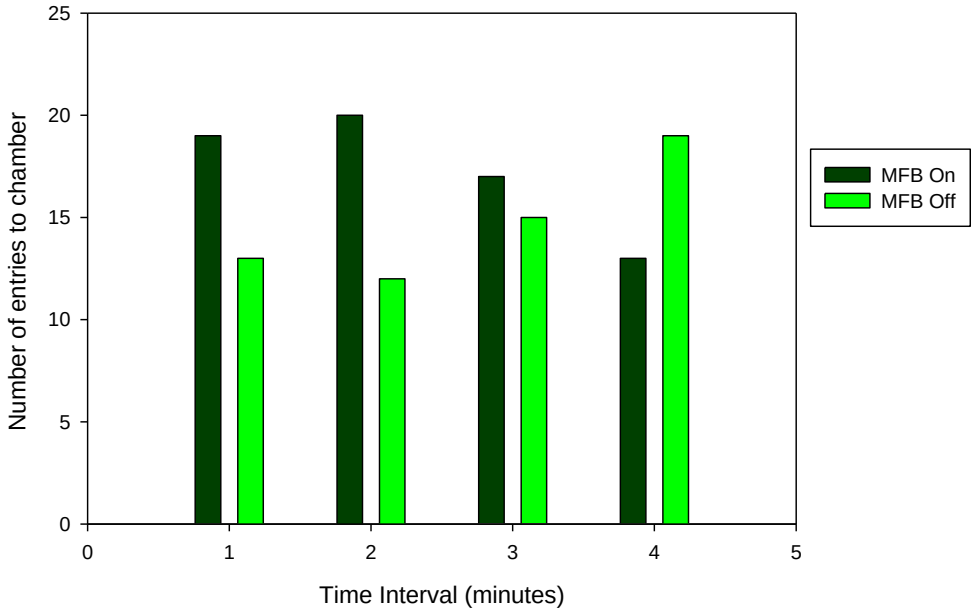


**Figure 3.1:** Number of times *Daphnia magna* was recorded as located in a MFB chamber when the MFB was 'on' compared with when the MFB was 'off' in Reference water

When all time points are combined to obtain a total number of location choices made by the *D. magna* for the full experiment, 69 *D. magna* were recorded in the 'on' chambers and 59 *D. magna* were recorded in the 'off' chambers. The Chi-square for this combined test is 0.7812 with a corresponding p-value of 0.3767.

**Table 3.1:** Chi-square values for one minute time intervals and total time for Daphnia magna in choice chambers

| Time (mins) | χ2 | df | p-value |
|---|---|---|---|
| 1 | 1.125 | 1 | 0.288 |
| 2 | 2.000 | 1 | 0.157 |
| 3 | 0.125 | 1 | 0.724 |
| 4 | 1.125 | 1 | 0.288 |
| Total | 0.781 | 1 | 0.377 |

As no preference or avoidance of 'on/off' chambers were noted, it can be said that the activation of the electrodes is not affecting *D. magna* behaviour and the use of MFB chambers should not, due to electrical field, bias results.

## 3.3 Behavioural Bioassays with Exposure to Solvent DMSO

### *3.3.1 Daphnia magna Response to DMSO Exposure*

*3.3.1.1 Beaker Visual Analysis*

Dimethyl sulfoxide (DMSO) was used as an organic solvent in order to ensure that TBT and atrazine were evenly distributed throughout the test water and that the contaminants did not adsorb to equipment surfaces (Bowman *et al.* 1981; De la Torre *et al.*, 1995). Initial bioassays were conducted to determine the concentration at which DMSO would have no detrimental effect on the organisms while still having a high enough concentration to operate as a solvent. In order for the reactions of the organisms to be useful in future biomonitoring systems, there must be no effect caused by the DMSO. It is unlikely that this solvent will be found in the natural environment in conjunction with either TBT or atrazine; therefore, it is crucial that the reactions elicited by the organisms are those directly associated with the contaminant(s) they are being exposed to. Visual analysis of organisms was conducted to determine which concentration of DMSO elicited no behavioural responses from *Daphnia magna*. It was observed that at both 0.5% and 1% DMSO, an adverse behavioural effect was present after 3 hours (Table 3.2).

**Table 3.2:** Behavioural Responses of *Daphna magna* when exposed to several concentrations of DMSO

| | **1% DMSO** | **0.5% DMSO** | **0.1%DMSO** |
|---|---|---|---|
| **Swimming height** | - 80% of daphnids confined to one section of beaker after 3 hours of exposure | - 60% of daphnids confined to one section of beaker after 3 hours of exposure | - No effect observed |
| **Swimming style** | - 60% of daphnids demonstrated short, jerky swimming movements after 3 hours of exposure | - 60% of daphnids demonstrated short, jerky swimming movements after 6 hours of exposure | - No effect observed |
| **Immobility** | - No effect observed | - No effect observed | - No effect observed |

However, no alterations in behaviour were observed when the organisms were exposed to 0.1% DMSO. These findings support the validity of the bioasssays conducted

by Marshall (2009), who also used 0.1% DMSO as a solvent for her studies. This concentration has also been investigated and deemed to be appropriate in other studies (Bowman *et al.,* 1981; De la Torre *et al.*, 1995; Barbosa *et al.,* 2003).

*3.3.1.2 MFB Chamber Analysis*

Once the visual analysis was complete, it remained to be confirmed that 0.1% DMSO could also be used in the MFB chambers without affecting behaviour. As the MFB may detect behavioural changes not apparent through human/visual observation, it had to be demonstrated that this carrier concentration was acceptable. Several previous studies with the MFB and *D. magna* used DMSO as a solvent for organophosphate pesticides (Ren *et al.,* 2007; Ren *et al.,* 2008). These studies used DMSO concentrations of less than 0.5% in the tests citing Sandbacka *et al.* (2000) that such concentrations would neither lead to acute toxicity to *D. magna* nor affect its mobility. Through other assessments conducted (the present study; Marshall, 2009) 0.5% DMSO concentrations did effect *D. magna* mobility and therefore 0.1% DMSO was applied here instead.

Analysis of *D. magna*'s behaviour after 1, 6 and 12 hours of exposure are shown in Figures 3.2, 3.3, and 3.4 respectively. In order to increase the number of replicates and determine comparability between bioassays conducted with the MFB, the effect of 0.1% DMSO on *D. magna* was done by conducting three identical experiments on May 7$^{th}$, 26$^{th}$, and 29$^{th}$ of 2010. After one hour of exposure (Figure 3.2), there were no significant differences between Reference and DMSO treatments on May 7$^{th}$ ($p = 0.266$), May 26$^{th}$ ($p = 0.431$), or May 29$^{th}$ ($p = 0.148$). There was, however, a significant difference of locomotive activity among dates ($p = 0.001$) with activity higher on May 26$^{th}$ than that on other dates for both the Reference and DMSO treatments (Appendix A). This difference was present despite control of organism age (20- 24 hours old), temperature of water (22 ± 1 ºC), time at which experiments were conducted (all test began at 12 noon), feeding time and rate, and external disturbances in the laboratory such as vibrations. The issue most likely lies with the organism activities as previous testing has shown high variability of *D. magna* in the MFB (Gerhardt *et al.,* 2003; Gerhardt *et al.,* 2005; Ren *et al.,* 2007).

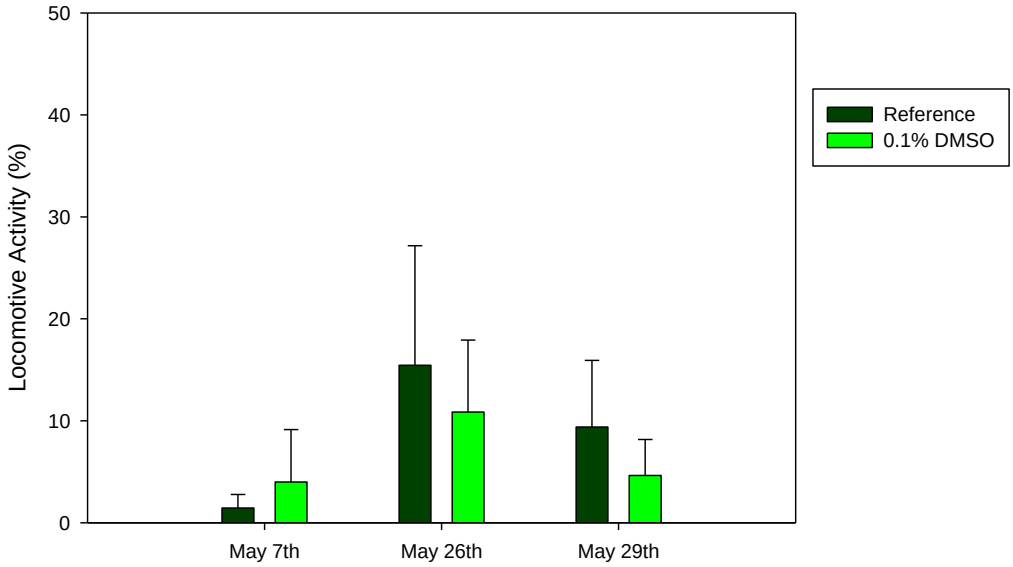


**Figure 3.2:** Locomotive activity (%) of *Daphnia magna* on three different days when exposed to 0.1% DMSO for 1 hour. Values plotted are mean ± standard deviation.

After 6 hours of exposure (Figure 3.3.), the overall locomotive activity of the organisms diminished slightly for May 26$^{th}$ in comparison to the first hour of exposure. This can be attributed to acclimation of the organisms to the MFB chambers. When first placed in the chambers, organisms may show increased activity as a result of transference stress. This decrease in activity is not due to any influence of the DMSO as the activities of both the Reference and DMSO saw a decrease. Between the Reference and DMSO treatments, there was no significant difference on May 7$^{th}$ ($p = 0.185$) or on May 26$^{th}$ ($p = 0.645$); however, May 29$^{th}$ saw a borderline difference ($p = 0.051$). When the three dates were analysed together using ANOVA, there was no overall significant difference between Reference and DMSO conditions ($p = 0.916$). This indicates that after 6 hours of exposure to 0.1% DMSO, there is no influence on *D. magna* activity that is detectable by the MFB.

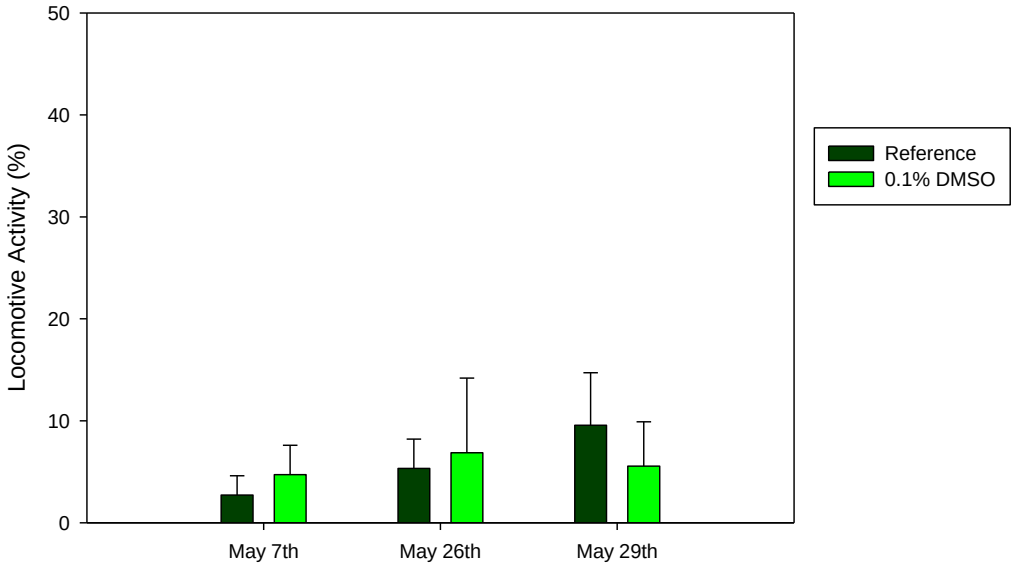


**Figure 3.3:** Locomotive activity (%) of *Daphnia magna* on three different days when exposed to 0.1% DMSO for 6 hours. Values plotted are mean ± standard deviation.

The "date effect" noted in the first hour of the test is not longer significant after 6 hours ($p = 0.122$) most likely because the organisms used on May 26$^{th}$ demonstrated a decrease in locomotive activity during this time. At 1 hour, the May 26$^{th}$ *D. magna* had an average locomotive activity of 15.4% in Reference and 10.9% in DMSO. After 6 hours, the same organisms had an average locomotive activity of 5.3% and 9.6% respectively. The decrease in locomotive activity is probably linked to the acclimatization of the organisms to the MFB chambers. When placed into the chambers, the *D. magna* were transferred using a sterile pipette with an opening of 5mm diameter. This transference can cause increased stress in the organisms and they must be given time to calm and return to normal swimming patterns. Two hours was given for this acclimatization. However, it would seem that the organisms on May 26$^{th}$ required more time to adjust. As several variables (see above) were controlled for to prevent such variations, it is difficult to say exactly what caused the difference in acclimatization requirement. Possibly it was caused by differences in temperature. Although the temperatures were within 2ºC on all three dates, *D. magna* are very sensitive to temperature and this may have affected acclimatization times required.

After 12 hours of exposure (Figure 3.4), there continued to be no significant difference between the Reference and DMSO conditions on May 7$^{th}$ ($p = 0.408$) and May 29$^{th}$ ($p = 0.486$). A slight significance was noted on May 26$^{th}$ ($p = 0.043$) between Reference and DMSO conditions, with the higher activity being seen in the Reference. This could be attributed to an influence of the DMSO on activity of the organisms; however, no trend in increasing or decreasing activity was seen to be caused by the DMSO. During the 1$^{st}$, 6$^{th}$ and 12$^{th}$ hours of detection over all three dates, there was no overall increase or decrease in locomotive activity that can be attributed to DMSO effect. In addition, there is absolutely no difference between Reference and DMSO conditions for the other two dates, May 7$^{th}$ and May 29$^{th}$, at 12 hours. Therefore this slight significance noted on May 26$^{th}$ at the 12$^{th}$ hour is most likely not caused by the DMSO but rather a normal fluctuation of locomotive activities.

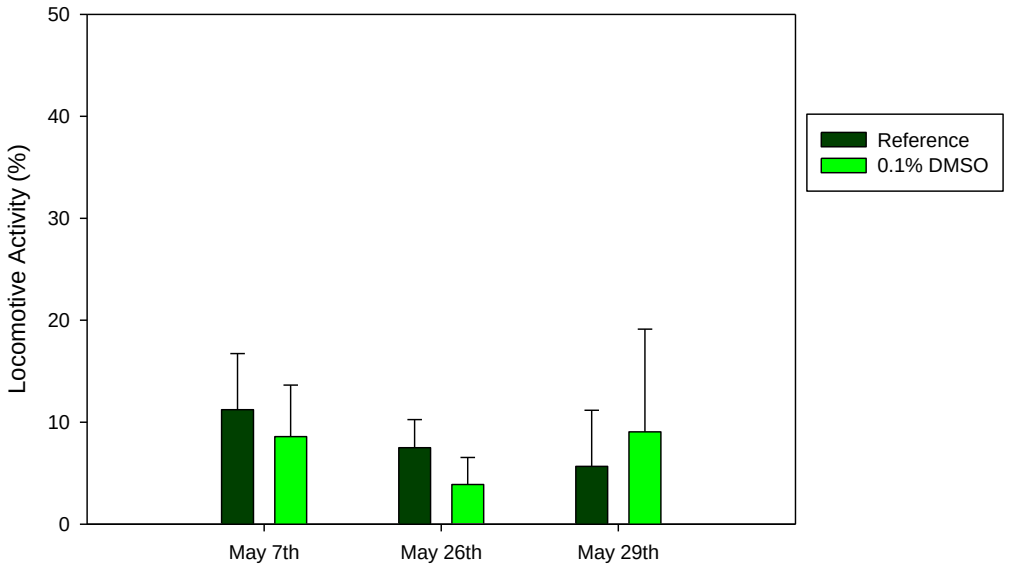


**Figure 3.4:** Locomotive activity (%) of *Daphnia magna* over three different days when exposed to 0.1% DMSO for 12 hours. Values plotted are mean ± standard deviation.

No significant difference was seen in *D. magna* locomotive activity between Reference conditions and exposure to the 0.1% DMSO solution when the three dates were analysed together over the full 12-hour experimental period ($p = 0.614$, 0.916, and 0.626 for 1, 6, and 12 hours, respectively). There was no general trend of increasing or decreasing activity caused by DMSO exposure and it is possible that this difference could

be attributed to the behavioural fluctuations of the organisms. The overall lack of significant differences between Reference and DMSO conditions agrees with the results determined in the "beaker" visual analysis bioassays. Because of these results, 0.1% DMSO was considered a safe solvent concentration for testing effects of TBT and atrazine on *D. magna* using the MFB.

### *3.3.2 Hyalella azteca Response to DMSO Exposure*

#### *3.3.2.1 Beaker Visual Analysis*

Visual analysis of *Hyalella azteca* was conducted to determine which concentration of DMSO elicited no behavioural responses. It was observed that at both 0.5% and 1% DMSO, an adverse behavioural effect was present after 3 hours expressed in shortened body length (Table 3.3). Immobility was also observed after 6 hours of exposure at a concentration of 1% DMSO. No alterations in either body length or immobility were observed when the organisms were exposed to 0.1% DMSO. These findings support the validity of bioasssays conducted by Marshall (2009), who also used 0.1% DMSO as a solvent for her studies.

**Table 3.3:** Behavioural Responses of *Hyalella azteca* when exposed to several concentrations of DMSO

| | **1% DMSO** | **0.5% DMSO** | **0.1%DMSO** |
|---|---|---|---|
| **Immobility** | - Immobility was observed in 50% of organisms after 6 hours of exposure | - No effect observed | - No effect observed |
| **Body Length** | - 70% of organisms demonstrated shortened body length after 3 hours of exposure | - 70% of organisms demonstrated shortened body length after 3 hours of exposure | - No effect observed |

#### *3.3.2.2 MFB Chamber Analysis*

As with the *Daphnia magna* MFB chamber analysis, tests were conducted to determine if 0.1% DMSO had an influence on *Hyalella azteca* detectable by the MFB. In this experiment with *H. azteca*, identical tests were conducted on two days (May 19$^{th}$ and 20$^{th}$) and gave comparable results.

Analysis of *H. azteca* behaviour after 1, 6 and 12 hours of exposure are shown in Figures 3.5, 3.6 and 3.7 respectively. Locomotive activity, in percent, represents the movement and swimming behaviours of the organisms as recorded by the MFB. The figures depict the mean of the lowest frequencies generated by the MFB, 0.5 Hz, with error bars of standard deviations. This frequency represents the largest movement made by the organisms and all subsequent frequencies follow the same pattern. After one hour of exposure to 0.1% DMSO (Figure 3.5), no significant difference was seen on either May 19$^{th}$ ($p = 0.065$) or May 20$^{th}$ ($p = 0.246$).

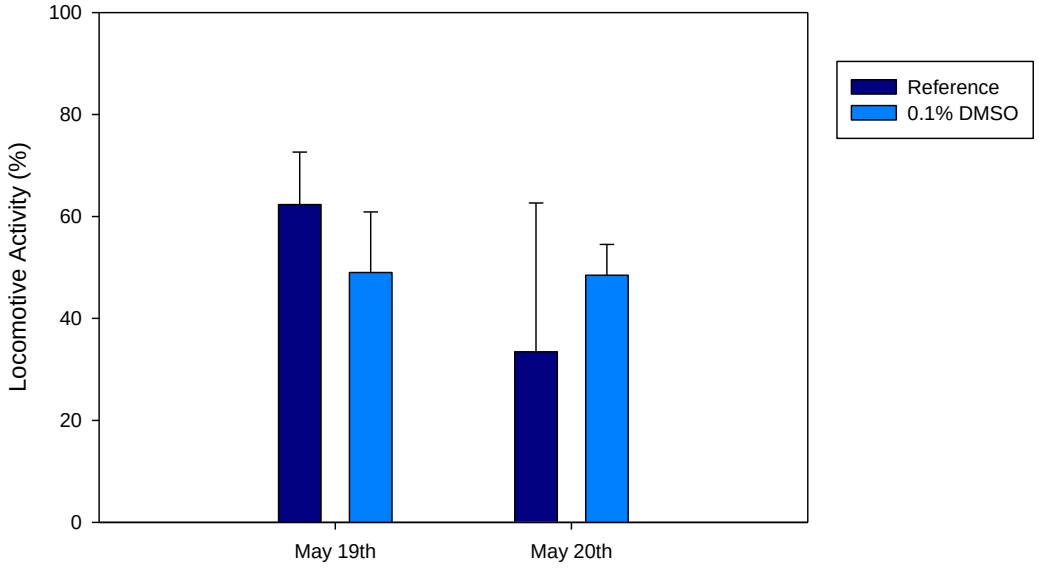


**Figure 3.5:** Locomotive activity (%) of *Hyalella azteca* during two days after 1 hour of exposure to 0.1% DMSO. Values plotted are mean ± standard deviation.

Reverse trends are seen, with locomotive activity being higher for the Reference than the DMSO treatment on May 19$^{th}$, while organisms used on May 20$^{th}$ had reversed locomotive activities. As no significant differences were present and no correlating trends are seen, it can be concluded that after one hour of exposure, 0.1% DMSO has no influence on the locomotive activity of *H. azteca*. This finding supports the "beaker" analysis outlined previously as well as previous work by Marshall (2009).

Although no effect was seen from 0.1% DMSO exposure, as with the *D. magna* experiments, a slightly significant difference was seen between locomotive activities

between dates May 19th and May 20th ($p = 0.045$). As with the *D. magna* experiment, a variety of variables were controlled in order to create reproducible data including organism size, temperature of test water, time at which experiments were conducted, feeding time and rate, and external disturbances in the laboratory such as vibrations or disturbing of the test vessels. In this case, the difference is most visible between the Reference activities with the May 19th organisms having an average activity of 62.3% while May 20th showed an average of 33.4%. However, these values had very large standard deviations of 10.3% for May 19th and 29.2% for May 20th. Because of these huge deviations, the difference noted between the dates is most likely attributed to normal fluctuations of organism locomotive activity, ranging form stationary at the bottom of the chamber to swimming freely throughout the water. Large standard deviations were attempted to be minimized through strict control of afore mentioned variables; however, it was not possible. Large deviations are normal to organisms as they have a variety of behaviours. However, such deviations can cause issues when differentiating between treatment effects.

It should be noted that the locomotive activities for *H. azteca* are quite a bit higher than those of *D. magna*. The magnitude of the readings generated by the MFB is proportional to the size of the organism inside the chamber (Gerhardt & Svensson, 1994). Larger organisms will generate larger impedance signals in the electric field generated within the chamber. Therefore, as *H. azteca* is larger in size than *D. magna*, the locomotive activities generated were much higher.

There was no significant difference present after 6 hours of exposure for May 19th ($p = 0.799$) or May 20th ($p = 0.297$) between the Reference and 0.1% DMSO conditions (Figure 3.6).

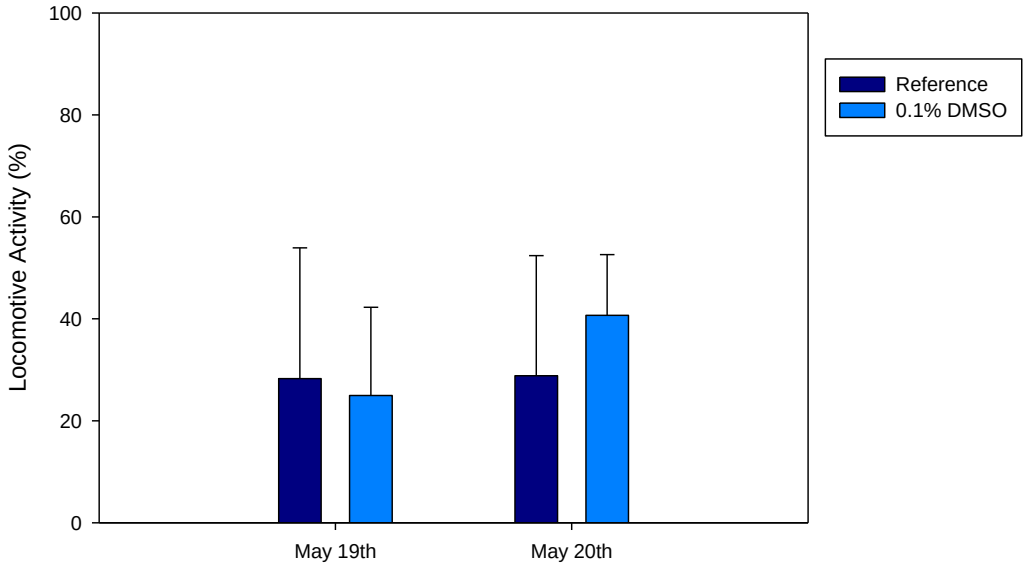


**Figure 3.6:** Locomotive activity (%) of *Hyalella azteca* during two days after 6 hours of exposure to 0.1% DMSO. Values are mean ± standard deviation.

The "date effect", as with *D. magna*, is no longer present ($p = 0.338$). After 6 hours, it can be noted that there is a decrease in overall locomotive activity. The most notable difference lies between the first hour of the May 19$^{th}$ Reference (activity = 62.3%) and the sixth hour of the same organisms (activity = 28.3%). This decrease is attributed to the acclimatization of the organisms to placement in the MFB chambers. As with the *D. magna*, *H. azteca* were transferred into the chambers using sterile pipettes with openings of 5mm in diameter (Environment Canada, 1997) and given 2 hours to acclimate (Gerhardt *et al.*, 1998; Kirkpartick *et al.*, 2006a). By comparing the first and sixth hour, it can be observed that organisms continued to settle after the 2-hour acclimation and demonstrate a decrease in locomotive activity as time passed. It is possible that a longer acclimation period was required as treatment effects may be hidden in the increased locomotive activity associated with transferring. However, early-warning signs are of importance and assessment of organism behaviour and locomotive activity cannot be made for the first time after 6 hours of exposure. This issue could be addressed with the introduction of a flow-through test system, rather than a batch experiment. In this way, organisms can be placed in the chambers and allowed to acclimatize for more than two hours under reference conditions. After such time, the contaminant or stressor

can be added to the water and the locomotive activity of the organism can be assessed purely based on contaminant effect. In addition, flow through systems are a better representation of an in field situation.

At the conclusion of the experiments, after 12 hours, there continued to be no significant difference between the Reference and DMSO conditions on May 19$^{th}$ ($p = 0.735$) or on May 20$^{th}$ ($p = 0.060$) (Fig. 3.7). The "date effect" still was not significant ($p = 0.706$). It can be concluded that, as with the *D. magna*, the *H. azteca* were not influenced by the presence of 0.1% DMSO.

There was still a large variability noted in the standard deviations, however, suggesting that the organisms were exhibiting different behaviours from each other throughout the hours under analysis. This was to be expected as organisms respond to a variety of different behaviours throughout the duration of the test, such as swimming, walking and remaining stationary for extended periods. However the presence of large standard deviations can cause treatment effects to be hidden in the large deviations.

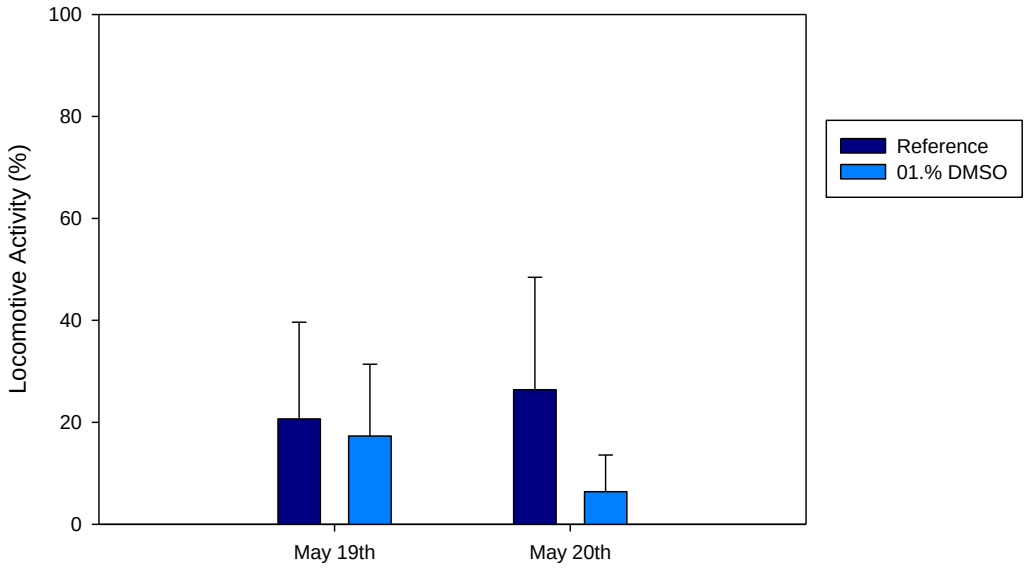


**Figure 3.7:** Locomotive activity (%) of *Hyalella azteca* during two days after 12 hours of exposure to 0.1% DMSO. Values are mean ± standard deviation.

### *3.3.3 Summary of D. magna and H. azteca exposure to 0.1% DMSO*

From the four experiments outlined and discussed above, it can be concluded that 0.1% DMSO has no influence on the behaviour and locomotive activity of either *D. magna* or *H. azteca* based on visual observations and through MFB analysis. Because of this, 0.1% DMSO could be used in the subsequent experiments as an organic carrier to ensure proper distribution of tributyltin and atrazine through the test waters.

## 3.4 Behavioural Bioassays Exposure to Contaminants TBT and Atrazine

### *3.4.1 Daphnia magna Exposed to Tributyltin (TBT)*

In the following results, averages of the lowest frequency (0.5 Hz) are presented as averages over an hour period. Six measurements are taken every hour. It is to be determined what early response behaviours are exhibited by *D. magna* when exposed to TBT. This will establish whether *D. magna* is indeed an adequate test species to be used to detect TBT in an automated system and whether the MFB is a good addition to the larger technology of the early-warning system. The $1^{st}$, $2^{nd}$, $6^{th}$ and $12^{th}$ hours of exposure were selected for graphing to identify if and when an early reaction can be seen from the organisms (Fig. 3.8a) A comparison between the two tests conducted is also shown to demonstrate any differences between the two dates (Fig. 3.8b).

During the first hour, a marginally significant difference was noted between the Reference and DMSO Reference treatments (Figure 3.8a, $p = 0.057$). No significant differences were present between the References and the three TBT treatment concentrations. The difference noted between the Reference and the 0.1% DMSO Reference was not expected as thorough testing had been previously done through visual observation in beakers and assessment using the MFB. Very large standard deviations were seen in all treatments, making identifying minor effects on activity very difficult. In both 10µg/L and 100µg/L TBT average activities were lower than the Reference value as expected from the mode of action of TBT; however, with the large standard deviations, these effects were not significant. There was a significant difference between the experiments conducted on two dates ($p < 0.001$). Comparison among TBT treatment

levels controlled for the variation among dates by using date as a blocking term in the ANOVA (Appendix B).

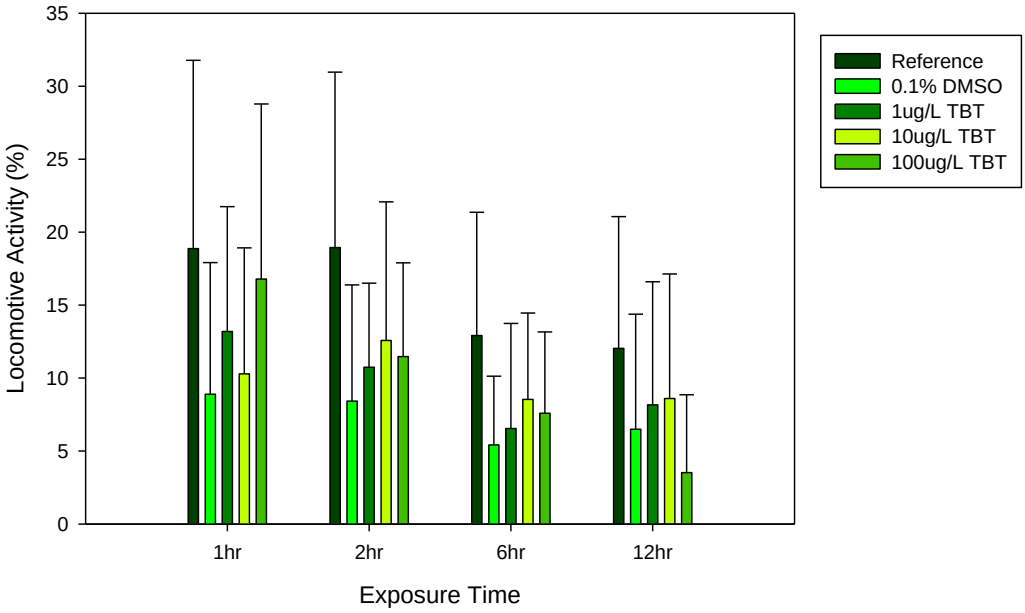


**Figure 3.8a:** Locomotive activity (%) of *Daphnia magna* over 12 hours of exposure to three concentrations of TBT. Values are mean ± standard deviation.

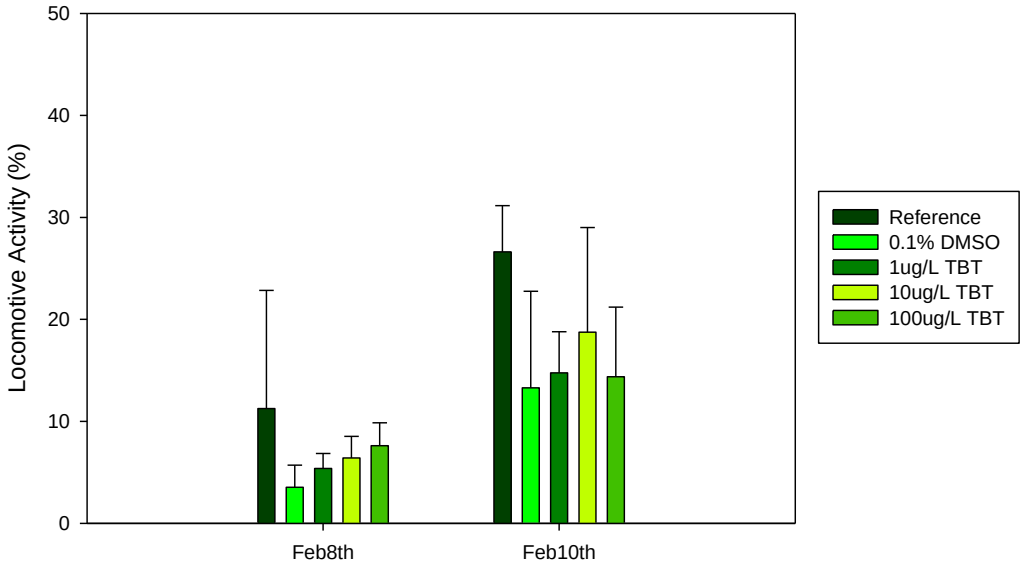


**Figure 3.8b:** Locomotive activity (%) of *Daphnia magna* after 2-hour of exposure to three concentrations of TBT – Date effect from Feb 8$^{th}$ and Feb 10$^{th}$. Values are mean ± standard deviation.

During the second hour of exposure to TBT, a strong "date effect" continued to be observed ($p < 0.001$) while the difference of locomotive activities continued to be significant between the Reference and DMSO Reference treatments ($p = 0.037$). No significant differences were noted among any of the TBT treatments, or upon comparison to Reference and DMSO Reference treatments.

After six hours of exposure, the difference between Reference and 0.1% DMSO treatments was no longer significant ($p = 0.069$). There were, in fact, no significant overall treatment effects during hour 6 ($p = 0.075$) or hour 12 ($p = 0.472$). However, there remained a "date effect" during both time periods ($p = 0.001$ and $p = 0.014$ for 6 hour and 12 hour, respectively), which was controlled for in assessment of treatment effects by using date as a blocking term (Appendix B).

These results are in contrast to visual observations of Marshall (2009) after 2 hours of exposure to TBT. In her experiments, ability to swim through the water column was strongly affected after both 1 and 2 hours of exposure. She also noted that swimming style was affected slightly at this time at 10µg/L and 100µg/L, while effects on immobilization were seen following longer exposure times. The observation of an effect of DMSO on activity is in contrast to previous findings in this study (Section 3.3) and others (de la Torre, 1995; Marshall, 2009). The unexpected results obtained may be related to the organism selected. Gerhardt *et al.* (2003) suggested *D. magna* may not be the ideal organism for use in early-warning biomitoring systems that do not use image analysis, such as the MFB, as specific movement patterns that are normally assessed, such as height in the water column and swimming style, cannot be directly assessed. Several other studies (Ren *et al.*, 2007; Ren *et al.*, 2008; Ren *et al.*, 2009) however have concluded that *D. magna* was indeed an appropriate organism for use in the MFB. *D. magna* is also a standard test organism for toxicity tests and its use allows for comparison between other biomonitors; hence, its application in the present experiments. It is possible that some of the behaviours elicited by *D. magna* under stress conditions are not able to be picked up by the MFB such as changes in locomotive activity.

After 6 hours of exposure, *D. magna* should be showing strong indicators of stress at the concentrations of TBT used. At 100µg/L TBT, it was observed by Marshall (2009) that 100% of exposed *D. magna* were unable to move throughout the water column. She

also observed that 80% of organisms had abnormal swimming style and 60% were considered immobile. It is possible that ability to swim through the water column is not measurable by the MFB. Swimming style may also be difficult to detect if a decrease in one behaviour type is compensated for with an increase of another behaviour type, therefore resulting in no or little change in overall locomotive activity. However, a presence of 60% immobile organisms should have been detected by the MFB. A decrease is seen in locomotive activity of Reference in comparison to 100µg/L TBT treatment, but it is not significant. Large standard deviations may be the cause of obscured TBT effect as the locomotive activity of 100µg/L TBT was 3.5% ± 5.3 while that of the Reference was 12.0% ± 9.0.

When comparing dates, the most notable difference between the two tests is the lower overall locomotive activities of the *D. magna* on February 8$^{th}$ compared with February 10$^{th}$, as noted in the date effects of all four times analysed. This occurred even though measures were taken to reduce any external causes for behaviour variability including age/size, light source intensity, disruption of the test vessels or MFB chambers, and diurnal rhythms of the organisms. This date effect was also seen in the previous bioassays conducted with *D. magna* exposed to 0.1% DMSO. In those situations, however, the date effect was no longer present after 6 hours of exposure. In this case, the date effect persists through the 12 hour duration of the test.

Throughout the four times analysed, and over the two experiments, no significant TBT effect was observed. This holds true even at the highest concentration of 100µg/L TBT, which has been shown to have strong influences on *D. magna* behaviour over short time periods in other toxicity bioassays (Marshall, 2009). This was not seen in this study. The only effect that was present was that of DMSO. From previous studies (Bowman et al, 1981; Barbosa et al, 2003) and previous experiments from this study, the use of 0.1% DMSO as an organic carrier/solvent has been shown to have no influence in *D. magna* behaviour. It is difficult to say exactly why DMSO had an influence on *D. magna* detectable by the MFB in this experiment but not in the previous ones. The initial experiments were repeated three times to ensure that no effect was caused by the DMSO at a concentration on 0.1%. The probable cause for the lack of TBT effect being noted while a DMSO effect was present is that *D. magna* elicits stress responses not measurable

by the MFB. This conclusion is disappointing because of previous assurance by several studies that *D. magna* was indeed applicable for use in the MFB (Gerhardt & Svensson, 1994; Gerhardt *et al.*, 2005; Gerhardt *et al.*, 2006; Ren *et al.*, 2007; Ren *et al.*, 2008; Ren *et al.*, 2009). One study stated that *D. magna* was not as applicable in long-term biomonitoring as *Gammarus pulex* (Gerhardt *et al.*, 2003) but the others strongly approved its usability. The use of *D. magna* for detection of TBT presence in Canadian freshwaters was shown to have great potential (Marshall, 2009); however, the above results to do not support this or allow for research progress to be made.

The above results do not support the use of *Daphnia magna* with MFB as an automated biomonitoring system as this combination was unable to detect changes in organism behaviour. High variability in activity of individual organisms, both within and between experiments, may be the cause of this. There is a known TBT effect on *D. magna* observed through visual observation which was not detected at all with the MFB. More studies must be conducted in order to determine whether the lack of reaction to the TBT concentrations was a result of the organisms or of the equipment.

### *3.4.2 Hyalella azteca Exposed to Tributyltin (TBT)*

The results of the Reference, DMSO Reference, and three concentrations of TBT treatments below are from 8 to 9 replicates. High numbers of replicates are required as organism behaviour is variable. *Hyalella azteca* were placed within the MFB chambers and into the test solutions two hours before recording and analysis began. This was done to allow the organisms to acclimatize to their new surroundings so as not to influence the recorded "treatment effects". A two-hour period has been previously determined as sufficient acclimatization time for benthic amphipods (Gerhardt *et al.*, 2003; Kirkpatrick *et al.*, 2006). There were no treatment effects apparent from *H. azteca* exposure to any concentrations of TBT after 1 hour in comparison to the References (Fig. 3.9).

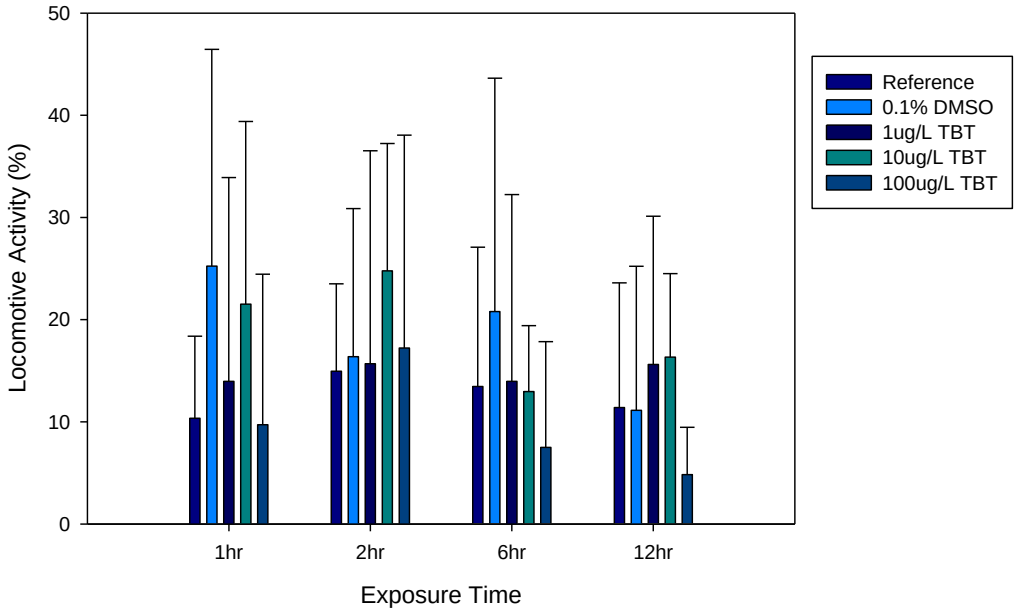


**Figure 3.9:** Locomotive activity (%) of *Hyalella azteca* comparing 1, 2, 6 and 12 hours of exposure to three concentrations of TBT. Values are mean ± standard deviation.

No significant differences were noted between the Reference and 1µg/L ($p = 0.998$), 10µg/L ($p = 0.763$) or 100µg/L ($p = 0.999$) of TBT after 1 hour. There was a strong significant difference between the two days over which this experiment was conducted, however ($p = 0.003$). After two hours of exposure to TBT, *H. azteca* remained uninfluenced by any of the concentrations, with no difference between the Reference and the three concentrations ($p = 0.761$). There was effect between the two test dates after 2 hours ($p = 0.105$), contrary to what was observed in the *D. magna* exposure to TBT. There remained no significant difference of treatment effect on *H. azteca* locomotive activity after 6 hours of exposure to TBT. The "date effect" was no longer present as it had been in the first hour of exposure ($p = 0.613$) and there were no significant differences between the reference and DMSO conditions ($p = 0.975$).

From previous studies, *H. azteca* began to show reduction in mobility at 100µg/L TBT exposure after 1 hour of exposure as well as a slight reduction in body length at this time and concentration. Although the highest concentration of TBT showed a lower locomotive activity than the other treatments, 9.7 ± 14.7%, it was very similar to that of

the Reference, 10.3 ± 8.0%. Very large standard deviations were seen with *H. azteca* behaviour as with the *D. magna*. The highest was for the first hour of exposure of *H. azteca* to a concentration of 1µg/L TBT being 14.0 ± 20%. The incredibly high standard deviations are assumedly from normal behaviour made by the organisms, as explained in the DMSO experiments. The organisms may be stationary, walking, or swimming and averaging out the locomotive activities read by the MFB can give large variation. However, this is not ideal as it is very difficult to determine when a treatment effect is indeed present.

After two hours, it seems that the locomotive activity of *H. azteca* is stabilizing with hardly any difference between the Reference (15.0 ± 8.4%), the DMSO Reference (16.4 ± 14.5%) and 1µg/L TBT treatment (15.7 ± 20.8%). Although the standard deviations are still high, these results were expected. However, there should have been an influence in locomotive activity for exposure to 10µg/L and 100µg/L TBT. Marshall (2009) stated that after 2 hours, almost 50% of organisms exposed to 10µg/L TBT were immobile while at 100µg/L almost 70% were. This should have caused an overall decrease in locomotive activity. Rather, no significant difference was noted. The high standard deviations could be the cause of no significance being noted.

After 6 hours of exposure, there should have been a strong treatment influence on *H. azteca*, especially at 100µg/L TBT. There was a slight decreasing trend seen with increasing concentration; however, no significant differences were present. Marshall (2009) stated that after 6 hours of exposure to 100µg/L TBT, 100% of organisms were exhibiting immobility and over 70% were immobile at exposure to 10µg/L TBT. This should have been readily picked up by the MFB, most notably the 100% where the standard deviations would have been much lower with such high numbers of organisms exhibiting the same response. It is possible that the contaminant effect is diminished in some way while using the MFB and the increased surface area presented by the chambers could add more surface area upon which the TBT molecules could adsorb. If the organisms were not being exposed to the true concentrations, they would not be exhibiting strong stress responses. It is possible that increased levels of DMSO are required for complete distribution and to prevent adsorption; however, even slightly higher levels of 0.5% DMSO have influence over organism behaviour. The adsorptive

abilities of TBT and the effect of higher concentrations of DMSO must be assessed in future research.

In comparing the three concentrations by means of each time of analysis, there are overall no general trends to be seen. The locomotive activity for organisms exposed to 100µ/L TBT is slightly lower, but no significant decreases were noted throughout the twelve-hour duration. The fairly consistent locomotive activities of both the Reference and 1µg/L TBT treatment demonstrates that an acclimatization period of 2 hours was sufficient for organisms to return to normal activity levels after transferring them into the MFB chambers. However, absolutely no significant differences were seen between any of the Reference or treatment conditions during the entire 12-hour duration of the test. This goes against previous testing which demonstrated a significant difference in the mobility of the organisms at exposure to both 10µg/L TBT and 100µg/L TBT after 2 hours (Marshall, 2009).

As no significant treatment effect was observed, the above results demonstrate that the MFB is not appropriate in detecting behavioural changes in *H. azteca* when exposed to TBT. This conclusion was not expected as an extensive literature search showed that a multitude of aquatic invertebrates should be applicable for contaminant testing using the MFB (Gerhardt & Svensson, 1994; Gerhardt, 1995; Gerhardt & Palmer, 1998; Gerhardt & Schmidt, 2002; Gerhardt *et al.*, 2002a; Gerhardt *et al.*, 2004; de Bisthoven *et al.*, 2004; Gerhardt *et al.*, 2005b; de Bisthoven *et al.*, 2006; Gerhardt *et al.* 2006; Kirkpatrick et al, 2006a; Kirkpatrick *et al.*, 2006b; Gerhardt *et al.*, 2007; Macedo-Sousa *et al.*, 2007; Sardo *et al.*, 2007; Ren *et al.*, 2007; Kienle *et al.*, 2009; Peeters *et al.*, 2009**)**, and it was the aim of this study to add *H. azteca* to the list of usable test species. This was thought possible as thorough testing had previously been conducted specifically on other species of amphipods, including *Gammarus pulex*, *Corophium volutator* and *Crangonyx pseudogracilis*, and deemed such organisms applicable in the MFB (Gerhardt, 1995; Gerhardt *et al.*, 1998; Gerhardt *et al.*, 2003; Kirkpatrick *et al.*, 2006a; Kirkpatrick *et al.*, 2006b).

### *3.4.3 Daphnia magna Exposed to Atrazine*

The results of the References and three concentrations of atrazine below are the observations made from 7 or 8 replicates acquired from two separate tests conducted over two days. As with the previous experiments analysing the effect of TBT, *D. magna* were placed into the MFB chambers and the test solutions and given two hours to acclimatize. After this period, the mean locomotive activities were averaged and analysed to detect any treatment effect of the three concentrations of atrazine on *D. magna* over a 12-hour period.

After the first hour, there were no significant differences between the Reference and any of the treatment conditions (Fig.3.10) (DMSO p = 0.113; 5µg/L p = 1.000; 50µg/L p = 0.545; 500µg/L p = 0.800). There was no significant difference within the treatment conditions either. There was no significant difference between the experiments conducted on two separate days (p = 0.146).

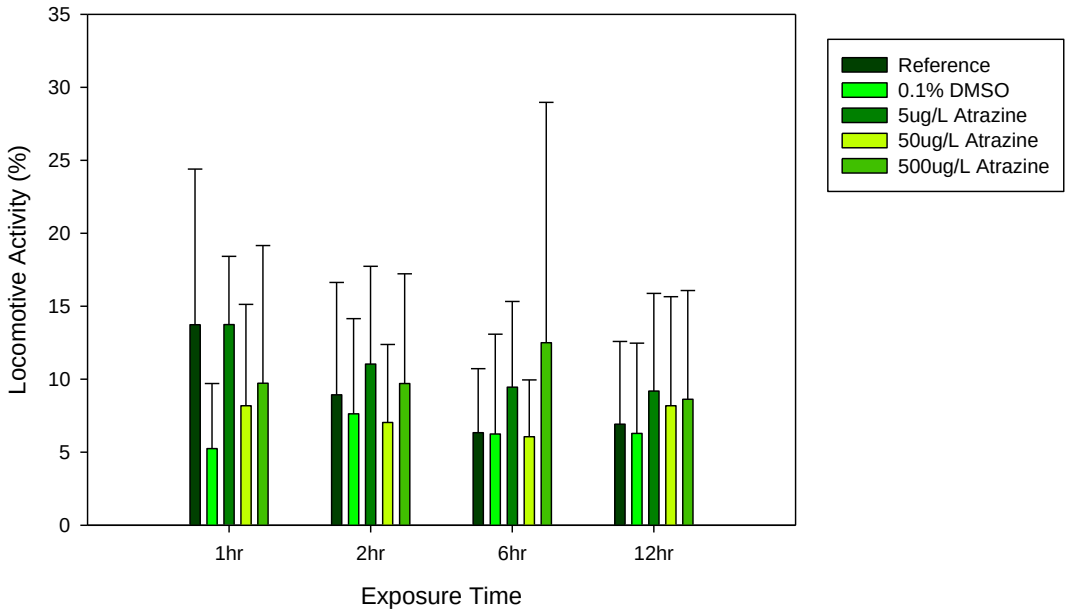


**Figure 3.10:** Locomotive activity (%) of *Daphnia magna* comparing 1, 2, 6 and 12 hours of exposure to three concentrations of atrazine. Values are mean ± standard deviation.

There continued to be no treatment effect over longer exposure periods of either two hours or six hours to any of the concentrations of atrazine. At two hours, a "date effect" did appear, with significant differences between the values from the two experiments (p = 0.015). This difference continued and was still present after six hours of

*D. magna* exposure to atrazine ($p = 0.014$). In comparing the mean locomotive activities of *D. magna* under exposure to varying concentrations of atrazine, no treatment effect was present at any point during the twelve-hour duration of the test. The significant difference between the two experiments conducted was still present at 12 hours and had increased in significance ($p < 0.001$).

According to a previous assessment in this study, the fact that no significant difference was noted between the Reference and the DMSO Reference of 0.1% was to be expected. However, after I hour of exposure, Marshall (2009) found that around 70% of *D. magna* were displaying changes in swimming height for concentrations of 50µg/L and 100µg/L atrazine. She also noted that at this time, over 60% of organisms were displaying abnormal swimming behaviour and over 30% were immobilized at exposure to 100µg/L atrazine. Since the highest concentration used in this experiment was 500µg/L, a response should have been elicited by the organisms.

It is surprising that after 6 hours of exposure to concentrations of atrazine as high as 500µg/L, no effect was seen. Marshall (2009) saw strong behavioural effects at both the second and sixth hour of exposure at concentrations of 5µg/L, 50µg/L, and 100µg/L of atrazine. At 2 hours of exposure, for example, it was noted that just over 50% of organisms exposed to 50µg/L were exhibiting inability to swim throughout the water column. That number rose to 80% after 6 hours. Swimming height may not be the ideal parameter for measurement in the MFB; however, strong influences were seen on mobility of *D. magna* with 80% of organisms being immobilized after 6 hours of exposure to 100µg/L. Immobility should be a good parameter for measurement in the MFB as the overall locomotive activity of the organisms would decrease. This was not the case. Actually, after 6 hours of exposure to 500µg/L of atrazine, *D. magna* showed an increase in overall locomotive activity, although this increase was not significantly different then the References or treatments. No trends are visible over any of the times or exposure concentrations. A slight decrease in locomotive activity can be seen as time passes for the Reference and 5µg/L treatment; however, the DMSO Reference and 50µg/L treatment show almost even values from beginning to end of the test. The standard deviations are also quite large, and any effects that could have been attributed to treatment exposure would not be observed through analysis of the data. From this

experiment, it can be determined that the MFB is not a good system for detection of behavioural changes in *D. magna* caused by exposure to the herbicide atrazine.

The issues which arose in the *D. magna* bioassays with atrazine as a stressor were similar to those of the TBT bioassays. The standard deviations were large, sometimes larger then their respective means and a deviation between experiments also appeared after one hour of exposure. As previously suggested, there may be issue with proper distribution of the contaminants through the test water. With the increased surface area created by the MFB chambers, 0.1% DMSO may not be sufficient for dispersal and increased adsorption could occur thereby removing the contaminant molecules from the test solution. It is strange that absolutely no effect was seen at such high concentrations of both TBT and atrazine after 12 hours of exposure.

### *3.4.4 Hyalella azteca Exposed to Atrazine*

The values of the references and treatments below have 8 or 9 replicates, acquired from two separate tests conducted over two days.

After two hours of acclimatization, the locomotive activity means of *Hyalella azteca* were taken (Fig. 3.11). There was no significant difference between any of the treatments, including the Reference, the DMSO Reference and the three atrazine concentrations. Although there was a slight increase in activity visible for the highest concentration of atrazine, it is not significantly different than the Reference ($p = 0.263$) or any of the other treatments.

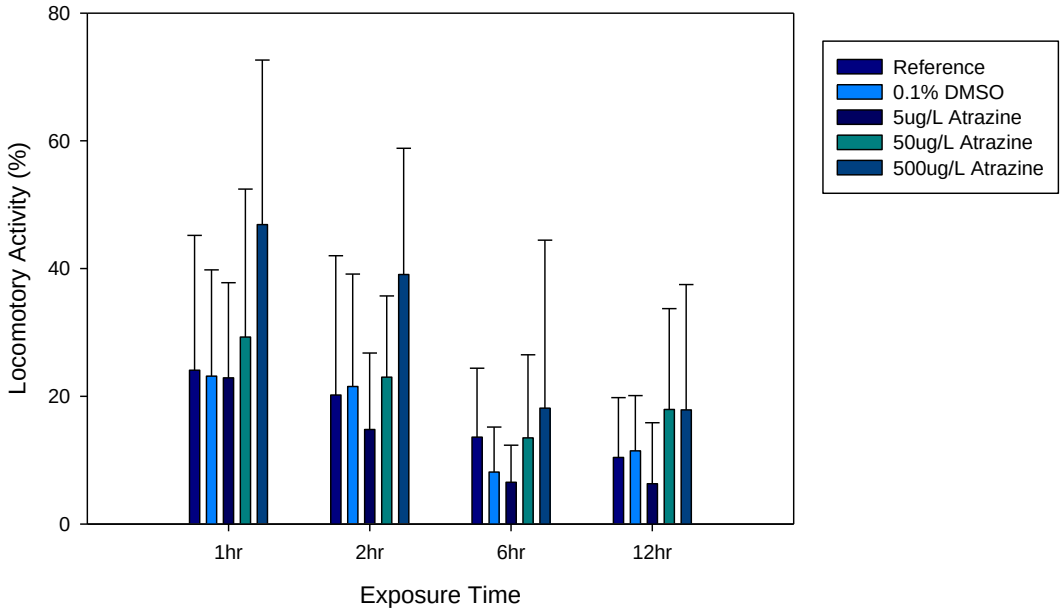


**Figure 3.11:** Locomotive activity (%) of *Hyalella azteca* comparing 1, 2, 6 and 12 hours of concentrations to three treatments of atrazine. Values are mean ± standard deviation.

Two hours of exposure to atrazine continued to show no significant difference between the Reference, the DMSO Reference and the three atrazine concentrations. Locomotive activities of *H. azteca* remained unaffected by atrazine through the sixth hour of exposure. There continued to be no significant difference present for all treatment and Reference conditions. Through the remainder of the experiment, up to twelve hours of exposure, no sign of stress was noted by the MFB and no "date effect" was observed in comparison of the two experiments conducted.

After 1 hour of exposure, Marshall (2009) noted that at 5µg/L atrazine over 50% of *H. azteca* were immobilized, with 60% being immobilized at exposure to both 50µg/L and 100µg/L. That effect was not observed in the present study by the MFB. There was actually an increase in locomotive activity noted, although not significantly different from other concentrations. Very high standard deviations were present here as with the TBT experiments, with locomotive activity of the Reference at 24.1 ± 21.1% and 29.3 ± 23.2% for organisms exposed to 50µg/L of atrazine. If the increase in activity seen at 500µg/L did not have such large deviations, it may be explained as an escape response elicited by the organisms.

Marshall (2009) noted that after 2 hours of exposure, the number of immobilized organisms rose to 65% for 5µg/L, and over 70% for 50µg/L and 100µg/L. The responses observed in this study contradict this, although a very slight decrease in locomotive activity is seen at 5µg/L exposure in comparison to the Reference and DMSO Reference. Again, this effect may have been more prominent if the standard deviations were not so high.

According to Marshall's research, after six and twelve hours of exposure to 100µg/L of atrazine, 100% percent of organisms were immobilized. Nothing to this affect was seen in the present study, even at a concentration as high as 500µg/L. The locomotive activity of *H. azteca* remained higher then that of the Reference for the duration of the study. The fact that absolutely no effect was seen at any of the exposure concentrations for the duration of the experiments suggests that the MFB was not able to detect any overall changes in locomotion from *H. azteca*, if any changes were exhibited.

A slightly stronger atrazine effect was noted on the *H. azteca* than with the TBT bioassay. Most notable was the overall higher locomotive activity level of the organisms at 500µg/L of atrazine. If the standard deviation bars are disregarded, there is a general trend visible with the increase of atrazine concentration. The Reference, DMSO and 5µg/L atrazine solutions had similar locomotive activities, while the 50µg/L presents a very slight increase in activity. However, none of these concentrations are significant in their differences to each other.

The issues present in this experiment are similar to those noted in the analysis of TBT effect on *H. azteca*. Very large standard deviations are noted, and no significant differences are seen even at the highest concentration. At exposure to 500µg/L of atrazine, the organisms have been shown to react within the first hour, with increasing distress behaviour continuing until mortality occurs (Marshall, 2009). The fact that no significant effect was seen could be the result of several issues. As mentioned, the MFB may not be able to detect the most sensitive behavioural responses to stress elicited by *H. azteca*. If most organisms within the chambers prefer to remain motionless and hidden in between the electrodes, it may be difficult to distinguish between reference and treatment conditions. It is also possible that the reactions elicited by individual organisms to contaminant exposure may occur at different times after initial contact. This may be

caused by such factors as the gender of the organism or molting stage. There is also the possibility that 0.1% DMSO was insufficient in preventing the adsorption of the contaminants to the surface of the chambers. If there was a reaction between the chamber material and the contaminant, the true concentration and exposure to the organisms would be much lower then anticipated resulting in little or no reactions being demonstrated by the organisms.

### *3.4.5 Summary of TBT and Atrazine exposure to D. magna and H. azteca*

Tributyltin exposure causes a slow shut down of many biological functions rather than a single organ system. As many cell types take up TBT, it is hard to pin point its primary mode of toxicity on organisms (Schmidt *et al.*, 2005). TBT works by preventing the breakdown of ATP to ADP; therefore, no energy is available for the muscles and locomotory activities are impaired (Alzieu, 1998). Because of this, TBT causes a decrease in overall locomotory activity (Schmidt *et al.*, 2005). In *Daphnia magna*, a decrease in locomotory activity should be seen as the secondary antennae are impaired by exposure to TBT which in turn affects the swimming height in the water column, the swimming style, and decrease mobility. For *Hyalella azteca*, a decrease in overall locomotory activity should have also been observed. This would be caused by both the reduction of energy available for the muscles as well as the primary escape response of the organism to burrow into the sediment and escape contaminants in the water column. In this study, no decrease in activity was seen for either organism at any of the three concentrations of TBT over the 12 hour duration of the test.

Atrazine causes depression of acetylcholinesterase (AChE) which functions as a neurotransmitter usually found at muscular junctions (Saglio & Trijasse, 1998; Forget *et al.*, 2003; Key *et al.*, 2003). With a decrease in AChE, there will be an increase in acetylcholine which acts as a stimulus for nerve and muscle fibre. With such stimulus, an initial increase in activity may be seen at low concentrations or short exposure times. Continued exposure to high levels of atrazine will result in immobilization and eventual death of the organism. In this study, neither an initial stimulated response nor a decrease in mobility was seen in *D. magna* or *H. azteca* at any of the three concentrations of atrazine over the 12 hour duration of the test.

The reason for lack of response noted by the MFB from *D. magna* and *H. azteca* when exposed to TBT and atrazine could be three fold: 1) the distribution of the contaminants through the test solutions; 2) the MFB apparatus and equipment; or 3) the behaviour elicited by the organisms.

Firstly, the even distribution of TBT and atrazine through the test solutions and the prevention of molecule adsorption are important in order for complete behavioural responses to be elicited from the organisms. If the organisms are not in contact with the full concentration of the contaminants, reduced behavioural responses will result. It is possible that the use of 0.1% DMSO as an organic solvent was insufficient to distribute evenly and prevent adsorption to the increased number of surfaces presented by the inclusion of the MFB chambers, resulting in reduced organism responses. This is most likely not the case as the use of concentrations lower than 0.5% have been used successfully with the MFB (Ren *et al.*, 2007; Ren *et al.*, 2008). In addition, an effect would have been expected at the highest concentrations after extended exposure to TBT and atrazine even if the contaminant had adsorbed to the chamber surfaces.

The second possible issue with the lack of significant responses could lie with the MFB itself. The specific MFB model purchased by this team at Ryerson University had 24 chambers and 24 ports available. However, two ports (# 14 and #22) were sporadic in their generation of any readings. These ports were excluded from all testing and experiments. The 24 chambers were tested and all were deemed reliable and able to generate reproducible results. As *D. magna* are organisms that swim continuously, the MFB readouts are fairly easy to assess for viability of the organisms. Locomotive activity was elicited by all organisms unless immobility or death occurred. *H. azteca*, however, are benthic amphipods which generally prefer to remain at the bottom of the water body and hidden under detritus. Often, *H. azteca* inside a chamber would find a little nook or space between the electrodes or between the electrodes and Nitex mesh, and remain there for extended periods during a test. During these times, no locomotive activity was picked up by the MFB as it should have been. Higher frequencies, associated with little movements such as leg or antennae, were also not picked up by the MFB. Therefore, for long stretches of time (2 – 3 hours), no activity at all was registered for some *H. azteca*

by the MFB, and a chamber was deemed inactive and eliminated from analysis (reducing replicates and statistical power) although a living unstressed organism was located inside.

Finally, the behaviour elicited by *D. magna* and *H. azteca* may not be ideal for assessment with the MFB. As identified by Marshall (2009), ability to swim through the water column, swimming style and immobility were the three most sensitive and reliable parameters for behavioural analysis of *D. magna* stress in order from greatest to least effective. These behaviours, the first two especially, may not be well detectable by the MFB chambers. MFB measurements are made based on size and amplitude of movements made and presented as locomotive activity of the organism. Immobility is theoretically a good parameter for MFB assessment; however, it is not the most sensitive elicited in *D. magna*. Immobility is caused by higher concentrations as acute toxicity or after longer exposure periods when total exhaustion occurs in the organism. It is height in the water column and swimming style which are good measures of short-term, low concentrations of contaminants. However, the MFB does not generate measurements this way. Movements are not classified based on type, but rather by amplitude of each action made. If the organism is unable to swim throughout the water column, but is still swimming at the bottom of the chamber, then the MFB may not register the difference. The same explanation can be applied to swimming style. If an organism is swimming abnormally, but the overall impedance created in the electrical field is the same, then the MFB will not register the difference. When it comes to *H. azteca*, its primary and most sensitive stress response is immobilization (Marshall, 2009). However, organisms used were seen to spend much of their time in the chambers not moving. This was seen in both Reference and treatment conditions. If the MFB is unable to differentiate between stationary organisms and immobilized or dead organisms, as stated above, then no stress response will be observed.

The lack of effect of TBT and atrazine on both *D. magna* and *H. azteca* detected by the MFB detailed in this study most likely results from a combination of the above issues. From the results presented in this study, it is clear that the MFB in not ready for application in an early-warning biomonitoring system using *D. magna* and *H. azteca* to monitor freshwater drinking water systems

# 4 Summary and Future Work

The goal of MFB use is for *in situ* application as an early-warning biomonitoring system (EWBS) for assessing drinking water quality. Employing multiple species simultaneously will increase the sensitivity of the system. As various species can react differently when exposed to the same contaminant, families of contaminants may be identified based on organisms' behavioural responses. Different responses can arise because the positions organisms inhabit in the water system will offer increased or decreased exposure to a given contaminant. For example, if the contaminant is slightly hydrophobic in nature, it will partition into the benthos more readily, thereby exposing sediment-dwelling organisms such as *Hyalella azteca* to higher concentrations then the water-column dwellers such as *Daphnia magna*. Behavioural responses can also differ based on the mode of action of a contaminant resulting in varying organism sensitivities. Thus, this study aimed to assess the applicability of two species, *D. magna* and *H. azteca*, for use in the MFB early-warning biomonitoring system and to assess the applicability of the MFB system as a component of the EWBS.

The contaminants used for analysis in this study were TBT and atrazine, two compounds that pose threats to the Great Lakes and Niagara regions. In order to ensure that even distribution of the contaminants occurred, DMSO was used as an organic solvent at a concentration of 0.1%. Through multiple visual and MFB automated bioassays, it was determined that at this concentration, DMSO had no influence over either of the organisms' behaviours.

It was previously established that *D. magna* and *H. azteca* were good indicator organisms for detecting TBT and atrazine (Marshall, 2009). These organisms were shown to exhibit specific and sensitive behavioural responses, even at low, environmentally-relevant concentrations. However, during the present study, the MFB was unable to detect these expected behavioural changes.

Behaviour is a sensitive parameter to be measured, which can be both an advantage and a restriction. Complications can transpire when organisms of the same species react differently when exposed to the same, or similar, conditions. It is understood that behaviour is a very variable response and reactions will fluctuate depending on age of the organism and the physical parameters of their environment.

Temperature, feeding patterns and light cycles must be particularly controlled during experimental procedures. This will minimize stress to the organisms not caused by contaminant exposure and aide in reducing false alarms.

Large standard deviations were presented by the MFB because of the variation of organism behaviour. When the variation of individuals within a single experiment is compounded with variations of organisms from another experiment, it is highly possible that any reactions that are exhibited could be concealed and not deemed significant. High variability creates issues when experiments are attempted to be reproduced, replicated and compared.

These findings are extremely disappointing because an exhaustive literature search of publications from scientists who had developed and implemented the MFB was conducted. This literature suggested that this costly technology would be supremely suitable for detecting behavioural responses of daphnids and amphipods exposed to common aquatic contaminants such as anti-fouling organics found of the hulls of ships or conventional herbicides. Additionally, a visit to the MFB manufacturers in Europe seemed to further verify the published findings. However, when the conflicting results from this study were constantly sent to the scientist responsible for the major portion of the development of the MFB, there were no replies forthcoming.

With regards to the entire project, future work required in order for the early-warning biomonitoring system to be applicable in the field is to compile a comprehensive library database which can be adjusted for toxicants of concern at a specific site. This database will be made up of the behavioural reactions elicited by multiple species of organisms when exposed to different concentrations of various toxicants. In addition, as toxicants rarely enter the environment in isolation, the reaction of multiple species to various chemical cocktails will also be included in the data base.

It is probable that organisms of the same species will react in the same manner to different contaminants or chemical cocktails. There are, after all, a limited number of stress responses that can be exhibited by a single species. Therefore, it is not possible to absolutely identify the species of contaminant passing through a water system. Rather, the general category to which it belongs can be suggested. The identification of contaminants can be facilitated through use of multiple species concurrently and selection

of which organisms to be used in a given location must be made by considering several factors. Such factors include sensitivities of a species to a contaminant of concern in a given area and whether the species is native to said area. Species must also be chosen to maximize coverage of different positions inhabited through the water column and encompass a range of different phyla. Through proper selection of species to be included in the EWBS, a more accurate assessment of water quality can be made. Using an EWBS will determine whether or not the water has been contaminated to such an extent that it is no longer potable. By narrowing down the possible substances that may have contaminated a water system, chemical analysis can follow in an efficient, rapid and more cost-effective manner.

With regards to the current study, however, before the library database can be compiled, reproducibility with the MFB experiments must be attained. Whether this is to be achieved through further control over variables such as temperature and size of the organisms or by the use of different species in the system must be determined. From this study, it can be resolved that the MFB is not ready to be applied in the field using *D. magna* and *H. azteca* as test species. The *D. magna* results from TBT and atrazine suggest that *D. magna* with the MFB is not a good combination since both chemicals elicit a response that can be detected by visual observation, but not one that can be detected by the MFB. This could be the organism, or it could be the system. The *H. azteca* results from the TBT and atrazine bioassays similarly suggest that *H. azteca* with the MFB is not a good combination for the same reasons given above. Again, it could be the organisms or it could be the system. That neither organism seems to give expected results in the MFB, but both do respond to the stressors as observed in visual analysis, builds a case that the MFB system itself is the problem and does not have the sensitivity to detect low concentrations of stressors. Therefore, it is probably not a good component for the overall miner's canary system.

Analysis of organism behaviour presents a sensitive, environmentally- relevant and cost-effective manner to detecting chemical contaminants in water systems. Behavioural biomonitoring should be used in conjunction with chemical testing in order to increase the rate at which contaminants are detected as well as reduce the cost of water monitoring. By using automated biological early-warning systems, remote supervision of

water quality can be conducted efficiently. However, more research is required in order to establish if and how the MFB can be used *in situ* as a freshwater drinking biomonitor.

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# Appendices

## *Appendix A: Dilution Calculations*

**TBT Stock Solution: 100mg/L TBT in DMSO**

A 1L volume of 100mg/L TBT in DMSO stock solution was prepared.

D =m/v

V = 100mgTBT / 1.103gcm$^{-3}$

V = 0.09066 cm$^3$

V = 0.09066 mL TBT in 1L DMSO

**TBT Stock Solution: 10mg/L TBT in DMSO**

A 100mL volume of 10mg/L TBT in DMSO stock solution was prepared.

$C_1V_1 = C_2V_2$

(100mg/L TBT in DMSO) $V_1$ = (10mg/L TBT in DMSO)(0.1L)

$V_1$ = 0.01L

The stock was made by adding 10mL of the 100mg/L TBT solution into 90mL of DMSO.

**TBT Stock Solution: 1mg/L TBT in DMSO**

A 100mL volume of 1mg/L TBT in DMSO stock solution was prepared.

$C_1V_1 = C_2V_2$

(10mg/L TBT in DMSO) $V_1$ = (1mg/L TBT in DMSO)(0.1L)

$V_1$ = 0.01L

The stock was made by adding 10mL of the 10mg/L TBT solution into 90mL of DMSO.

**Atrazine Stock Solution: 500mg/L Atrazine in DMSO**

A 1L volume of 100mg/L TBT in DMSO stock solution was prepared.

D =m/v

V = 100mgAtrazine / 1.187gcm$^{-3}$

V = 0.084 cm$^3$

V = 0.084 mL Atrazine in 1L DMSO

**Atrazine Stock Solution: 50mg/L Atrazine in DMSO**

A 100mL volume of 50mg/L TBT in DMSO stock solution was prepared.

$C_1V_1 = C_2V_2$

(500mg/L atrazine in DMSO) $V_1$ = (50mg/L atrazine in DMSO)(0.1L)

$V_1 = 0.01L$

The stock was made by adding 10mL of the 500mg/L TBT solution into 90mL of DMSO.

**Atrazine Stock Solution: 5mg/L Atrazine in DMSO**

A 100mL volume of 5mg/L TBT in DMSO stock solution was prepared.

$C_1V_1 = C_2V_2$

(50mg/L atrazine in DMSO) $V_1$ = (5mg/L atrazine in DMSO)(0.1L)

$V_1 = 0.01L$

The stock was made by adding 10mL of the 50mg/L TBT solution into 90mL of DMSO.

Final concentrations of *Daphnia magna* and *Hyalella azteca* TBT test solutions used in MFB bioassays

| Test Concentration | Total Volume | TBT Stock Used | Volume of Stock Added |
|---|---|---|---|
| 0.1% DMSO | 100mL | DMSO | 100µg/L |
| 1µg/L | 100mL | 1mg/L | 100µg/L |
| 10µg/L | 100mL | 10mg/L | 100µg/L |
| 100µg/L | 100mL | 100mg/L | 100µg/L |

Final concentrations of *Daphnia magna* and *Hyalella azteca* atrazine test solutions used in MFB bioassays

| Test Concentration | Total Volume | Atrazine Stock Used | Volume of Stock Added |
|---|---|---|---|
| 0.1% DMSO | 100mL | DMSO | 100µg/L |
| 5µg/L | 100mL | 5mg/L | 100µg/L |
| 50µg/L | 100mL | 50mg/L | 100µg/L |
| 500µg/L | 100mL | 500mg/L | 100µg/L |

## *Appendix B: Statistical Analysis*

### *Daphnia magna*: Reference and 0.1% DMSO comparison

**May 7th**

**1st hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 19.593 | 1 | 19.593 | 1.390 | 0.266 |
| Error | 140.926 | 10 | 14.093 | | |

**6th hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 12.000 | 1 | 12.000 | 2.024 | 0.185 |
| Error | 59.296 | 10 | 5.930 | | |

**12th hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 20.891 | 1 | 20.891 | 0.747 | 0.408 |
| Error | 279.579 | 10 | 27.958 | | |

**May 10th**

**1st hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 32.859 | 1 | 32.859 | 7.212 | 0.023 |
| Error | 45.560 | 10 | 4.556 | | |

**May 26th**

**1st hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 63.021 | 1 | 63.021 | 0.673 | 0.431 |
| Error | 936.616 | 10 | 93.662 | | |

**6th hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 7.002 | 1 | 7.002 | 0.226 | 0.645 |
| Error | 310.412 | 10 | 31.041 | | |

**12th hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 39.120 | 1 | 39.120 | 5.340 | 0.043 |
| Error | 73.259 | 10 | 7.326 | | |

**May 29th**

**1st hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 67.688 | 1 | 67.688 | 2.453 | 0.148 |
| Error | 275.949 | 10 | 27.595 | | |

**6th hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 81.815 | 1 | 81.815 | 4.925 | 0.051 |
| Error | 166.120 | 10 | 16.612 | | |

**12th hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 34.454 | 1 | 34.454 | 0.523 | 0.486 |
| Error | 658.981 | 10 | 65.898 | | |

**May 7th, 10th, 26th & 29th**

**1st hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 9.022 | 1 | 9.022 | 0.258 | 0.614 |
| DATE | 717.774 | 3 | 239.258 | 6.841 | 0.001 |
| TREATMENT$*DATE | 174.138 | 3 | 58.046 | 1.660 | 0.191 |
| Error | 1399.051 | 40 | 34.976 | | |

Tukey HSD Multiple Comparisons.
Matrix of pairwise comparison probabilities:

| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | 1.000 | | | |
| 2 | 0.762 | 1.000 | | |
| 3 | 0.001 | 0.009 | 1.000 | |
| 4 | 0.299 | 0.855 | 0.068 | 1.000 |

**6th hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 0.223 | 1 | 0.223 | 0.011 | 0.916 |
| DATE | 89.847 | 2 | 44.924 | 2.259 | 0.122 |
| TREATMENT$*DATE | 66.779 | 2 | 33.390 | 1.679 | 0.204 |
| Error | 596.671 | 30 | 19.889 | | |

Tukey HSD Multiple Comparisons.
Matrix of pairwise comparison probabilities:

| | 1 | 2 | 3 |
|---|---|---|---|
| 1 | 1.000 | | |
| 2 | 0.404 | 1.000 | |
| 3 | 0.106 | 0.705 | 1.000 |

**12th hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 8.186 | 1 | 8.186 | 0.243 | 0.626 |
| DATE | 107.792 | 2 | 53.896 | 1.598 | 0.219 |
| TREATMENT$*DATE | 86.279 | 2 | 43.140 | 1.279 | 0.293 |
| Error | 1011.819 | 30 | 33.727 | | |

Tukey HSD Multiple Comparisons.
Matrix of pairwise comparison probabilities:

| | 1 | 2 | 3 |
|---|---|---|---|
| 1 | 1.000 | | |
| 2 | 0.195 | 1.000 | |
| 3 | 0.538 | 0.764 | 1.000 |

***Hyalella azteca*: Reference and 0.1% DMSO comparison**

**May 19th**

**1st hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 533.333 | 1 | 533.333 | 4.306 | 0.065 |
| Error | 1238.722 | 10 | 123.872 | | |

**6th hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 32.780 | 1 | 32.780 | 0.069 | 0.799 |
| Error | 4783.838 | 10 | 478.384 | | |

**12th hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 33.891 | 1 | 33.891 | 0.121 | 0.735 |
| Error | 2789.634 | 10 | 278.963 | | |

**May 20th**

**1st hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 675.000 | 1 | 675.000 | 1.521 | 0.246 |
| Error | 4436.491 | 10 | 443.649 | | |

**6th hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 422.058 | 1 | 422.058 | 1.212 | 0.297 |
| Error | 3481.801 | 10 | 348.180 | | |

**12th hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 1203.336 | 1 | 1203.336 | 4.481 | 0.060 |
| Error | 2685.412 | 10 | 268.541 | | |

**May 19th & 20th**

**1st hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 4.167 | 1 | 4.167 | 0.015 | 0.905 |
| DATE | 1295.560 | 1 | 1295.560 | 4.566 | 0.045 |
| TREATMENT$*DATE | 1204.167 | 1 | 1204.167 | 4.244 | 0.053 |
| Error | 5675.213 | 20 | 283.761 | | |

**6th hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 109.796 | 1 | 109.796 | 0.266 | 0.612 |
| DATE | 397.449 | 1 | 397.449 | 0.962 | 0.338 |
| TREATMENT$*DATE | 345.042 | 1 | 345.042 | 0.835 | 0.372 |
| Error | 8265.639 | 20 | 413.282 | | |

**12th hour of exposure in Reference and 0.1% DMSO**

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 820.560 | 1 | 820.560 | 2.997 | 0.099 |
| DATE | 40.042 | 1 | 40.042 | 0.146 | 0.706 |
| TREATMENT$*DATE | 416.667 | 1 | 416.667 | 1.522 | 0.232 |
| Error | 5475.046 | 20 | 273.752 | | |

## *Daphnia magna* Statistical Analysis for three treatments of TBT

## 1st hour of exposure

**Analysis of Variance**

| Source | Type III SS | df | Mean Squares | F-Ratio | p-Value |
|---|---|---|---|---|---|
| TREATMENT$ | 512.488 | 4 | 128.122 | 2.492 | 0.066 |
| DATE | 2,003.701 | 1 | 2,003.701 | 38.971 | 0.000 |
| TREATMENT$*DATE | 147.069 | 4 | 36.767 | 0.715 | 0.589 |
| Error | 1,439.627 | 28 | 51.415 | | |

**Analysis of Variance**

| Source | Type III SS | df | Mean Squares | F-Ratio | p-Value |
|---|---|---|---|---|---|
| TREATMENT$ | 526.597 | 4 | 131.649 | 2.655 | 0.051 |
| DATE | 1,971.669 | 1 | 1,971.669 | 39.764 | 0.000 |
| Error | 1,586.696 | 32 | 49.584 | | |

**Tukey's Honestly-Significant-Difference Test**

| TREATMENT$(i) | TREATMENT$(j) | Difference | p-Value | 95% Confidence Interval | |
|---|---|---|---|---|---|
| | | | | Lower | Upper |
| 100ug/L TBT | 10ug/L TBT | 5.461 | 0.572 | -5.069 | 15.991 |
| 100ug/L TBT | 1ug/L TBT | 3.595 | 0.873 | -7.280 | 14.471 |
| 100ug/L TBT | DMSO | 6.857 | 0.348 | -3.673 | 17.387 |
| 100ug/L TBT | Reference | -3.122 | 0.911 | -13.652 | 7.408 |
| 10ug/L TBT | 1ug/L TBT | -1.866 | 0.986 | -12.396 | 8.664 |
| 10ug/L TBT | DMSO | 1.396 | 0.995 | -8.777 | 11.569 |
| 10ug/L TBT | Reference | -8.583 | 0.131 | -18.756 | 1.590 |
| 1ug/L TBT | DMSO | 3.262 | 0.897 | -7.268 | 13.792 |
| 1ug/L TBT | Reference | -6.717 | 0.369 | -17.248 | 3.813 |
| DMSO | Reference | -9.979 | 0.057 | -20.152 | 0.194 |

## 2nd hour of exposure

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 519.565 | 4 | 129.891 | 2.716 | 0.050 |
| DATE | 1076.690 | 1 | 1076.690 | 22.513 | 0.000 |
| TREATMENT$*DATE | 80.187 | 4 | 20.047 | 0.419 | 0.793 |
| Error | 1339.120 | 28 | 47.826 | | |

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 529.308 | 4 | 132.327 | 2.983 | 0.034 |
| DATE | 1115.594 | 1 | 1115.594 | 25.152 | 0.000 |
| Error | 1419.307 | 32 | 44.353 | | |

Tukey HSD Multiple Comparisons.
Matrix of pairwise comparison probabilities:

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | 1.000 | | | | |
| 2 | 0.992 | 1.000 | | | |
| 3 | 0.999 | 0.955 | 1.000 | | |
| 4 | 0.951 | 0.749 | 0.990 | 1.000 | |
| 5 | 0.206 | 0.373 | 0.128 | 0.037 | 1.000 |

## 6th hour of exposure

| Analysis of Variance | | | | | |
|---|---|---|---|---|---|
| **Source** | **Type III SS** | **df** | **Mean Squares** | **F-Ratio** | **p-Value** |
| TREATMENT$ | 284.068 | 4 | 71.017 | 2.126 | 0.104 |
| DATE | 448.863 | 1 | 448.863 | 13.439 | 0.001 |
| TREATMENT$*DATE | 20.994 | 4 | 5.249 | 0.157 | 0.958 |
| Error | 935.206 | 28 | 33.400 | | |

| Analysis of Variance | | | | | |
|---|---|---|---|---|---|
| **Source** | **Type III SS** | **df** | **Mean Squares** | **F-Ratio** | **p-Value** |
| TREATMENT$ | 280.562 | 4 | 70.141 | 2.347 | 0.075 |
| DATE | 440.262 | 1 | 440.262 | 14.734 | 0.001 |
| Error | 956.200 | 32 | 29.881 | | |

| Tukey's Honestly-Significant-Difference Test | | | | | |
|---|---|---|---|---|---|
| **TREATMENT$(i)** | **TREATMENT$(j)** | **Difference** | **p-Value** | **95% Confidence Interval** | |
| | | | | **Lower** | **Upper** |
| 100ug/L TBT | 10ug/L TBT | -1.435 | 0.986 | -9.609 | 6.740 |
| 100ug/L TBT | 1ug/L TBT | 1.048 | 0.996 | -7.395 | 9.490 |
| 100ug/L TBT | DMSO | 1.690 | 0.975 | -6.484 | 9.865 |
| 100ug/L TBT | Reference | -5.810 | 0.266 | -13.984 | 2.365 |
| 10ug/L TBT | 1ug/L TBT | 2.482 | 0.903 | -5.692 | 10.657 |
| 10ug/L TBT | DMSO | 3.125 | 0.782 | -4.772 | 11.022 |
| 10ug/L TBT | Reference | -4.375 | 0.508 | -12.272 | 3.522 |
| 1ug/L TBT | DMSO | 0.643 | 0.999 | -7.532 | 8.817 |
| 1ug/L TBT | Reference | -6.857 | 0.136 | -15.032 | 1.317 |
| DMSO | Reference | -7.500 | 0.069 | -15.397 | 0.397 |

## 12th hour of exposure

| Analysis of Variance | | | | | |
|---|---|---|---|---|---|
| **Source** | **Type III SS** | **df** | **Mean Squares** | **F-Ratio** | **p-Value** |
| TREATMENT$ | 198.051 | 4 | 49.513 | 0.810 | 0.529 |
| DATE | 370.069 | 1 | 370.069 | 6.053 | 0.020 |
| TREATMENT$*DATE | 25.215 | 4 | 6.304 | 0.103 | 0.981 |
| Error | 1,834.153 | 30 | 61.138 | | |

| Analysis of Variance | | | | | |
|---|---|---|---|---|---|
| **Source** | **Type III SS** | **df** | **Mean Squares** | **F-Ratio** | **p-Value** |
| TREATMENT$ | 198.051 | 4 | 49.513 | 0.905 | 0.472 |
| DATE | 370.069 | 1 | 370.069 | 6.767 | 0.014 |
| Error | 1,859.368 | 34 | 54.687 | | |

| Tukey's Honestly-Significant-Difference Test | | | | | |
|---|---|---|---|---|---|
| **TREATMENT$(i)** | **TREATMENT$(j)** | **Difference** | **p-Value** | **95% Confidence Interval** | |
| | | | | **Lower** | **Upper** |

| Analysis of Variance | | | | | | |
|---|---|---|---|---|---|---|
| Source | Type III SS | df | Mean Squares | | F-Ratio | p-Value |
| 100ug/L TBT | 10ug/L TBT | -2.375 | 0.967 | -13.022 | 8.272 | |
| 100ug/L TBT | 1ug/L TBT | -2.604 | 0.954 | -13.252 | 8.043 | |
| 100ug/L TBT | DMSO | -0.979 | 0.999 | -11.627 | 9.668 | |
| 100ug/L TBT | Reference | -6.521 | 0.411 | -17.168 | 4.127 | |
| 10ug/L TBT | 1ug/L TBT | -0.229 | 1.000 | -10.877 | 10.418 | |
| 10ug/L TBT | DMSO | 1.396 | 0.995 | -9.252 | 12.043 | |
| 10ug/L TBT | Reference | -4.146 | 0.794 | -14.793 | 6.502 | |
| 1ug/L TBT | DMSO | 1.625 | 0.992 | -9.022 | 12.272 | |
| 1ug/L TBT | Reference | -3.917 | 0.826 | -14.564 | 6.731 | |
| DMSO | Reference | -5.542 | 0.570 | -16.189 | 5.106 | |

## *Hyalella azteca* Statistical Analysis for three treatments of TBT

## 1st hour of exposure

| Analysis of Variance | | | | | |
|---|---|---|---|---|---|
| Source | Type III SS | df | Mean Squares | F-Ratio | p-Value |
| TREATMENT$ | 1,183.404 | 4 | 295.851 | 1.181 | 0.339 |
| DATE | 2,538.820 | 1 | 2,538.820 | 10.131 | 0.003 |
| TREATMENT$*DATE | 771.487 | 4 | 192.872 | 0.770 | 0.553 |
| Error | 7,768.427 | 31 | 250.594 | | |

| Analysis of Variance | | | | | |
|---|---|---|---|---|---|
| Source | Type III SS | df | Mean Squares | F-Ratio | p-Value |
| TREATMENT$ | 1,232.918 | 4 | 308.230 | 1.263 | 0.303 |
| DATE | 2,409.667 | 1 | 2,409.667 | 9.876 | 0.003 |
| Error | 8,539.914 | 35 | 243.998 | | |

| Tukey's Honestly-Significant-Difference Test | | | | | |
|---|---|---|---|---|---|
| TREATMENT$(i) | TREATMENT$(j) | Difference | p-Value | 95% Confidence Interval | |
| | | | | Lower | Upper |
| 100ugL | 10ugL | -11.799 | 0.633 | -36.053 | 12.456 |
| 100ugL | 1ugL | -4.244 | 0.984 | -27.436 | 18.947 |
| 100ugL | DMSO | -14.660 | 0.401 | -38.330 | 9.009 |
| 100ugL | Reference | -2.563 | 0.998 | -26.817 | 21.691 |
| 10ugL | 1ugL | 7.554 | 0.845 | -13.749 | 28.857 |
| 10ugL | DMSO | -2.862 | 0.996 | -24.684 | 18.961 |
| 10ugL | Reference | 9.236 | 0.763 | -13.219 | 31.691 |
| 1ugL | DMSO | -10.416 | 0.600 | -31.051 | 10.219 |
| 1ugL | Reference | 1.681 | 0.999 | -19.621 | 22.984 |
| DMSO | Reference | 12.097 | 0.518 | -9.725 | 33.920 |

## 2nd hour of exposure

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 490.918 | 4 | 122.730 | 0.465 | 0.761 |
| DATE | 734.779 | 1 | 734.779 | 2.784 | 0.105 |
| TREATMENT$*DATE | 638.394 | 4 | 159.599 | 0.605 | 0.662 |
| Error | 8180.915 | 31 | 263.900 | | |

Tukey HSD Multiple Comparisons.

Matrix of pairwise comparison probabilities:

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | 1.000 | | | | |
| 2 | 0.909 | 1.000 | | | |
| 3 | 1.000 | 0.763 | 1.000 | | |
| 4 | 1.000 | 0.803 | 1.000 | 1.000 | |
| 5 | 1.000 | 0.817 | 1.000 | 1.000 | 1.000 |

## 6th hour of exposure

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 659.732 | 4 | 164.933 | 0.569 | 0.687 |
| DATE | 75.799 | 1 | 75.799 | 0.261 | 0.613 |
| TREATMENT$*DATE | 259.055 | 4 | 64.764 | 0.223 | 0.923 |
| Error | 8986.752 | 31 | 289.895 | | |

Tukey HSD Multiple Comparisons.
Matrix of pairwise comparison probabilities:

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | 1.000 | | | | |
| 2 | 0.975 | 1.000 | | | |
| 3 | 0.946 | 1.000 | 1.000 | | |
| 4 | 0.597 | 0.886 | 0.914 | 1.000 | |
| 5 | 0.977 | 1.000 | 1.000 | 0.889 | 1.000 |

## 12th hour of exposure

**Analysis of Variance**

| Source | Type III SS | df | Mean Squares | F-Ratio | p-Value |
|---|---|---|---|---|---|
| TREATMENT$ | 631.008 | 4 | 157.752 | 1.054 | 0.396 |
| DATE | 59.517 | 1 | 59.517 | 0.398 | 0.533 |
| TREATMENT$*DATE | 407.904 | 4 | 101.976 | 0.681 | 0.610 |
| Error | 4,639.537 | 31 | 149.662 | | |

**Analysis of Variance**

| Source | Type III SS | df | Mean Squares | F-Ratio | p-Value |
|---|---|---|---|---|---|
| TREATMENT$ | 595.681 | 4 | 148.920 | 1.033 | 0.404 |
| DATE | 53.546 | 1 | 53.546 | 0.371 | 0.546 |
| Error | 5,047.441 | 35 | 144.213 | | |

**Tukey's Honestly-Significant-Difference Test**

| TREATMENT$(i) | TREATMENT$(j) | Difference | p-Value | 95% Confidence Interval | |
|---|---|---|---|---|---|
| | | | | Lower | Upper |
| 100ugL | 10ugL | -11.500 | 0.405 | -30.146 | 7.146 |
| 100ugL | 1ugL | -10.783 | 0.424 | -28.613 | 7.046 |
| 100ugL | DMSO | -6.424 | 0.847 | -24.621 | 11.773 |
| 100ugL | Reference | -6.275 | 0.869 | -24.921 | 12.372 |
| 10ugL | 1ugL | 0.717 | 1.000 | -15.661 | 17.094 |
| 10ugL | DMSO | 5.076 | 0.906 | -11.701 | 21.853 |

| Analysis of Variance | | | | | | |
|---|---|---|---|---|---|---|
| Source | Type III SS | df | Mean Squares | | F-Ratio | p-Value |
| 10ugL | Reference | 5.225 | 0.907 | -12.038 | 22.489 | |
| 1ugL | DMSO | 4.359 | 0.932 | -11.505 | 20.223 | |
| 1ugL | Reference | 4.509 | 0.932 | -11.869 | 20.886 | |
| DMSO | Reference | 0.150 | 1.000 | -16.627 | 16.926 | |

## *Daphnia magna* Statistical Analysis for three treatments of Atrazine

### 1st hour of exposure

| Analysis of Variance | | | | | |
|---|---|---|---|---|---|
| Source | Type III SS | df | Mean Squares | F-Ratio | p-Value |
| TREATMENT$ | 504.143 | 4 | 126.036 | 2.150 | 0.096 |
| DATE | 129.625 | 1 | 129.625 | 2.212 | 0.146 |
| TREATMENT$*DATE | 203.944 | 4 | 50.986 | 0.870 | 0.492 |
| Error | 1,992.747 | 34 | 58.610 | | |

| Tukey's Honestly-Significant-Difference Test | | | | | |
|---|---|---|---|---|---|
| TREATMENT$(i) | TREATMENT$(j) | Difference | p-Value | 95% Confidence Interval | |
| | | | | Lower | Upper |
| 500ug/L | 50ug/L | 1.542 | 0.994 | -9.343 | 12.426 |
| 500ug/L | 5ug/L | -4.021 | 0.827 | -14.905 | 6.863 |
| 500ug/L | DMSO | 4.479 | 0.727 | -5.847 | 14.805 |
| 500ug/L | Reference | -4.004 | 0.800 | -14.330 | 6.322 |
| 50ug/L | 5ug/L | -5.563 | 0.592 | -16.447 | 5.322 |
| 50ug/L | DMSO | 2.937 | 0.925 | -7.388 | 13.263 |
| 50ug/L | Reference | -5.546 | 0.545 | -15.872 | 4.780 |
| 5ug/L | DMSO | 8.500 | 0.150 | -1.826 | 18.826 |
| 5ug/L | Reference | 0.017 | 1.000 | -10.309 | 10.342 |
| DMSO | Reference | -8.483 | 0.113 | -18.218 | 1.252 |

### 2nd hour of exposure

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 85.309 | 4 | 21.327 | 0.492 | 0.742 |
| DATE | 284.198 | 1 | 284.198 | 6.554 | 0.015 |
| TREATMENT$*DATE | 87.427 | 4 | 21.857 | 0.504 | 0.733 |
| Error | 1474.425 | 34 | 43.365 | | |

Tukey HSD Multiple Comparisons.
Matrix of pairwise comparison probabilities:

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | 1.000 | | | | |
| 2 | 0.926 | 1.000 | | | |
| 3 | 0.994 | 0.743 | 1.000 | | |
| 4 | 0.963 | 1.000 | 0.810 | 1.000 | |
| 5 | 0.999 | 0.973 | 0.961 | 0.992 | 1.000 |

## 6th hour of exposure

**Analysis of Variance**

| Source | Type III SS | df | Mean Squares | F-Ratio | p-Value |
|---|---|---|---|---|---|
| TREATMENT$ | 267.357 | 4 | 66.839 | 1.181 | 0.337 |
| DATE | 379.867 | 1 | 379.867 | 6.711 | 0.014 |
| TREATMENT$*DATE | 594.588 | 4 | 148.647 | 2.626 | 0.052 |
| Error | 1,924.590 | 34 | 56.606 | | |

**Tukey's Honestly-Significant-Difference Test**

| TREATMENT$(i) | TREATMENT$(j) | Difference | p-Value | 95% Confidence Interval | |
|---|---|---|---|---|---|
| | | | | Lower | Upper |
| 500ug/L | 50ug/L | 6.438 | 0.518 | -5.218 | 18.093 |
| 500ug/L | 5ug/L | 3.042 | 0.944 | -8.614 | 14.697 |
| 500ug/L | DMSO | 6.250 | 0.495 | -4.808 | 17.308 |
| 500ug/L | Reference | 6.167 | 0.509 | -4.891 | 17.224 |
| 50ug/L | 5ug/L | -3.396 | 0.918 | -15.052 | 8.260 |
| 50ug/L | DMSO | -0.188 | 1.000 | -11.245 | 10.870 |
| 50ug/L | Reference | -0.271 | 1.000 | -11.329 | 10.787 |
| 5ug/L | DMSO | 3.208 | 0.919 | -7.849 | 14.266 |
| 5ug/L | Reference | 3.125 | 0.926 | -7.933 | 14.183 |
| DMSO | Reference | -0.083 | 1.000 | -10.509 | 10.342 |

## 12th hour of exposure

**Analysis of Variance**

| Source | Type III SS | df | Mean Squares | F-Ratio | p-Value |
|---|---|---|---|---|---|
| TREATMENT$ | 52.620 | 4 | 13.155 | 0.475 | 0.754 |
| DATE | 655.102 | 1 | 655.102 | 23.669 | 0.000 |
| TREATMENT$*DATE | 182.524 | 4 | 45.631 | 1.649 | 0.185 |
| Error | 941.019 | 34 | 27.677 | | |

**Tukey's Honestly-Significant-Difference Test**

| TREATMENT$(i) | TREATMENT$(j) | Difference | p-Value | 95% Confidence Interval | |
|---|---|---|---|---|---|
| | | | | Lower | Upper |
| 500ug/L | 50ug/L | 0.438 | 1.000 | -7.347 | 8.222 |
| 500ug/L | 5ug/L | -0.563 | 1.000 | -8.347 | 7.222 |
| 500ug/L | DMSO | 2.342 | 0.892 | -5.043 | 9.726 |
| 500ug/L | Reference | 1.708 | 0.963 | -5.676 | 9.093 |
| 50ug/L | 5ug/L | -1.000 | 0.996 | -8.784 | 6.784 |
| 50ug/L | DMSO | 1.904 | 0.946 | -5.480 | 9.289 |
| 50ug/L | Reference | 1.271 | 0.988 | -6.114 | 8.655 |
| 5ug/L | DMSO | 2.904 | 0.792 | -4.480 | 10.289 |
| 5ug/L | Reference | 2.271 | 0.902 | -5.114 | 9.655 |
| DMSO | Reference | -0.633 | 0.999 | -7.596 | 6.329 |

## *Hyalella azteca* Statistical Analysis for three treatments of Atrazine

### 1st hour of exposure

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 3126.664 | 4 | 781.666 | 1.822 | 0.151 |
| DATE | 1683.741 | 1 | 1683.741 | 3.925 | 0.057 |
| TREATMENT$*DATE | 534.490 | 4 | 133.623 | 0.311 | 0.868 |
| Error | 12869.007 | 30 | 428.967 | | |

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 3028.310 | 4 | 757.078 | 1.920 | 0.130 |
| DATE | 1663.979 | 1 | 1663.979 | 4.221 | 0.048 |
| Error | 13403.498 | 34 | 394.221 | | |

Tukey HSD Multiple Comparisons.
Matrix of pairwise comparison probabilities:

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | 1.000 | | | | |
| 2 | 0.448 | 1.000 | | | |
| 3 | 0.225 | 0.985 | 1.000 | | |
| 4 | 0.162 | 0.976 | 1.000 | 1.000 | |
| 5 | 0.263 | 0.995 | 1.000 | 1.000 | 1.000 |

### 2nd hour of exposure

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 2623.542 | 4 | 655.885 | 1.951 | 0.128 |
| DATE | 13.384 | 1 | 13.384 | 0.040 | 0.843 |
| TREATMENT$*DATE | 431.165 | 4 | 107.791 | 0.321 | 0.862 |
| Error | 10085.682 | 30 | 336.189 | | |

Tukey HSD Multiple Comparisons.
Matrix of pairwise comparison probabilities:

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | 1.000 | | | | |
| 2 | 0.418 | 1.000 | | | |
| 3 | 0.095 | 0.888 | 1.000 | | |
| 4 | 0.291 | 1.000 | 0.943 | 1.000 | |
| 5 | 0.310 | 0.999 | 0.959 | 1.000 | 1.000 |

## 6th hour of exposure

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 679.472 | 4 | 169.868 | 0.850 | 0.505 |
| DATE | 0.024 | 1 | 0.024 | 0.000 | 0.991 |
| TREATMENT$*DATE | 1438.815 | 4 | 359.704 | 1.799 | 0.155 |
| Error | 5998.215 | 30 | 199.940 | | |

Tukey HSD Multiple Comparisons.
Matrix of pairwise comparison probabilities:

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | 1.000 | | | | |
| 2 | 0.964 | 1.000 | | | |
| 3 | 0.533 | 0.883 | 1.000 | | |
| 4 | 0.624 | 0.946 | 0.999 | 1.000 | |
| 5 | 0.988 | 1.000 | 0.820 | 0.898 | 1.000 |

## 12th hour of exposure

| Source | Sum-of-Squares | df | Mean-Square | F-ratio | P |
|---|---|---|---|---|---|
| TREATMENT$ | 736.536 | 4 | 184.134 | 0.944 | 0.452 |
| DATE | 2.462 | 1 | 2.462 | 0.013 | 0.911 |
| TREATMENT$*DATE | 349.869 | 4 | 87.467 | 0.448 | 0.773 |
| Error | 5850.800 | 30 | 195.027 | | |

Tukey HSD Multiple Comparisons.
Matrix of pairwise comparison probabilities:

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | 1.000 | | | | |
| 2 | 1.000 | 1.000 | | | |
| 3 | 0.506 | 0.501 | 1.000 | | |
| 4 | 0.887 | 0.883 | 0.940 | 1.000 | |
| 5 | 0.888 | 0.885 | 0.955 | 1.000 | 1.000 |

## *Appendix C: Data obtained from Preference, DMSO, TBT and Atrazine bioassays conducted with Daphnia magna and Hyalella azteca*

### Daphnia magna – On/Off Preference

| Time Interval (minutes) | On | Off | Chi-square | p value |
|---|---|---|---|---|
| **1** | 19 | 13 | 1.125 | 0.288 |
| **2** | 20 | 12 | 2 | 0.157 |
| **3** | 17 | 15 | 0.125 | 0.724 |
| **4** | 13 | 19 | 1.125 | 0.288 |
| **Total** | 69 | 59 | 0.78125 | 0.3767 |

### Daphnia magna – DMSO

### May 7th, 10th, 26th and 29th – 1st hour

| Treatment | Date | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | 1 | 0 | 0.333333 | 0 | 0 | 0 | 0 |
| **Reference** | 1 | 2 | 2 | 1.333333 | 0.5 | 0 | 0 |
| **Reference** | 1 | 2.833333 | 2.666667 | 1.166667 | 0.333333 | 0 | 0 |
| **Reference** | 1 | 0.333333 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 1 | 0.5 | 0.5 | 0.5 | 0 | 0 | 0 |
| **Reference** | 1 | 3 | 2.5 | 1 | 0.5 | 0 | 0 |
| **DMSO** | 1 | 3.833333 | 3.666667 | 3 | 1.833333 | 0 | 0 |
| **DMSO** | 1 | 0 | 0.333333 | 0 | 0 | 0 | 0 |
| **DMSO** | 1 | 2.333333 | 3.333333 | 2.833333 | 2.333333 | 0 | 0 |
| **DMSO** | 1 | 2.333333 | 2.166667 | 1.5 | 0.5 | 0 | 0 |
| **DMSO** | 1 | 14.16667 | 12.66667 | 8.166667 | 4.5 | 0 | 0 |
| **DMSO** | 1 | 1.333333 | 1.5 | 1.5 | 0 | 0 | 0 |
| **Reference** | 2 | 5.571429 | 6.142857 | 4.714286 | 3 | 0.714286 | 0.428571 |
| **Reference** | 2 | 3.142857 | 3 | 2.857143 | 2.285714 | 0.571429 | 0.428571 |
| **Reference** | 2 | 4.714286 | 5 | 4.428571 | 3.428571 | 0.428571 | 0.285714 |
| **Reference** | 2 | 2 | 1.833333 | 2.333333 | 1.833333 | 0.333333 | 0 |
| **Reference** | 2 | 1 | 0.833333 | 1.333333 | 0.5 | 0 | 0 |
| **Reference** | 2 | 4.166667 | 4.166667 | 3.833333 | 2.333333 | 0 | 0 |
| **DMSO** | 2 | 6.285714 | 7.285714 | 6.428571 | 6.142857 | 2.285714 | 0.714286 |
| **DMSO** | 2 | 8 | 9.166667 | 8 | 5.333333 | 1 | 0.333333 |
| **DMSO** | 2 | 3.5 | 4 | 3.166667 | 2 | 0 | 0.333333 |
| **DMSO** | 2 | 4.333333 | 4.666667 | 4.333333 | 3.333333 | 0.333333 | 0 |
| **DMSO** | 2 | 9.833333 | 9.166667 | 6.666667 | 3.833333 | 1.666667 | 0.666667 |
| **DMSO** | 2 | 8.5 | 9.5 | 9 | 6.833333 | 2.5 | 1.333333 |
| **Reference** | 3 | 30.83333 | 26.33333 | 19.33333 | 9.166667 | 1.666667 | 0.333333 |
| **Reference** | 3 | 5.666667 | 4.5 | 2.666667 | 2 | 0 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Reference** | 3 | 30 | 25.5 | 16.16667 | 8 | 0.5 | 0 |
| **Reference** | 3 | 8.833333 | 9 | 6.666667 | 3.5 | 0.333333 | 0 |
| **Reference** | 3 | 10.66667 | 9.666667 | 7 | 3.666667 | 0.333333 | 0 |
| **Reference** | 3 | 6.666667 | 6.666667 | 4.666667 | 2.333333 | 0.666667 | 0 |
| **DMSO** | 3 | 12.83333 | 13.16667 | 9.666667 | 5.666667 | 2 | 0 |
| **DMSO** | 3 | 5.5 | 5.5 | 4 | 2.333333 | 0 | 0 |
| **DMSO** | 3 | 2.833333 | 3.5 | 2.5 | 1 | 0 | 0 |
| **DMSO** | 3 | 21.66667 | 21.66667 | 17.33333 | 10 | 1.666667 | 0 |
| **DMSO** | 3 | 15.33333 | 14.33333 | 10.5 | 5 | 0.5 | 0.333333 |
| **DMSO** | 3 | 7 | 7.333333 | 6.5 | 4.833333 | 0.833333 | 0 |
| **Reference** | 4 | 9.666667 | 8.666667 | 6 | 2.833333 | 0 | 0 |
| **Reference** | 4 | 5.833333 | 5 | 4.666667 | 2.666667 | 0 | 0.333333 |
| **Reference** | 4 | 20.16667 | 19.16667 | 15 | 7.166667 | 0 | 0 |
| **Reference** | 4 | 12.66667 | 13.5 | 11.16667 | 6.833333 | 1.833333 | 0.5 |
| **Reference** | 4 | 6.833333 | 7 | 5.333333 | 3 | 0 | 0 |
| **Reference** | 4 | 1.166667 | 1.166667 | 0.666667 | 0 | 0 | 0 |
| **DMSO** | 4 | 1.833333 | 2 | 1.5 | 1.666667 | 0 | 0 |
| **DMSO** | 4 | 7.333333 | 6.333333 | 6.166667 | 3.5 | 0.333333 | 0 |
| **DMSO** | 4 | 1.333333 | 1.333333 | 1.333333 | 1.166667 | 0 | 0 |
| **DMSO** | 4 | 7.666667 | 6.5 | 5.166667 | 2 | 0.5 | 0 |
| **DMSO** | 4 | 8.5 | 9 | 7.333333 | 4 | 0.333333 | 0.333333 |
| **DMSO** | 4 | 1.166667 | 1 | 0.833333 | 0.5 | 0 | 0 |

**May 7th, 26th and 29th – 6th hour**

| **Treatment** | **Date** | **0.5** | **1** | **1.5** | **2** | **2.5** | **3** |
|---|---|---|---|---|---|---|---|
| **Reference** | 1 | 1.666667 | 1.666667 | 1 | 0.5 | 0 | 0 |
| **Reference** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 1 | 1.666667 | 1.666667 | 1 | 0.333333 | 0 | 0 |
| **Reference** | 1 | 4.5 | 3.833333 | 3.333333 | 1 | 0 | 0 |
| **Reference** | 1 | 4.666667 | 4.666667 | 4.166667 | 2.666667 | 0.333333 | 0 |
| **Reference** | 1 | 3.833333 | 3.333333 | 2.666667 | 0.5 | 0 | 0 |
| **DMSO** | 1 | 5.5 | 5.666667 | 5 | 3 | 0.833333 | 0 |
| **DMSO** | 1 | 1.333333 | 1.5 | 1.166667 | 0.666667 | 0 | 0 |
| **DMSO** | 1 | 5.166667 | 6.333333 | 5 | 4.333333 | 0.833333 | 0 |
| **DMSO** | 1 | 9.666667 | 8.666667 | 8 | 5 | 0 | 0 |
| **DMSO** | 1 | 4 | 3.333333 | 2.666667 | 1.333333 | 0 | 0 |
| **DMSO** | 1 | 2.666667 | 2.666667 | 2 | 1.333333 | 0.333333 | 0 |
| **Reference** | 3 | 2.666667 | 2.166667 | 1 | 0.333333 | 0 | 0 |
| **Reference** | 3 | 6.666667 | 5.666667 | 5 | 2.166667 | 0 | 0 |
| **Reference** | 3 | 9.5 | 9 | 6.333333 | 3.666667 | 0.333333 | 0.333333 |
| **Reference** | 3 | 6.833333 | 5.5 | 3.5 | 2 | 0 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Reference** | 3 | 4.5 | 4 | 2.333333 | 1.5 | 0 | 0 |
| **Reference** | 3 | 1.833333 | 1.5 | 1.333333 | 0.5 | 0 | 0 |
| **DMSO** | 3 | 17.33333 | 16.83333 | 11.83333 | 7.166667 | 2 | 0 |
| **DMSO** | 3 | 14.16667 | 12.66667 | 10.16667 | 7.666667 | 1.833333 | 0.333333 |
| **DMSO** | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 3 | 6.166667 | 6.166667 | 4.166667 | 1.833333 | 0.5 | 0 |
| **DMSO** | 3 | 3.5 | 2.833333 | 2.166667 | 0.833333 | 0 | 0 |
| **Reference** | 4 | 15.5 | 15.66667 | 14.5 | 9.833333 | 2.5 | 0.5 |
| **Reference** | 4 | 9 | 12 | 10.16667 | 9 | 3.666667 | 1.5 |
| **Reference** | 4 | 15.33333 | 16.66667 | 12 | 7.333333 | 1.666667 | 0.5 |
| **Reference** | 4 | 9.666667 | 9.333333 | 7.666667 | 4.666667 | 1.333333 | 0 |
| **Reference** | 4 | 3.833333 | 3.833333 | 3.333333 | 2.333333 | 0.333333 | 0 |
| **Reference** | 4 | 4 | 4 | 3 | 1 | 0 | 0 |
| **DMSO** | 4 | 4.333333 | 6 | 5 | 2.833333 | 0.333333 | 0 |
| **DMSO** | 4 | 7.5 | 7.333333 | 5 | 2.333333 | 0 | 0 |
| **DMSO** | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 4 | 8.333333 | 8.166667 | 7 | 4.166667 | 0 | 0 |
| **DMSO** | 4 | 11.5 | 11.16667 | 7.833333 | 3.5 | 0.333333 | 0 |
| **DMSO** | 4 | 1.666667 | 1.333333 | 1.833333 | 1.166667 | 0 | 0 |

**May 7th, 26th and 29th – 12th hour**

| **Treatment** | **Date** | **0.5** | **1** | **1.5** | **2** | **2.5** | **3** |
|---|---|---|---|---|---|---|---|
| **Reference** | 1 | 10.66667 | 9.333333 | 7 | 4.333333 | 0 | 0 |
| **Reference** | 1 | 6 | 7.333333 | 6.333333 | 4.833333 | 1.833333 | 0.5 |
| **Reference** | 1 | 19.5 | 19.5 | 16.16667 | 10 | 1.333333 | 0.666667 |
| **Reference** | 1 | 7.833333 | 7.333333 | 6.333333 | 2.833333 | 0 | 0.333333 |
| **Reference** | 1 | 7 | 6.5 | 6.166667 | 4.166667 | 0 | 0 |
| **Reference** | 1 | 16.33333 | 15.33333 | 11.83333 | 6.666667 | 2.166667 | 0 |
| **DMSO** | 1 | 11.83333 | 13.5 | 12.5 | 10.16667 | 3.333333 | 2.166667 |
| **DMSO** | 1 | 15.33333 | 16 | 13.33333 | 7.666667 | 2.5 | 0.666667 |
| **DMSO** | 1 | 8.5 | 9 | 7.666667 | 5.833333 | 1.5 | 0.333333 |
| **DMSO** | 1 | 5 | 5.166667 | 4.5 | 3.333333 | 0.833333 | 0 |
| **DMSO** | 1 | 9.833333 | 9.5 | 7.166667 | 4.833333 | 0 | 0 |
| **DMSO** | 1 | 1 | 1.166667 | 2 | 1 | 0 | 0 |
| **Reference** | 3 | 8.166667 | 7.333333 | 5 | 2.333333 | 0 | 0 |
| **Reference** | 3 | 6.5 | 6.833333 | 5.5 | 3.166667 | 0.333333 | 0.666667 |
| **Reference** | 3 | 11.16667 | 10.5 | 7.833333 | 4.666667 | 1.5 | 0 |
| **Reference** | 3 | 8.666667 | 7.166667 | 4.333333 | 2.5 | 0 | 0 |
| **Reference** | 3 | 7.666667 | 7 | 5.333333 | 2.333333 | 0 | 0 |
| **Reference** | 3 | 2.833333 | 2.833333 | 2.166667 | 1.5 | 0 | 0 |
| **DMSO** | 3 | 1.5 | 1.5 | 1.333333 | 0 | 0 | 0 |
| **DMSO** | 3 | 5.5 | 5.166667 | 3.833333 | 1.5 | 0 | 0 |
| **DMSO** | 3 | 0 | 0 | 0 | 0 | 0 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **DMSO** | 3 | 7 | 6.333333 | 5 | 3.166667 | 0.333333 | 0 |
| **DMSO** | 3 | 4 | 4.333333 | 2 | 0.333333 | 0 | 0 |
| **DMSO** | 3 | 5.333333 | 4.833333 | 3.833333 | 1.166667 | 0 | 0 |
| **Reference** | 4 | 5.5 | 5.166667 | 4.166667 | 2.166667 | 0 | 0 |
| **Reference** | 4 | 10.33333 | 9.666667 | 6.5 | 3.5 | 0 | 0 |
| **Reference** | 4 | 13.66667 | 13.83333 | 11.33333 | 8 | 0.5 | 0.833333 |
| **Reference** | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 4 | 4.5 | 4.666667 | 4.166667 | 2.5 | 0 | 0 |
| **Reference** | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 4 | 2 | 1.833333 | 1.5 | 1 | 0 | 0 |
| **DMSO** | 4 | 2.666667 | 2.666667 | 1.333333 | 0.333333 | 0 | 0 |
| **DMSO** | 4 | 0.666667 | 0.833333 | 0.333333 | 0 | 0 | 0 |
| **DMSO** | 4 | 21.33333 | 16 | 11 | 5.333333 | 0.666667 | 0 |
| **DMSO** | 4 | 22.5 | 19.16667 | 12.66667 | 5.833333 | 0.5 | 0 |
| **DMSO** | 4 | 5.166667 | 5 | 3.5 | 2.166667 | 0 | 0 |

***Hyalella azteca* – DMSO**

**May 19th and 20th – 1st hour**

| Treatment | Date | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | 1 | 59.33333 | 42.83333 | 22.83333 | 9.666667 | 2.833333 | 0.5 |
| **Reference** | 1 | 76 | 69.5 | 37.5 | 11.83333 | 2.333333 | 0.833333 |
| **Reference** | 1 | 71.83333 | 63.83333 | 42 | 17.83333 | 3.666667 | 0 |
| **Reference** | 1 | 51.5 | 40.16667 | 24.66667 | 11 | 1.5 | 0.333333 |
| **Reference** | 1 | 64.16667 | 50 | 29.33333 | 13.16667 | 1.833333 | 0 |
| **Reference** | 1 | 51.16667 | 41.16667 | 22.83333 | 9.666667 | 0.5 | 0 |
| **DMSO** | 1 | 39.66667 | 33.5 | 23.5 | 11.83333 | 2.166667 | 0.666667 |
| **DMSO** | 1 | 62.83333 | 40.5 | 22.83333 | 12.16667 | 7.333333 | 6.333333 |
| **DMSO** | 1 | 47 | 36.83333 | 21.16667 | 11.5 | 2.333333 | 0.333333 |
| **DMSO** | 1 | 52.33333 | 38.16667 | 18.16667 | 9.5 | 3.333333 | 0.833333 |
| **DMSO** | 1 | 32 | 25 | 12.5 | 5 | 0.5 | 0.333333 |
| **DMSO** | 1 | 60.16667 | 45.5 | 20.33333 | 6.666667 | 0.5 | 0 |
| **Reference** | 2 | 73.16667 | 58.66667 | 31.16667 | 13.5 | 3 | 0.5 |
| **Reference** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 2 | 35.16667 | 27.5 | 13.5 | 5 | 1.333333 | 0.333333 |
| **Reference** | 2 | 4.666667 | 3.166667 | 2.666667 | 1.166667 | 0 | 0 |
| **Reference** | 2 | 59.83333 | 44.5 | 26.5 | 10 | 1.333333 | 0 |
| **Reference** | 2 | 28 | 22.16667 | 10.66667 | 4.166667 | 1 | 0.666667 |
| **DMSO** | 2 | 51.16667 | 40.33333 | 20.83333 | 9 | 1 | 0.333333 |
| **DMSO** | 2 | 40.83333 | 30.83333 | 18 | 7.333333 | 2.166667 | 0 |
| **DMSO** | 2 | 54.66667 | 41.83333 | 22.33333 | 10.33333 | 3.833333 | 2 |
| **DMSO** | 2 | 46.33333 | 33.5 | 17.16667 | 8.166667 | 2.333333 | 1 |
| **DMSO** | 2 | 42.83333 | 33.33333 | 16.5 | 6.5 | 1.5 | 0.333333 |
| **DMSO** | 2 | 55 | 41.16667 | 18.66667 | 9.833333 | 2.166667 | 1 |

**May 19th and 20th – 6th hour**

| Treatment | Date | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | 1 | 11.66667 | 8.666667 | 4.5 | 1.833333 | 0 | 0 |
| **Reference** | 1 | 69.33333 | 58.66667 | 29.66667 | 12.83333 | 4.666667 | 2.166667 |
| **Reference** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 1 | 11.66667 | 9.5 | 6.833333 | 2.166667 | 0.5 | 0 |
| **Reference** | 1 | 34 | 27.16667 | 16.5 | 6.333333 | 1 | 0 |
| **Reference** | 1 | 43 | 34.83333 | 19.16667 | 7.166667 | 0.5 | 0 |
| **DMSO** | 1 | 19.33333 | 15.66667 | 9.333333 | 3.666667 | 0.333333 | 0 |
| **DMSO** | 1 | 38.66667 | 25.5 | 12.66667 | 5.5 | 1.833333 | 0.833333 |
| **DMSO** | 1 | 39.33333 | 28.83333 | 17.83333 | 8.5 | 3.333333 | 2.5 |
| **DMSO** | 1 | 8.166667 | 5.5 | 2.666667 | 1.333333 | 0.333333 | 0 |
| **DMSO** | 1 | 2.5 | 2.5 | 1.666667 | 0.333333 | 0 | 0 |
| **DMSO** | 1 | 41.83333 | 35.66667 | 19.33333 | 8 | 3.333333 | 0 |
| **Reference** | 2 | 60 | 46.83333 | 22.33333 | 11.5 | 4.166667 | 0.833333 |
| **Reference** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 2 | 52 | 41.83333 | 22.33333 | 10.16667 | 2.5 | 0 |
| **Reference** | 2 | 8.5 | 6.666667 | 3.666667 | 1.166667 | 0 | 0 |
| **Reference** | 2 | 28.33333 | 19.5 | 9.333333 | 2 | 0.333333 | 0 |
| **Reference** | 2 | 24.16667 | 17 | 8.166667 | 3 | 0 | 0 |
| **DMSO** | 2 | 44 | 31.33333 | 19.5 | 7.5 | 1.833333 | 0 |
| **DMSO** | 2 | 57.66667 | 43.33333 | 26.66667 | 11 | 3 | 0 |
| **DMSO** | 2 | 45.66667 | 36 | 22.83333 | 13.5 | 5.166667 | 2.166667 |
| **DMSO** | 2 | 30.33333 | 19.66667 | 9 | 5.333333 | 1.166667 | 0 |
| **DMSO** | 2 | 24.16667 | 19.5 | 11.5 | 4.333333 | 0.333333 | 0 |
| **DMSO** | 2 | 42.33333 | 29.5 | 14.66667 | 7.666667 | 0.833333 | 0 |

**May 19th and 20th – 12th hour averages**

| Treatment | Date | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | 1 | 22.83333 | 16 | 9.666667 | 4.166667 | 1 | 0.333333 |
| **Reference** | 1 | 49.33333 | 43.33333 | 24.66667 | 12.66667 | 3.833333 | 0.5 |
| **Reference** | 1 | 31.5 | 26 | 16.83333 | 6.666667 | 1.166667 | 0.5 |
| **Reference** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 1 | 20.33333 | 17.33333 | 10.66667 | 5.166667 | 1.5 | 0 |
| **Reference** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 1 | 26.16667 | 22 | 14.16667 | 6.666667 | 0.5 | 0 |
| **DMSO** | 1 | 10.66667 | 6.166667 | 3.666667 | 2.333333 | 0.833333 | 0.666667 |
| **DMSO** | 1 | 37.33333 | 30.66667 | 19.5 | 10.16667 | 3.833333 | 1 |
| **DMSO** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 1 | 6 | 5.5 | 2.666667 | 1.666667 | 0 | 0 |
| **DMSO** | 1 | 23.66667 | 18.33333 | 10.16667 | 4.666667 | 0.666667 | 0 |
| **Reference** | 2 | 69 | 54 | 30.5 | 13 | 3.333333 | 0.333333 |
| **Reference** | 2 | 18.83333 | 15.83333 | 10.83333 | 5 | 1.5 | 0 |
| **Reference** | 2 | 19 | 17 | 13 | 8.833333 | 2 | 0.666667 |
| **Reference** | 2 | 28.66667 | 20 | 10.66667 | 4.333333 | 0.666667 | 0 |
| **Reference** | 2 | 16.5 | 12.66667 | 5 | 1.833333 | 0 | 0 |
| **Reference** | 2 | 6.5 | 5.5 | 2.5 | 0 | 0 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **DMSO** | 2 | 9.666667 | 8.333333 | 6.666667 | 3.333333 | 0 | 0 |
| **DMSO** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 2 | 14.5 | 11.16667 | 8.5 | 4 | 0.666667 | 0 |
| **DMSO** | 2 | 14.16667 | 10.5 | 6.666667 | 2 | 0.5 | 0 |

***Daphnia magna* – TBT**

**Feb. 8th and 10th – 1st hour means**

| Treatment | Date | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | **1** | 4.166667 | 4 | 3.833333 | 1.5 | 0.333333 | 0 |
| **Reference** | **1** | 8.333333 | 8.166667 | 7 | 3.333333 | 0.333333 | 0 |
| **Reference** | **1** | 26.83333 | 23.16667 | 18 | 13.83333 | 1.666667 | 1.666667 |
| **Reference** | **1** | 0.333333 | 0.5 | 0 | 0.333333 | 0 | 0 |
| **Reference** | **2** | 30.5 | 27.66667 | 18.16667 | 8 | 1 | 0 |
| **Reference** | **2** | 24 | 26 | 20.5 | 11.83333 | 3.5 | 1.333333 |
| **Reference** | **2** | 21.83333 | 24.83333 | 22.33333 | 18.33333 | 6.333333 | 3 |
| **Reference** | **2** | 35 | 32.83333 | 27 | 17.33333 | 4 | 0.5 |
| **DMSO** | **1** | 0.333333 | 0.333333 | 0.666667 | 0.666667 | 0 | 0 |
| **DMSO** | **1** | 0.666667 | 0.666667 | 0.5 | 0.333333 | 0 | 0 |
| **DMSO** | **1** | 8.333333 | 7.833333 | 7.166667 | 4.5 | 0 | 0 |
| **DMSO** | **1** | 3.5 | 3.333333 | 3.166667 | 2.166667 | 0 | 0 |
| **DMSO** | **2** | 27.66667 | 33.66667 | 31.66667 | 23 | 13.16667 | 6.833333 |
| **DMSO** | **2** | 7.333333 | 6.333333 | 4.166667 | 0.833333 | 0 | 0 |
| **DMSO** | **2** | 7.833333 | 10.83333 | 12.83333 | 10.5 | 4.333333 | 2.5 |
| **DMSO** | **2** | 15.5 | 17.33333 | 15.33333 | 11 | 3.666667 | 1 |
| **1ug/L TBT** | **1** | 1.5 | 1.5 | 1.166667 | 0.833333 | 0.5 | 0 |
| **1ug/L TBT** | **1** | 5.833333 | 6 | 5.166667 | 3 | 0 | 0 |
| **1ug/L TBT** | **1** | 7.333333 | 8.166667 | 7.333333 | 5.333333 | 0.833333 | 0 |
| **1ug/L TBT** | **2** | 19.33333 | 23.5 | 21.16667 | 14.33333 | 4.666667 | 1.833333 |
| **1ug/L TBT** | **2** | 25 | 24.83333 | 22 | 16.66667 | 4 | 1 |
| **1ug/L TBT** | **2** | 19.16667 | 19.66667 | 16.16667 | 10.16667 | 3.333333 | 0.5 |
| **1ug/L TBT** | **2** | 14.16667 | 15 | 13.33333 | 9.166667 | 3.166667 | 0.833333 |
| **10ug/L TBT** | **1** | 6 | 6 | 5.666667 | 3.166667 | 0.5 | 0 |
| **10ug/L TBT** | **1** | 9.5 | 9.666667 | 8 | 5.166667 | 0 | 0 |
| **10ug/L TBT** | **1** | 3.833333 | 3.666667 | 2.666667 | 1.166667 | 0 | 0 |
| **10ug/L TBT** | **1** | 3.166667 | 3 | 3.166667 | 1.333333 | 0 | 0 |
| **10ug/L TBT** | **2** | 20.66667 | 21.5 | 18.5 | 13.66667 | 5.833333 | 1.666667 |
| **10ug/L TBT** | **2** | 23 | 29.16667 | 28.66667 | 21.16667 | 9.833333 | 3.5 |
| **10ug/L TBT** | **2** | 0 | 0 | 0 | 0 | 0 | 0 |
| **10ug/L TBT** | **2** | 16.16667 | 17.33333 | 16.66667 | 12.5 | 3.166667 | 0.833333 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **100ug/L TBT** | **1** | 4.166667 | 5 | 4 | 2.5 | 0.666667 | 0.5 |
| **100ug/L TBT** | **1** | 9.333333 | 8.833333 | 8.166667 | 5.166667 | 0.666667 | 0 |
| **100ug/L TBT** | **1** | 2.666667 | 2.333333 | 2.5 | 1.166667 | 0 | 0 |
| **100ug/L TBT** | **2** | 17.5 | 14.33333 | 11.83333 | 6.5 | 0 | 0 |
| **100ug/L TBT** | **2** | 23.83333 | 27.5 | 25.5 | 17.33333 | 6.166667 | 3.166667 |
| **100ug/L TBT** | **2** | 25 | 29.5 | 26.5 | 18.5 | 7.5 | 3.833333 |
| **100ug/L TBT** | **2** | 35 | 38.33333 | 31.83333 | 19.33333 | 6.666667 | 1.666667 |

**Feb. 8th and 10th – 2nd hour means**

| **Treatment** | **Date** | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | **1** | 5.5 | 5.333333 | 4.5 | 3.166667 | 0 | 0 |
| **Reference** | **1** | 4.833333 | 4.666667 | 4.333333 | 2 | 0 | 0 |
| **Reference** | **1** | 30.33333 | 28 | 23 | 16.66667 | 3.333333 | 1.333333 |
| **Reference** | **1** | 4.333333 | 4.5 | 4 | 1.666667 | 0 | 0 |
| **Reference** | **2** | 26.66667 | 23.83333 | 15.16667 | 7.166667 | 1.166667 | 0 |
| **Reference** | **2** | 24.5 | 24.5 | 22.16667 | 13.33333 | 4.666667 | 1.333333 |
| **Reference** | **2** | 32.5 | 32.5 | 27.16667 | 15.66667 | 3 | 0.333333 |
| **Reference** | **2** | 22.83333 | 22.5 | 20 | 14 | 4.833333 | 2 |
| **DMSO** | **1** | 6.833333 | 6.666667 | 4.833333 | 2.333333 | 0 | 0 |
| **DMSO** | **1** | 1.833333 | 1.666667 | 1.166667 | 0 | 0 | 0 |
| **DMSO** | **1** | 2.666667 | 2.833333 | 2.833333 | 2 | 0.333333 | 0 |
| **DMSO** | **1** | 2.833333 | 3 | 2.333333 | 1.333333 | 0 | 0 |
| **DMSO** | **2** | 23.33333 | 25 | 21 | 13.83333 | 6.333333 | 2 |
| **DMSO** | **2** | 2.166667 | 2.166667 | 1.833333 | 0.666667 | 0 | 0 |
| **DMSO** | **2** | 11.16667 | 11.83333 | 11.16667 | 9.666667 | 2.5 | 0.666667 |
| **DMSO** | **2** | 16.5 | 15.83333 | 11.66667 | 7 | 2.833333 | 0.5 |
| **1ug/L TBT** | **1** | 7 | 8.833333 | 7.833333 | 4.833333 | 1.333333 | 0 |
| **1ug/L TBT** | **1** | 5 | 4.833333 | 4 | 2.333333 | 0 | 0 |
| **1ug/L TBT** | **1** | 4.166667 | 4.666667 | 4.666667 | 3.333333 | 0.333333 | 0 |
| **1ug/L TBT** | **2** | 11 | 10.33333 | 8.666667 | 3.833333 | 0 | 0 |
| **1ug/L TBT** | **2** | 17.66667 | 18.66667 | 15.66667 | 8.666667 | 2.666667 | 0.333333 |
| **1ug/L TBT** | **2** | 18.5 | 18.66667 | 16.33333 | 11.83333 | 3 | 0.833333 |
| **1ug/L TBT** | **2** | 11.83333 | 14 | 13 | 10.5 | 5 | 2 |
| **10ug/L TBT** | **1** | 6 | 5.333333 | 3.166667 | 2 | 0 | 0 |
| **10ug/L TBT** | **1** | 9 | 9 | 7.833333 | 4 | 0.333333 | 0 |
| **10ug/L TBT** | **1** | 6.5 | 6.166667 | 5.333333 | 2.5 | 0.5 | 0.5 |
| **10ug/L TBT** | **1** | 4.166667 | 4 | 3.833333 | 3.333333 | 0.666667 | 0.333333 |
| **10ug/L TBT** | **2** | 28.5 | 27.66667 | 23.16667 | 15 | 5 | 1.5 |
| **10ug/L TBT** | **2** | 21.83333 | 22.83333 | 19.83333 | 11.33333 | 3.666667 | 2 |
| **10ug/L TBT** | **2** | 4.333333 | 4.333333 | 3.5 | 1 | 0 | 0 |
| **10ug/L TBT** | **2** | 20.33333 | 22.33333 | 20 | 16.83333 | 5.666667 | 3.166667 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **100ug/L TBT** | **1** | 7.666667 | 8.666667 | 8.166667 | 4.333333 | 1.5 | 0.5 |
| **100ug/L TBT** | **1** | 5.333333 | 5 | 3.333333 | 2.166667 | 0 | 0 |
| **100ug/L TBT** | **1** | 9.833333 | 11.83333 | 10.66667 | 7.666667 | 1.166667 | 0 |
| **100ug/L TBT** | **2** | 23.33333 | 22.33333 | 17 | 10.16667 | 1.666667 | 0 |
| **100ug/L TBT** | **2** | 11.33333 | 11.66667 | 9.333333 | 6 | 1.833333 | 1.166667 |
| **100ug/L TBT** | **2** | 6.333333 | 6.333333 | 4.833333 | 2.166667 | 0 | 0 |
| **100ug/L TBT** | **2** | 16.5 | 16.5 | 13.16667 | 8.666667 | 2.333333 | 0 |

**Feb. 8th and 10th – 6th hour means**

| **Treatment** | **Date** | **0.5** | **1** | **1.5** | **2** | **2.5** | **3** |
|---|---|---|---|---|---|---|---|
| **Reference** | **1** | 6 | 5.833333 | 4.833333 | 3.166667 | 0 | 0 |
| **Reference** | **1** | 1.666667 | 1.833333 | 1 | 0.333333 | 0 | 0 |
| **Reference** | **1** | 25 | 21.83333 | 17.83333 | 13.66667 | 3.333333 | 1.833333 |
| **Reference** | **1** | 4.166667 | 3.333333 | 3.333333 | 1.666667 | 0 | 0 |
| **Reference** | **2** | 21.16667 | 17.5 | 12.16667 | 5.833333 | 1.333333 | 0 |
| **Reference** | **2** | 15.83333 | 15.33333 | 11.33333 | 6.833333 | 2 | 0 |
| **Reference** | **2** | 11.66667 | 11 | 9.5 | 6.166667 | 1 | 0.333333 |
| **Reference** | **2** | 17.83333 | 16.16667 | 12.66667 | 6.5 | 1.5 | 0 |
| **DMSO** | **1** | 4.166667 | 3.833333 | 3.5 | 1.333333 | 0 | 0 |
| **DMSO** | **1** | 2.833333 | 2.833333 | 2.5 | 1.166667 | 0 | 0 |
| **DMSO** | **1** | 1 | 0.833333 | 1 | 0.666667 | 0 | 0 |
| **DMSO** | **1** | 2 | 2.166667 | 1.333333 | 0.5 | 0 | 0 |
| **DMSO** | **2** | 13.83333 | 14 | 14.5 | 8.666667 | 1.333333 | 0.5 |
| **DMSO** | **2** | 2.5 | 2.333333 | 1.833333 | 0.666667 | 0 | 0 |
| **DMSO** | **2** | 5.5 | 5.833333 | 5 | 2.833333 | 0.333333 | 0 |
| **DMSO** | **2** | 11.5 | 12 | 9.833333 | 6 | 1.166667 | 0 |
| **1ug/L TBT** | **1** | 2.166667 | 2.333333 | 1.666667 | 0.833333 | 0 | 0 |
| **1ug/L TBT** | **1** | 2.5 | 2.5 | 2.5 | 1.5 | 0 | 0 |
| **1ug/L TBT** | **1** | 1.666667 | 1.666667 | 1.666667 | 1.666667 | 0 | 0 |
| **1ug/L TBT** | **2** | 1 | 1.333333 | 1 | 0.5 | 0 | 0 |
| **1ug/L TBT** | **2** | 14.5 | 13.33333 | 11.5 | 6 | 1 | 0 |
| **1ug/L TBT** | **2** | 19 | 17.33333 | 13.5 | 7.666667 | 2 | 0.5 |
| **1ug/L TBT** | **2** | 5 | 5.5 | 4.666667 | 3.166667 | 0.833333 | 0.5 |
| **10ug/L TBT** | **1** | 9.333333 | 8 | 5.333333 | 3.333333 | 0.5 | 0 |
| **10ug/L TBT** | **1** | 10.33333 | 9.666667 | 8.166667 | 5.5 | 0 | 0 |
| **10ug/L TBT** | **1** | 1.5 | 1.333333 | 1 | 0.5 | 0 | 0 |
| **10ug/L TBT** | **1** | 3.666667 | 3.333333 | 3 | 2 | 0 | 0 |
| **10ug/L TBT** | **2** | 20.33333 | 19.16667 | 14.66667 | 9.666667 | 2 | 0 |
| **10ug/L TBT** | **2** | 8.833333 | 9 | 7.166667 | 4.833333 | 1.333333 | 0 |
| **10ug/L TBT** | **2** | 3.666667 | 3.5 | 2.833333 | 0 | 0 | 0 |
| **10ug/L TBT** | **2** | 10.66667 | 9.333333 | 6.166667 | 2.333333 | 0.5 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **100ug/L TBT** | **1** | 2.5 | 2.333333 | 2.5 | 1.333333 | 0 | 0 |
| **100ug/L TBT** | **1** | 0.833333 | 0.833333 | 0 | 0.333333 | 0 | 0 |
| **100ug/L TBT** | **1** | 4.166667 | 4.666667 | 3 | 0.5 | 0 | 0 |
| **100ug/L TBT** | **2** | 16.16667 | 15.16667 | 11.5 | 7.333333 | 3.833333 | 1.5 |
| **100ug/L TBT** | **2** | 7.833333 | 6.333333 | 3.666667 | 2.333333 | 0 | 0.333333 |
| **100ug/L TBT** | **2** | 8.833333 | 7.666667 | 5.833333 | 3.833333 | 1.166667 | 0 |
| **100ug/L TBT** | **2** | 12.83333 | 12.83333 | 10.16667 | 7.5 | 1.333333 | 0.5 |

**Feb. 8th and 10th – 12th hour means**

| **Treatment** | **Date** | **0.5** | **1** | **1.5** | **2** | **2.5** | **3** |
|---|---|---|---|---|---|---|---|
| **Reference** | **1** | 1.166667 | 1.166667 | 0.833333 | 0.833333 | 0 | 0 |
| **Reference** | **1** | 2.666667 | 2.833333 | 1.833333 | 0.333333 | 0 | 0 |
| **Reference** | **1** | 25.5 | 24.33333 | 19 | 13.66667 | 3.333333 | 2 |
| **Reference** | **1** | 2.666667 | 2.666667 | 2.166667 | 1.166667 | 0 | 0 |
| **Reference** | **2** | 15.5 | 12.83333 | 11.33333 | 7 | 1.666667 | 0.333333 |
| **Reference** | **2** | 14.33333 | 12.66667 | 8.666667 | 3.833333 | 0 | 0 |
| **Reference** | **2** | 13.83333 | 11.33333 | 9.333333 | 4.833333 | 0.833333 | 0.333333 |
| **Reference** | **2** | 20.66667 | 18.33333 | 15 | 9.333333 | 2.5 | 0 |
| **DMSO** | **1** | 6.833333 | 6 | 4 | 2.166667 | 0 | 0 |
| **DMSO** | **1** | 1 | 1 | 0.833333 | 0.5 | 0 | 0 |
| **DMSO** | **1** | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | **1** | 7.333333 | 6.5 | 4.166667 | 3 | 0.833333 | 0 |
| **DMSO** | **2** | 11.33333 | 10.83333 | 7.333333 | 5.5 | 1 | 0.333333 |
| **DMSO** | **2** | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | **2** | 2.333333 | 2.333333 | 1.833333 | 1.333333 | 0 | 0 |
| **DMSO** | **2** | 23.16667 | 20.5 | 13.16667 | 6.5 | 0.833333 | 0 |
| **1ug/L TBT** | **1** | 4 | 3.833333 | 2.833333 | 1.5 | 0 | 0 |
| **1ug/L TBT** | **1** | 4.333333 | 4.5 | 4.166667 | 2.166667 | 0.333333 | 0.333333 |
| **1ug/L TBT** | **1** | 0.666667 | 1.333333 | 2 | 1.833333 | 0.333333 | 0 |
| **1ug/L TBT** | **2** | 7.833333 | 6.666667 | 4.333333 | 2.333333 | 0 | 0 |
| **1ug/L TBT** | **2** | 15.33333 | 13.16667 | 8.666667 | 4.333333 | 0 | 0 |
| **1ug/L TBT** | **2** | 23.66667 | 19.83333 | 13.83333 | 5.333333 | 2 | 0.5 |
| **1ug/L TBT** | **2** | 1.333333 | 1.166667 | 0.833333 | 0.5 | 0 | 0 |
| **10ug/L TBT** | **1** | 7.833333 | 6.5 | 4.5 | 3 | 0.833333 | 0.333333 |
| **10ug/L TBT** | **1** | 3.833333 | 3.833333 | 3 | 2 | 0 | 0 |
| **10ug/L TBT** | **1** | 1 | 1.166667 | 1.5 | 1.166667 | 0 | 0 |
| **10ug/L TBT** | **1** | 6 | 6.833333 | 4.5 | 4 | 0.333333 | 0 |
| **10ug/L TBT** | **2** | 10.83333 | 9.166667 | 7.166667 | 4.5 | 1.333333 | 0 |
| **10ug/L TBT** | **2** | 26.66667 | 28.83333 | 20 | 15.33333 | 5 | 0.333333 |
| **10ug/L TBT** | **2** | 0 | 0 | 0 | 0 | 0 | 0 |
| **10ug/L TBT** | **2** | 12.66667 | 9.666667 | 8.333333 | 7 | 3.166667 | 1.333333 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **100ug/L TBT** | **1** | 2.166667 | 1.666667 | 2 | 1.166667 | 0 | 0 |
| **100ug/L TBT** | **1** | 0.5 | 0.666667 | 0.5 | 0.666667 | 0 | 0 |
| **100ug/L TBT** | **1** | 0 | 0 | 0 | 0 | 0 | 0 |
| **100ug/L TBT** | **2** | 0 | 0 | 0 | 0 | 0 | 0 |
| **100ug/L TBT** | **2** | 13.33333 | 13 | 10.16667 | 6 | 1 | 0 |
| **100ug/L TBT** | **2** | 8.666667 | 8.166667 | 6.333333 | 2.5 | 0 | 0 |
| **100ug/L TBT** | **2** | 0 | 0 | 0 | 0 | 0 | 0 |

***Hyalella azteca* – TBT**

**March 23rd and 26th – 1st hour means**

| Treatment | Date | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | 1 | 16.83333 | 14.5 | 9.333333 | 4.166667 | 1 | 0 |
| **Reference** | 1 | 15.66667 | 12.66667 | 8 | 4.5 | 0.333333 | 0 |
| **Reference** | 1 | 22 | 14.33333 | 7.333333 | 2.833333 | 0 | 0 |
| **Reference** | 2 | 2.166667 | 1.5 | 0.666667 | 0.5 | 0 | 0 |
| **Reference** | 2 | 12.83333 | 10.16667 | 6.666667 | 2.833333 | 0.5 | 0 |
| **Reference** | 2 | 2.333333 | 2 | 1.333333 | 0.5 | 0 | 0 |
| **Reference** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 2 | 11 | 10.16667 | 8.166667 | 8.166667 | 1 | 0.5 |
| **DMSO** | 1 | 46.83333 | 38.33333 | 25.33333 | 8.833333 | 0.833333 | 0 |
| **DMSO** | 1 | 18.83333 | 12 | 6.666667 | 2.333333 | 0 | 0 |
| **DMSO** | 1 | 64.83333 | 51.5 | 24.16667 | 10 | 1 | 0 |
| **DMSO** | 1 | 9.5 | 6.666667 | 3.333333 | 2.166667 | 0 | 0 |
| **DMSO** | 1 | 40.33333 | 30.66667 | 16.66667 | 9.666667 | 2.333333 | 0 |
| **DMSO** | 2 | 12 | 8.666667 | 5.166667 | 2 | 0.5 | 0 |
| **DMSO** | 2 | 4.5 | 3.333333 | 1.166667 | 0.5 | 0 | 0 |
| **DMSO** | 2 | 26.16667 | 22.66667 | 12 | 3.833333 | 0 | 0 |
| **DMSO** | 2 | 4.166667 | 2.666667 | 1.5 | 0.833333 | 0 | 0 |
| **1ugL** | 1 | 47.83333 | 37.33333 | 21.5 | 9.833333 | 1 | 0.333333 |
| **1ugL** | 1 | 1.5 | 1.666667 | 1 | 0.5 | 0 | 0 |
| **1ugL** | 1 | 9 | 7.666667 | 4 | 1.666667 | 0.5 | 0 |
| **1ugL** | 1 | 13.33333 | 12 | 7 | 3.166667 | 0.5 | 0 |
| **1ugL** | 1 | 2.833333 | 2.333333 | 1.666667 | 0.833333 | 0 | 0 |
| **1ugL** | 2 | 9.166667 | 7.333333 | 3.833333 | 0.666667 | 0 | 0 |
| **1ugL** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1ugL** | 2 | 2.333333 | 2.166667 | 1.5 | 0.833333 | 0 | 0 |
| **1ugL** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1ugL** | 2 | 53.66667 | 46 | 28.83333 | 16.5 | 2.833333 | 1.5 |
| **10ugL** | 1 | 53.33333 | 37.5 | 19.33333 | 10.33333 | 2.5 | 0 |
| **10ugL** | 1 | 13.33333 | 9.333333 | 3.666667 | 1 | 0 | 0 |
| **10ugL** | 1 | 45.16667 | 42.33333 | 32.16667 | 18 | 4.5 | 0.333333 |
| **10ugL** | 1 | 18.83333 | 14.66667 | 8 | 3.666667 | 0 | 0 |
| **10ugL** | 2 | 2.833333 | 2.5 | 1.5 | 1.333333 | 0 | 0 |
| **10ugL** | 2 | 16.33333 | 14.66667 | 8.5 | 5.166667 | 2 | 0.5 |
| **10ugL** | 2 | 10.66667 | 7.5 | 5.333333 | 1.833333 | 0 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **10ugL** | 2 | 11.66667 | 9 | 5 | 2.333333 | 0 | 0 |
| **100ugL** | 1 | 1.166667 | 0.833333 | 0.5 | 0 | 0 | 0 |
| **100ugL** | 1 | 25.66667 | 15.16667 | 7 | 1 | 0 | 0 |
| **100ugL** | 1 | 31.5 | 26.83333 | 16.5 | 8.333333 | 1.666667 | 0 |
| **100ugL** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **100ugL** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **100ugL** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |

**March 23rd and 26th – 2nd hour means**

| Treatment | Date | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | 1 | 2hr | 9.166667 | 6.5 | 3.333333 | 1.5 | 0 |
| **Reference** | 1 | 2hr | 29.33333 | 26.33333 | 16.66667 | 8.833333 | 0 |
| **Reference** | 1 | 2hr | 20.33333 | 14.16667 | 6.833333 | 3.166667 | 0.833333 |
| **Reference** | 2 | 2hr | 17 | 15.33333 | 11.16667 | 5.833333 | 0.5 |
| **Reference** | 2 | 2hr | 17.66667 | 14.5 | 8.333333 | 3.833333 | 0.333333 |
| **Reference** | 2 | 2hr | 13.33333 | 10.33333 | 6.666667 | 3.5 | 0.833333 |
| **Reference** | 2 | 2hr | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 2 | 2hr | 12.83333 | 12.33333 | 10.33333 | 8.333333 | 1.5 |
| **DMSO** | 1 | 2hr | 40.83333 | 33.33333 | 21.5 | 8.833333 | 0.5 |
| **DMSO** | 1 | 2hr | 15.16667 | 11.83333 | 6.333333 | 1.833333 | 0 |
| **DMSO** | 1 | 2hr | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 1 | 2hr | 11.33333 | 8.5 | 5.166667 | 2.5 | 0 |
| **DMSO** | 1 | 2hr | 28.33333 | 20.83333 | 13.5 | 6.5 | 0 |
| **DMSO** | 2 | 2hr | 13.16667 | 9.166667 | 7.5 | 3.5 | 0.833333 |
| **DMSO** | 2 | 2hr | 6 | 5 | 3.166667 | 1.166667 | 0 |
| **DMSO** | 2 | 2hr | 32.5 | 26.5 | 14.5 | 5.333333 | 0.333333 |
| **DMSO** | 2 | 2hr | 0 | 0 | 0 | 0 | 0 |
| **1ugL** | 1 | 2hr | 40.83333 | 31.33333 | 18.66667 | 8 | 1.666667 |
| **1ugL** | 1 | 2hr | 0 | 0 | 0 | 0 | 0 |
| **1ugL** | 1 | 2hr | 18 | 14.16667 | 7.833333 | 3.333333 | 0 |
| **1ugL** | 1 | 2hr | 12.5 | 10.33333 | 6 | 2.333333 | 0 |
| **1ugL** | 1 | 2hr | 0 | 0 | 0 | 0 | 0 |
| **1ugL** | 2 | 2hr | 12.33333 | 10.33333 | 4.833333 | 1.833333 | 0 |
| **1ugL** | 2 | 2hr | 0 | 0 | 0 | 0 | 0 |
| **1ugL** | 2 | 2hr | 10.16667 | 9.666667 | 5.333333 | 2.5 | 0.833333 |
| **1ugL** | 2 | 2hr | 0 | 0 | 0 | 0 | 0 |
| **1ugL** | 2 | 2hr | 63 | 57.5 | 40.16667 | 21 | 6.5 |
| **10ugL** | 1 | 2hr | 43.33333 | 28.83333 | 15.16667 | 6.166667 | 1.166667 |
| **10ugL** | 1 | 2hr | 9.166667 | 7 | 2.833333 | 0.833333 | 0.5 |
| **10ugL** | 1 | 2hr | 41.16667 | 38.66667 | 34.33333 | 14.83333 | 3.333333 |
| **10ugL** | 1 | 2hr | 24.33333 | 18.66667 | 12.66667 | 6.333333 | 1.333333 |
| **10ugL** | 2 | 2hr | 17 | 13.66667 | 7.5 | 3.833333 | 0.333333 |
| **10ugL** | 2 | 2hr | 26.66667 | 21.66667 | 14.16667 | 7 | 1.5 |
| **10ugL** | 2 | 2hr | 24.66667 | 20.5 | 12.33333 | 5.333333 | 1 |
| **10ugL** | 2 | 2hr | 11.83333 | 10 | 5.333333 | 1.666667 | 0 |
| **100ugL** | 1 | 2hr | 3.833333 | 3.333333 | 2.333333 | 1.166667 | 0 |
| **100ugL** | 1 | 2hr | 30 | 17.66667 | 6.666667 | 1.833333 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **100ugL** | 1 | 2hr | 52.33333 | 43.83333 | 26.66667 | 15.33333 | 2.333333 |
| **100ugL** | 2 | 2hr | 17.16667 | 13.33333 | 8.833333 | 3.833333 | 0 |
| **100ugL** | 2 | 2hr | 0 | 0 | 0 | 0 | 0 |
| **100ugL** | 2 | 2hr | 0 | 0 | 0 | 0 | 0 |

**March 23rd and 26th – 6th hour means**

| Treatment | Date | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | 1 | 22 | 16.5 | 11.33333 | 6.666667 | 1.333333 | 0 |
| **Reference** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 1 | 10 | 7.5 | 3.833333 | 0.5 | 0 | 0 |
| **Reference** | 2 | 20.5 | 17.83333 | 11 | 5 | 0.833333 | 0 |
| **Reference** | 2 | 3.166667 | 3 | 2.333333 | 1.666667 | 0 | 0 |
| **Reference** | 2 | 39.83333 | 32.33333 | 21.66667 | 11.66667 | 2.833333 | 0 |
| **Reference** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 2 | 12.16667 | 10.16667 | 10.66667 | 7.833333 | 0.833333 | 1.333333 |
| **DMSO** | 1 | 36.66667 | 27.5 | 14.16667 | 7.333333 | 0.333333 | 0 |
| **DMSO** | 1 | 34.5 | 26 | 13.16667 | 4.333333 | 0 | 0 |
| **DMSO** | 1 | 10.33333 | 7.666667 | 4.333333 | 2.333333 | 0.5 | 0 |
| **DMSO** | 1 | 22.83333 | 18 | 10 | 4.666667 | 0.5 | 0 |
| **DMSO** | 1 | 7 | 5.666667 | 4.333333 | 1.166667 | 0.333333 | 0 |
| **DMSO** | 2 | 6.5 | 4.666667 | 3.333333 | 1.5 | 0 | 0 |
| **DMSO** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 2 | 69.33333 | 60 | 38.33333 | 20.5 | 5 | 0 |
| **DMSO** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1ugL** | 1 | 33.33333 | 29.33333 | 16.66667 | 6 | 0.5 | 0 |
| **1ugL** | 1 | 2.166667 | 1.833333 | 1.333333 | 0 | 0 | 0 |
| **1ugL** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1ugL** | 1 | 17.66667 | 14 | 6.666667 | 2.833333 | 0 | 0 |
| **1ugL** | 1 | 20.5 | 16.66667 | 10.83333 | 4.666667 | 0.5 | 0.333333 |
| **1ugL** | 2 | 3.833333 | 3.666667 | 2.5 | 1.5 | 0 | 0 |
| **1ugL** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1ugL** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1ugL** | 2 | 7 | 5.833333 | 4.5 | 2.833333 | 0.666667 | 0.5 |
| **1ugL** | 2 | 55.16667 | 50.33333 | 37 | 19.16667 | 5.666667 | 2 |
| **10ugL** | 1 | 9.666667 | 7.166667 | 4.166667 | 1.5 | 0.833333 | 0 |
| **10ugL** | 1 | 12.33333 | 8.5 | 4.5 | 2.5 | 0 | 0 |
| **10ugL** | 1 | 22 | 18.66667 | 15.66667 | 10.16667 | 2.5 | 1.166667 |
| **10ugL** | 1 | 9.333333 | 7.666667 | 5.666667 | 2.166667 | 0.333333 | 0 |
| **10ugL** | 2 | 12 | 9.5 | 4.333333 | 1.833333 | 0.5 | 0 |
| **10ugL** | 2 | 21.5 | 16.66667 | 13.5 | 6.833333 | 0 | 0 |
| **10ugL** | 2 | 14.33333 | 12.33333 | 7.333333 | 2.833333 | 0 | 0 |
| **10ugL** | 2 | 2.5 | 2 | 1.166667 | 0.333333 | 0 | 0 |
| **100ugL** | 1 | 10.33333 | 8.833333 | 6.166667 | 3 | 0.333333 | 0 |
| **100ugL** | 1 | 27.16667 | 14.66667 | 7.666667 | 2.166667 | 0 | 0 |
| **100ugL** | 1 | 4.166667 | 2.5 | 2 | 1.166667 | 0 | 0 |
| **100ugL** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **100ugL** | 2 | 3.333333 | 2.833333 | 1.666667 | 1 | 0 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **100ugL** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |

**March 23rd and 26th – 12th hour means**

| Treatment | Date | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 1 | 8.666667 | 7 | 3.5 | 1.333333 | 0.5 | 0 |
| **Reference** | 2 | 12.83333 | 10.83333 | 6.833333 | 3 | 0.5 | 0 |
| **Reference** | 2 | 30 | 24.83333 | 16 | 6.666667 | 1.333333 | 0 |
| **Reference** | 2 | 28.66667 | 22.66667 | 13 | 5 | 0 | 0 |
| **Reference** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 2 | 11 | 11.16667 | 10.66667 | 9.166667 | 0.833333 | 0.666667 |
| **DMSO** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 1 | 5.166667 | 3.333333 | 1.666667 | 0.5 | 0 | 0 |
| **DMSO** | 1 | 16.5 | 11.83333 | 5.833333 | 2.666667 | 0.5 | 0 |
| **DMSO** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 1 | 21.33333 | 15.16667 | 9 | 4.333333 | 1.5 | 0 |
| **DMSO** | 2 | 8.166667 | 6.833333 | 2.833333 | 1.166667 | 0.333333 | 0 |
| **DMSO** | 2 | 6 | 4.833333 | 2.833333 | 1.333333 | 0 | 0 |
| **DMSO** | 2 | 43 | 35.33333 | 20 | 7.666667 | 0.333333 | 0 |
| **DMSO** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1ugL** | 1 | 34.16667 | 28.83333 | 14.5 | 6.666667 | 0.5 | 0 |
| **1ugL** | 1 | 0.666667 | 0.666667 | 0.5 | 0 | 0 | 0 |
| **1ugL** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1ugL** | 1 | 19.33333 | 14.83333 | 7.5 | 2.333333 | 0 | 0 |
| **1ugL** | 1 | 32.83333 | 26.83333 | 14.66667 | 8.166667 | 1.333333 | 0 |
| **1ugL** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1ugL** | 2 | 10.5 | 7.833333 | 5 | 1.833333 | 0 | 0 |
| **1ugL** | 2 | 9.166667 | 7.666667 | 4.5 | 1.666667 | 0 | 0 |
| **1ugL** | 2 | 12.66667 | 10.33333 | 4.833333 | 2.5 | 0 | 0 |
| **1ugL** | 2 | 36.83333 | 35.66667 | 26.5 | 14.66667 | 1.833333 | 0 |
| **10ugL** | 1 | 27.16667 | 19 | 9.666667 | 4.5 | 0.833333 | 0 |
| **10ugL** | 1 | 22.83333 | 14.83333 | 6.333333 | 1.333333 | 0 | 0 |
| **10ugL** | 1 | 10.16667 | 8.833333 | 6.833333 | 4.166667 | 0 | 0 |
| **10ugL** | 1 | 8.666667 | 6.333333 | 3.5 | 1.5 | 0 | 0 |
| **10ugL** | 2 | 8.166667 | 5.833333 | 3.833333 | 2 | 0.333333 | 0 |
| **10ugL** | 2 | 24.66667 | 22.66667 | 18.66667 | 10.66667 | 1.666667 | 0.333333 |
| **10ugL** | 2 | 20.33333 | 17.33333 | 8 | 4.166667 | 0.666667 | 0 |
| **10ugL** | 2 | 8.666667 | 6.833333 | 2.666667 | 0.833333 | 0.333333 | 0 |
| **100ugL** | 1 | 2.5 | 2.166667 | 1.166667 | 0.5 | 0 | 0 |
| **100ugL** | 1 | 8.5 | 5.5 | 2.166667 | 0.666667 | 0 | 0 |
| **100ugL** | 1 | 6 | 5.166667 | 4.333333 | 2.333333 | 0 | 0 |
| **100ugL** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **100ugL** | 2 | 11.5 | 10.16667 | 6.833333 | 3.833333 | 1.833333 | 0 |
| **100ugL** | 2 | 0.5 | 0.5 | 0 | 0 | 0 | 0 |

***Daphnia magna* – Atrazine**

**April 6th and 7th – 1st hour means**

| Treatment | Date | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | 6 | 6 | 4.333333 | 3.5 | 0.333333 | 0.333333 | 0 |
| **Reference** | 6.5 | 6.333333 | 4.833333 | 2.333333 | 0.666667 | 0 | 0 |
| **Reference** | 12.16667 | 11.16667 | 8.833333 | 4.833333 | 0.333333 | 0.333333 | 0 |
| **Reference** | 6.5 | 5.5 | 5.666667 | 3.333333 | 0.5 | 0 | 0 |
| **Reference** | 32.83333 | 37.83333 | 35.33333 | 25.33333 | 12.66667 | 5 | 2.833333 |
| **Reference** | 12.66667 | 11.83333 | 7.833333 | 3 | 0 | 0 | 0 |
| **Reference** | 16.66667 | 17.83333 | 15.5 | 11.5 | 3.833333 | 1.5 | 0.333333 |
| **Reference** | 2.833333 | 3.333333 | 3.333333 | 2.166667 | 0 | 0 | 0 |
| **Reference** | 32.16667 | 32.16667 | 26.66667 | 16.66667 | 4 | 1 | 0 |
| **Reference** | 9 | 8 | 5.833333 | 1.833333 | 0 | 0 | 0 |
| **DMSO** | 1.333333 | 1.333333 | 1.166667 | 0.5 | 0 | 0 | 0 |
| **DMSO** | 5.666667 | 5.333333 | 3.166667 | 1.666667 | 0.333333 | 0 | 0 |
| **DMSO** | 5 | 6 | 4.666667 | 3.166667 | 1 | 0.5 | 0 |
| **DMSO** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 12.83333 | 12.5 | 7.666667 | 4.166667 | 0.5 | 0.333333 | 0 |
| **DMSO** | 1.833333 | 2 | 1.5 | 0.833333 | 0 | 0 | 0 |
| **DMSO** | 8.666667 | 7.5 | 6 | 3.333333 | 1.166667 | 0 | 0 |
| **DMSO** | 2.166667 | 1.833333 | 1.5 | 0 | 0 | 0 | 0 |
| **DMSO** | 11.66667 | 11.66667 | 6.5 | 3 | 0 | 0 | 0 |
| **DMSO** | 3.333333 | 3 | 2.5 | 1.833333 | 1 | 0 | 0 |
| **5ug/L** | 18.16667 | 17.66667 | 14.5 | 8.666667 | 1.333333 | 0 | 0 |
| **5ug/L** | 6.166667 | 5.5 | 5.166667 | 3 | 0 | 0 | 0 |
| **5ug/L** | 14 | 14.5 | 11.33333 | 8.166667 | 1.5 | 0.666667 | 0 |
| **5ug/L** | 17 | 17.16667 | 14 | 8.833333 | 1.333333 | 0 | 0 |
| **5ug/L** | 14.66667 | 17 | 13.83333 | 11.66667 | 6 | 1.5 | 1.333333 |
| **5ug/L** | 10.16667 | 8.666667 | 6.833333 | 3 | 1 | 0 | 0 |
| **5ug/L** | 19.83333 | 20 | 17.16667 | 10 | 3.5 | 0.5 | 0 |
| **5ug/L** | 10 | 8.5 | 5 | 2.666667 | 0.333333 | 0 | 0 |
| **50ug/L** | 1 | 0.833333 | 0.833333 | 0 | 0 | 0 | 0 |
| **50ug/L** | 5.833333 | 4.833333 | 3.833333 | 2 | 0 | 0 | 0 |
| **50ug/L** | 3.333333 | 3 | 1.5 | 1 | 0 | 0 | 0 |
| **50ug/L** | 16.83333 | 16.66667 | 14 | 7.666667 | 1.166667 | 1 | 0.333333 |
| **50ug/L** | 7.833333 | 7.333333 | 6 | 3 | 0.666667 | 0 | 0 |
| **50ug/L** | 20.83333 | 18.66667 | 14.83333 | 6.333333 | 0.5 | 0 | 0 |
| **50ug/L** | 4.666667 | 5.166667 | 3.333333 | 1.666667 | 0.333333 | 0 | 0 |
| **50ug/L** | 5.166667 | 4.833333 | 4.333333 | 2.5 | 0.333333 | 0 | 0 |
| **500ug/L** | 9.5 | 7.833333 | 6.166667 | 1.833333 | 0 | 0 | 0 |
| **500ug/L** | 1 | 1 | 0.5 | 0.5 | 0 | 0 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **500ug/L** | 1.666667 | 1.666667 | 0.833333 | 0 | 0 | 0 | 0 |
| **500ug/L** | 2.5 | 2.5 | 2.166667 | 0.333333 | 0 | 0 | 0 |
| **500ug/L** | 12.33333 | 11.5 | 8.833333 | 4.833333 | 0.5 | 0 | 0 |
| **500ug/L** | 14 | 12 | 9 | 4.666667 | 0.5 | 0.333333 | 0 |
| **500ug/L** | 29.66667 | 29 | 22.66667 | 12.33333 | 5.333333 | 0.666667 | 0.333333 |
| **500ug/L** | 7.166667 | 7 | 6 | 2.833333 | 0.333333 | 0 | 0 |

## April 6th and 7th – 2nd hour means

| Treatment | Date | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | 1 | 3.666667 | 3.666667 | 2.333333 | 0.833333 | 0 | 0 |
| **Reference** | 1 | 4.333333 | 5 | 5 | 3.5 | 0 | 0 |
| **Reference** | 1 | 8.666667 | 8.5 | 7.166667 | 3.833333 | 0 | 0 |
| **Reference** | 1 | 5.166667 | 4.666667 | 4.333333 | 2.333333 | 0.5 | 0 |
| **Reference** | 1 | 23.16667 | 24.83333 | 21.33333 | 14.33333 | 4.5 | 1.666667 |
| **Reference** | 2 | 17.33333 | 15.33333 | 10.66667 | 4.833333 | 0.666667 | 0 |
| **Reference** | 2 | 6.833333 | 7.5 | 7.5 | 5.333333 | 1.333333 | 0.5 |
| **Reference** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 2 | 17.5 | 18.5 | 15.33333 | 11.16667 | 2.333333 | 0.833333 |
| **Reference** | 2 | 2.666667 | 3 | 2 | 1.5 | 0 | 0 |
| **DMSO** | 1 | 0.666667 | 0.5 | 0.5 | 0.5 | 0 | 0 |
| **DMSO** | 1 | 4.5 | 4 | 3.333333 | 1.333333 | 0 | 0 |
| **DMSO** | 1 | 0.833333 | 0.833333 | 0.666667 | 0.5 | 0 | 0 |
| **DMSO** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 1 | 19.16667 | 16.5 | 10.66667 | 5.333333 | 0 | 0 |
| **DMSO** | 2 | 11.5 | 10.66667 | 8.166667 | 4.166667 | 0 | 0 |
| **DMSO** | 2 | 13.5 | 11.5 | 9.666667 | 4.5 | 0.666667 | 0 |
| **DMSO** | 2 | 5.666667 | 4.833333 | 3.166667 | 1 | 0 | 0 |
| **DMSO** | 2 | 13.33333 | 11.33333 | 7.5 | 2.666667 | 0.333333 | 0 |
| **DMSO** | 2 | 7.166667 | 7.333333 | 7 | 5 | 2.666667 | 0.5 |
| **5ug/L** | 1 | 3.333333 | 3.166667 | 2.833333 | 1.5 | 0 | 0 |
| **5ug/L** | 1 | 4.666667 | 3.833333 | 3 | 2.166667 | 0.333333 | 0.333333 |
| **5ug/L** | 1 | 7.333333 | 5.666667 | 4.5 | 2.666667 | 0.333333 | 0 |
| **5ug/L** | 1 | 14.83333 | 15 | 11.33333 | 6 | 1.333333 | 0.5 |
| **5ug/L** | 2 | 15 | 18.33333 | 16.83333 | 12.16667 | 5 | 2 |
| **5ug/L** | 2 | 22.83333 | 20.66667 | 15 | 7.333333 | 1.666667 | 0.333333 |
| **5ug/L** | 2 | 14 | 12.5 | 9.833333 | 6.166667 | 0.5 | 0.5 |
| **5ug/L** | 2 | 6.333333 | 6 | 4 | 2.166667 | 0 | 0 |
| **50ug/L** | 1 | 1.833333 | 1.666667 | 1 | 0.333333 | 0 | 0 |
| **50ug/L** | 1 | 3 | 3 | 2 | 0.833333 | 0 | 0 |
| **50ug/L** | 1 | 2.666667 | 2 | 1.5 | 1 | 0 | 0 |
| **50ug/L** | 1 | 8.333333 | 8 | 7.5 | 4 | 0.666667 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **50ug/L** | 2 | 12.33333 | 10.66667 | 7 | 3.833333 | 0.5 | 0 |
| **50ug/L** | 2 | 17 | 16 | 12 | 5.166667 | 0.5 | 0 |
| **50ug/L** | 2 | 4.166667 | 4.666667 | 4.5 | 2.833333 | 1 | 0 |
| **50ug/L** | 2 | 7 | 6.5 | 5 | 3.5 | 0.833333 | 0 |
| **500ug/L** | 1 | 16.33333 | 16.66667 | 13.5 | 7.166667 | 1.333333 | 0.333333 |
| **500ug/L** | 1 | 4.833333 | 4.333333 | 3.833333 | 2.166667 | 0.5 | 0 |
| **500ug/L** | 1 | 2.5 | 2.166667 | 1.5 | 0.833333 | 0 | 0 |
| **500ug/L** | 1 | 0.5 | 0.5 | 0.5 | 0.5 | 0 | 0 |
| **500ug/L** | 2 | 9.833333 | 8.666667 | 6.5 | 3 | 0.333333 | 0 |
| **500ug/L** | 2 | 5.833333 | 5.166667 | 3.166667 | 1 | 0 | 0 |
| **500ug/L** | 2 | 17.16667 | 18 | 17.16667 | 11.5 | 5 | 2.666667 |
| **500ug/L** | 2 | 20.66667 | 18.66667 | 12.83333 | 6.833333 | 1.333333 | 0.5 |

## April 6th and 7th – 6th hour means

| Treatment | Date | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | 4.666667 | 4.333333 | 3.833333 | 1.333333 | 0.333333 | 0 | 0 |
| **Reference** | 4.5 | 4.333333 | 3.666667 | 1.5 | 0 | 0 | 0 |
| **Reference** | 5.833333 | 6.5 | 5.333333 | 3.333333 | 0.833333 | 0 | 0 |
| **Reference** | 7.166667 | 7.5 | 7.666667 | 5.166667 | 1.5 | 0.333333 | 0 |
| **Reference** | 9 | 8.5 | 6.333333 | 2.666667 | 0.5 | 0 | 0 |
| **Reference** | 7.333333 | 6.833333 | 5 | 3.166667 | 0.833333 | 0.5 | 0 |
| **Reference** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 11 | 10.5 | 8.333333 | 4.5 | 0 | 0 | 0 |
| **Reference** | 13.83333 | 12.5 | 8.5 | 3 | 0 | 0.5 | 0 |
| **DMSO** | 2.333333 | 2.333333 | 1.666667 | 0.5 | 0 | 0 | 0 |
| **DMSO** | 6.5 | 6.5 | 4.166667 | 2.333333 | 0 | 0 | 0 |
| **DMSO** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 21.83333 | 17 | 12 | 6.333333 | 0.333333 | 0 | 0 |
| **DMSO** | 6 | 4.833333 | 4.333333 | 2.166667 | 0 | 0 | 0 |
| **DMSO** | 12.66667 | 10.16667 | 7.833333 | 5.333333 | 0.833333 | 0.833333 | 0 |
| **DMSO** | 2.666667 | 2.333333 | 0.5 | 0.833333 | 0 | 0 | 0 |
| **DMSO** | 9 | 8.833333 | 4.833333 | 2.166667 | 0 | 0 | 0 |
| **DMSO** | 1.5 | 1.5 | 2 | 1 | 0.333333 | 0 | 0 |
| **5ug/L** | 17.16667 | 19 | 12.16667 | 5.166667 | 1 | 0 | 0 |
| **5ug/L** | 4.333333 | 3.666667 | 3.666667 | 3 | 0.666667 | 0 | 0 |
| **5ug/L** | 4.666667 | 5.166667 | 5.166667 | 4.333333 | 1.166667 | 0.333333 | 0 |
| **5ug/L** | 5.166667 | 4.333333 | 3.166667 | 2.666667 | 0 | 0 | 0 |
| **5ug/L** | 16.33333 | 15 | 10.33333 | 5.166667 | 0 | 0 | 0 |
| **5ug/L** | 2.833333 | 2.833333 | 1.833333 | 0.5 | 0 | 0 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **5ug/L** | 11.33333 | 10.33333 | 7.833333 | 5 | 1.333333 | 0 | 0 |
| **5ug/L** | 13.83333 | 13.5 | 8.333333 | 3 | 0.333333 | 0 | 0 |
| **50ug/L** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **50ug/L** | 4 | 4 | 3 | 1 | 0 | 0 | 0 |
| **50ug/L** | 2.5 | 2.166667 | 1.5 | 1 | 0 | 0 | 0 |
| **50ug/L** | 6.833333 | 8.833333 | 6.666667 | 4.333333 | 2 | 0.5 | 0.5 |
| **50ug/L** | 11.33333 | 10.33333 | 8 | 4.5 | 0.666667 | 0 | 0 |
| **50ug/L** | 10.83333 | 10.83333 | 8.666667 | 6 | 1.5 | 0 | 0 |
| **50ug/L** | 7.166667 | 6.5 | 4.333333 | 2 | 0.333333 | 0 | 0 |
| **50ug/L** | 5.833333 | 5.5 | 4.5 | 2.333333 | 0 | 0 | 0 |
| **500ug/L** | 8.166667 | 8.333333 | 7.166667 | 4 | 0.5 | 0 | 0 |
| **500ug/L** | 1 | 1 | 0.666667 | 0.333333 | 0 | 0 | 0 |
| **500ug/L** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **500ug/L** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **500ug/L** | 9.5 | 9.333333 | 4.833333 | 2.5 | 0 | 0 | 0 |
| **500ug/L** | 11.83333 | 11.16667 | 8.666667 | 5.166667 | 1.5 | 0 | 0 |
| **500ug/L** | 49.5 | 49.33333 | 35.5 | 17.83333 | 5 | 0.833333 | 0 |
| **500ug/L** | 20 | 18.33333 | 13.5 | 7 | 1.166667 | 0 | 0 |

**April 6th and 7th – 12th hour means**

| Treatment | Date | 0.5 | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | 4.833333 | 4.666667 | 3 | 1.333333 | 0 | 0 | 0 |
| **Reference** | 10.16667 | 9.166667 | 8.666667 | 4.5 | 0.5 | 0 | 0 |
| **Reference** | 3.333333 | 3.666667 | 3 | 1.833333 | 0 | 0 | 0 |
| **Reference** | 5 | 3.833333 | 3.833333 | 1.833333 | 0 | 0 | 0 |
| **Reference** | 9.166667 | 9 | 6.666667 | 3.833333 | 0.5 | 0 | 0 |
| **Reference** | 10.83333 | 10.33333 | 8.333333 | 4.5 | 0.833333 | 0 | 0 |
| **Reference** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 18.83333 | 19.83333 | 14.5 | 8.5 | 2.5 | 0 | 0 |
| **Reference** | 7 | 6 | 5 | 2.166667 | 0.5 | 0.5 | 0 |
| **DMSO** | 1 | 1.166667 | 1.166667 | 0.5 | 0 | 0 | 0 |
| **DMSO** | 0.333333 | 0.333333 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 1.666667 | 1.333333 | 1.333333 | 1.5 | 0.666667 | 0 | 0 |
| **DMSO** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 11.16667 | 9.833333 | 9 | 6.166667 | 0.5 | 0.5 | 0 |
| **DMSO** | 0.333333 | 0.333333 | 0 | 0.333333 | 0 | 0 | 0 |
| **DMSO** | 15.16667 | 13.83333 | 11.5 | 4.333333 | 0.666667 | 0.333333 | 0 |
| **DMSO** | 12.33333 | 11 | 7.5 | 3.333333 | 0 | 0 | 0 |
| **DMSO** | 12.83333 | 11.16667 | 6.5 | 3.166667 | 0.5 | 0 | 0 |
| **DMSO** | 8 | 6.833333 | 5.166667 | 3.833333 | 1.166667 | 0 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **5ug/L** | 1.166667 | 1 | 1 | 0 | 0 | 0 | 0 |
| **5ug/L** | 1.166667 | 0.666667 | 0.5 | 0.333333 | 0 | 0 | 0 |
| **5ug/L** | 9.5 | 8.666667 | 7.833333 | 5.166667 | 1.333333 | 0.666667 | 0 |
| **5ug/L** | 9 | 6.666667 | 7.166667 | 3.333333 | 0.333333 | 0 | 0 |
| **5ug/L** | 14.66667 | 14 | 10.16667 | 5.833333 | 0.666667 | 0 | 0 |
| **5ug/L** | 19.5 | 19.33333 | 15.16667 | 10.16667 | 3 | 0.333333 | 0.5 |
| **5ug/L** | 14.16667 | 13 | 9.333333 | 5.833333 | 0.833333 | 0 | 0 |
| **5ug/L** | 4.333333 | 3.833333 | 2.833333 | 0.5 | 0 | 0 | 0 |
| **50ug/L** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **50ug/L** | 1.333333 | 1.333333 | 0.833333 | 0.5 | 0 | 0 | 0 |
| **50ug/L** | 0.5 | 0.333333 | 0.333333 | 0.333333 | 0 | 0 | 0 |
| **50ug/L** | 9.833333 | 9.166667 | 6.833333 | 4.166667 | 0.5 | 0 | 0 |
| **50ug/L** | 19.33333 | 17.66667 | 12.83333 | 7.666667 | 0.333333 | 0 | 0 |
| **50ug/L** | 5.833333 | 6.166667 | 3.333333 | 1.333333 | 0 | 0 | 0 |
| **50ug/L** | 16.5 | 16.5 | 13.16667 | 8.5 | 1.333333 | 0 | 0 |
| **50ug/L** | 12.16667 | 12.5 | 10.5 | 6.333333 | 0.666667 | 0 | 0 |
| **500ug/L** | 5.5 | 5.833333 | 4 | 2.5 | 0.5 | 0 | 0 |
| **500ug/L** | 3.833333 | 3.333333 | 3.666667 | 1.5 | 0 | 0 | 0 |
| **500ug/L** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **500ug/L** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **500ug/L** | 16.5 | 14.33333 | 10 | 4.5 | 1 | 0.5 | 0 |
| **500ug/L** | 16 | 15.33333 | 11.16667 | 6 | 0.333333 | 0.333333 | 0 |
| **500ug/L** | 18.16667 | 15.16667 | 11.66667 | 5.5 | 1.333333 | 0 | 0 |
| **500ug/L** | 9 | 8.166667 | 6.333333 | 3.333333 | 0.5 | 0 | 0 |

***Hyalella azteca* – Atrazine**

**April 12th and June 20th – 1st hour means**

| Treatment | Date | 0.5Hz | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | 1 | 13.66667 | 11.16667 | 7 | 4.333333 | 0.833333 | 0 |
| **Reference** | 1 | 9.833333 | 10.83333 | 8.5 | 4.5 | 0 | 0 |
| **Reference** | 1 | 1.333333 | 1.333333 | 1.333333 | 0.666667 | 0 | 0 |
| **Reference** | 1 | 36.66667 | 27.66667 | 15.66667 | 8.166667 | 1 | 0.333333 |
| **Reference** | 1 | 42 | 32 | 18.83333 | 8.333333 | 1.833333 | 0 |
| **Reference** | 2 | 11.16667 | 8.333333 | 4.833333 | 2.5 | 0.333333 | 0 |
| **Reference** | 2 | 63.5 | 47.83333 | 23.83333 | 12 | 3 | 0.833333 |
| **Reference** | 2 | 14.5 | 11.16667 | 8.833333 | 3.833333 | 0.666667 | 0 |
| **DMSO** | 1 | 33.33333 | 24 | 14.83333 | 6.333333 | 1.833333 | 0.333333 |
| **DMSO** | 1 | 17.33333 | 13 | 7.666667 | 2.666667 | 0 | 0 |
| **DMSO** | 1 | 16.16667 | 10.83333 | 6.166667 | 2.5 | 0 | 0 |
| **DMSO** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **DMSO** | 1 | 42.5 | 33.66667 | 18.66667 | 8.666667 | 3.333333 | 0 |
| **DMSO** | 2 | 41.83333 | 32.33333 | 22.5 | 11.5 | 5.166667 | 2.666667 |
| **DMSO** | 2 | 10.66667 | 8 | 3.833333 | 1.166667 | 0 | 0 |
| **DMSO** | 2 | 5.833333 | 5.833333 | 3.833333 | 1.833333 | 0 | 0 |
| **DMSO** | 2 | 40.83333 | 57.16667 | 49.5 | 44.83333 | 32.33333 | 20.33333 |
| **5ug/L** | 1 | 34.16667 | 29.83333 | 19.66667 | 10 | 2 | 0 |
| **5ug/L** | 1 | 24 | 19.83333 | 11.33333 | 6.333333 | 1.333333 | 0.333333 |
| **5ug/L** | 1 | 14 | 11.16667 | 7.5 | 3 | 0.5 | 0 |
| **5ug/L** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **5ug/L** | 2 | 46.5 | 38.33333 | 23.66667 | 10.33333 | 2.333333 | 0.833333 |
| **5ug/L** | 2 | 16.66667 | 13.33333 | 8.833333 | 4.5 | 0.833333 | 0 |
| **5ug/L** | 2 | 24.83333 | 18.66667 | 9.333333 | 3.666667 | 0 | 0 |
| **50ug/L** | 1 | 41.5 | 38.16667 | 25.33333 | 12 | 2.666667 | 0 |
| **50ug/L** | 1 | 10.16667 | 8.5 | 5.5 | 2.5 | 0 | 0 |
| **50ug/L** | 1 | 10 | 9 | 4.833333 | 1.666667 | 0 | 0 |
| **50ug/L** | 1 | 14.33333 | 12.16667 | 7.833333 | 3 | 0.5 | 0 |
| **50ug/L** | 2 | 48.33333 | 37.33333 | 21.16667 | 8.666667 | 2.5 | 1.333333 |
| **50ug/L** | 2 | 43.33333 | 35.33333 | 18.66667 | 9.166667 | 0.833333 | 0.5 |
| **50ug/L** | 2 | 1.166667 | 1.166667 | 0.666667 | 0.833333 | 0 | 0 |
| **50ug/L** | 2 | 65.33333 | 50.66667 | 29.66667 | 11.83333 | 3.666667 | 0 |
| **500ug/L** | 1 | 59.66667 | 48.16667 | 27.66667 | 12.66667 | 2.833333 | 0 |
| **500ug/L** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **500ug/L** | 1 | 19.83333 | 16.33333 | 8.166667 | 2 | 0 | 0 |
| **500ug/L** | 1 | 64.16667 | 54 | 34.83333 | 13.83333 | 3.333333 | 0.333333 |
| **500ug/L** | 2 | 39.5 | 30.33333 | 18.5 | 10.66667 | 3.5 | 0.833333 |
| **500ug/L** | 2 | 71.16667 | 56.83333 | 31.33333 | 14.16667 | 5.666667 | 1.333333 |
| **500ug/L** | 2 | 49.5 | 39 | 22.33333 | 11.33333 | 1.666667 | 0 |
| **500ug/L** | 2 | 71.33333 | 57.33333 | 34.66667 | 17.66667 | 6.166667 | 0.5 |

## April 12th and June 20th – 2nd hour means

| Treatment | Date | 0.5Hz | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | 1 | 13.5 | 11.16667 | 7 | 4.5 | 1.5 | 0 |
| **Reference** | 1 | 9.833333 | 8.5 | 6.333333 | 3.166667 | 0.333333 | 0 |
| **Reference** | 1 | 0 | 0.5 | 0.5 | 0.5 | 0.333333 | 0 |
| **Reference** | 1 | 28.66667 | 21.33333 | 11.5 | 4 | 0 | 0 |
| **Reference** | 1 | 39.16667 | 28.66667 | 15.66667 | 8 | 2 | 0.333333 |
| **Reference** | 2 | 5.333333 | 5.166667 | 3.166667 | 0.833333 | 0 | 0 |
| **Reference** | 2 | 62.66667 | 44.33333 | 23.16667 | 8.833333 | 2.833333 | 0 |
| **Reference** | 2 | 2.333333 | 1.833333 | 1.166667 | 0.833333 | 0.333333 | 0 |
| **DMSO** | 1 | 33.5 | 25.83333 | 15.33333 | 7.5 | 2.666667 | 1 |
| **DMSO** | 1 | 13.16667 | 10.83333 | 7.666667 | 3.5 | 0 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **DMSO** | 1 | 14.5 | 10 | 4.333333 | 0.833333 | 0 | 0 |
| **DMSO** | 1 | 8 | 6 | 2.833333 | 0.833333 | 0 | 0 |
| **DMSO** | 1 | 51.33333 | 39.66667 | 23 | 10 | 3.333333 | 1.666667 |
| **DMSO** | 2 | 25.16667 | 20.33333 | 12.83333 | 7.5 | 3.666667 | 0 |
| **DMSO** | 2 | 1 | 0.5 | 0 | 0 | 0 | 0 |
| **DMSO** | 2 | 4.833333 | 4.166667 | 3.333333 | 1.5 | 0 | 0 |
| **DMSO** | 2 | 42.33333 | 52.5 | 49.66667 | 40 | 27.83333 | 22.16667 |
| **5ug/L** | 1 | 29.5 | 22.33333 | 13.66667 | 5.666667 | 0.333333 | 0 |
| **5ug/L** | 1 | 2.5 | 2 | 1 | 0.333333 | 0 | 0 |
| **5ug/L** | 1 | 33.16667 | 26.33333 | 15.5 | 8.666667 | 2.5 | 1.166667 |
| **5ug/L** | 1 | 7.5 | 5.5 | 4.333333 | 1.833333 | 0.833333 | 0 |
| **5ug/L** | 2 | 15.33333 | 13.83333 | 9.5 | 5 | 1.333333 | 0 |
| **5ug/L** | 2 | 9.166667 | 7.166667 | 4.5 | 2.166667 | 0.5 | 0 |
| **5ug/L** | 2 | 6.5 | 4.666667 | 2.833333 | 0.833333 | 0 | 0 |
| **50ug/L** | 1 | 30.33333 | 25.5 | 14.33333 | 7.5 | 0 | 0 |
| **50ug/L** | 1 | 14 | 13.83333 | 8.833333 | 4 | 0.666667 | 0 |
| **50ug/L** | 1 | 20.83333 | 18.83333 | 12.33333 | 4.5 | 0.833333 | 0 |
| **50ug/L** | 1 | 16.16667 | 13.83333 | 9.833333 | 3.833333 | 0.5 | 0 |
| **50ug/L** | 2 | 31.33333 | 23.16667 | 12.5 | 3.5 | 0.333333 | 0 |
| **50ug/L** | 2 | 32.16667 | 26 | 15.16667 | 7.333333 | 1.5 | 0.333333 |
| **50ug/L** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **50ug/L** | 2 | 39.16667 | 29.66667 | 16.83333 | 7.5 | 1.5 | 0 |
| **500ug/L** | 1 | 54.16667 | 43.16667 | 23 | 12 | 1.333333 | 0 |
| **500ug/L** | 1 | 7.5 | 5.5 | 4.333333 | 1.833333 | 0.833333 | 0 |
| **500ug/L** | 1 | 19 | 15.66667 | 7.666667 | 3.5 | 0.333333 | 0 |
| **500ug/L** | 1 | 57.83333 | 45.16667 | 27.16667 | 12.5 | 3.833333 | 0 |
| **500ug/L** | 2 | 34.83333 | 30.33333 | 16.16667 | 9.5 | 3.166667 | 0.333333 |
| **500ug/L** | 2 | 52.16667 | 43.5 | 25.83333 | 12.33333 | 3.5 | 0.333333 |
| **500ug/L** | 2 | 27.5 | 22.16667 | 12 | 6 | 0 | 0 |
| **500ug/L** | 2 | 59.66667 | 46.83333 | 29.33333 | 16 | 3.5 | 0 |

## April 12th and June 20th – 6th hour means

| Treatment | Date | 0.5Hz | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | 1 | 15.83333 | 13.16667 | 8.333333 | 3.833333 | 1.833333 | 0 |
| **Reference** | 1 | 8.666667 | 7.666667 | 6 | 2.166667 | 0 | 0 |
| **Reference** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 1 | 3.5 | 3.333333 | 1.833333 | 1.666667 | 0 | 0 |
| **Reference** | 1 | 24.16667 | 18.66667 | 10.16667 | 3.5 | 0.333333 | 0.5 |
| **Reference** | 2 | 27.83333 | 21.33333 | 9.5 | 3.5 | 1.333333 | 0 |
| **Reference** | 2 | 24 | 19.66667 | 10.5 | 3.833333 | 0.5 | 0 |
| **Reference** | 2 | 4.833333 | 4.666667 | 4.666667 | 1.833333 | 0 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **DMSO** | 1 | 13.66667 | 11 | 7.5 | 3.5 | 0.5 | 0.333333 |
| **DMSO** | 1 | 7.666667 | 5.666667 | 3.333333 | 0.833333 | 0 | 0 |
| **DMSO** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 1 | 6.666667 | 5.666667 | 3.333333 | 0.5 | 0.333333 | 0 |
| **DMSO** | 1 | 2.333333 | 1.833333 | 1.833333 | 2.333333 | 0 | 0 |
| **DMSO** | 2 | 7.5 | 5.833333 | 3.333333 | 2.833333 | 0 | 0 |
| **DMSO** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 2 | 15.33333 | 13 | 7.833333 | 3.833333 | 0.333333 | 0 |
| **DMSO** | 2 | 20.16667 | 42.83333 | 45.5 | 41 | 32.33333 | 22.5 |
| **5ug/L** | 1 | 10.5 | 8.166667 | 5.5 | 2.5 | 0 | 0 |
| **5ug/L** | 1 | 1.666667 | 1.166667 | 1.333333 | 0 | 0 | 0 |
| **5ug/L** | 1 | 11 | 9 | 6.166667 | 3 | 0 | 0 |
| **5ug/L** | 1 | 0.333333 | 0.333333 | 0 | 0.333333 | 0 | 0 |
| **5ug/L** | 2 | 14.16667 | 11.83333 | 7.5 | 4.166667 | 0.333333 | 0 |
| **5ug/L** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **5ug/L** | 2 | 8.166667 | 5.5 | 3 | 1 | 0 | 0 |
| **50ug/L** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **50ug/L** | 1 | 14 | 11 | 5.666667 | 2.166667 | 0.833333 | 0.333333 |
| **50ug/L** | 1 | 8.166667 | 6.833333 | 4.166667 | 2.5 | 0.333333 | 0 |
| **50ug/L** | 1 | 14 | 12 | 7.666667 | 3.833333 | 0.333333 | 0 |
| **50ug/L** | 2 | 42.33333 | 30.66667 | 16.66667 | 5.166667 | 0.333333 | 0 |
| **50ug/L** | 2 | 16.83333 | 13.16667 | 7.833333 | 3.333333 | 0 | 0.333333 |
| **50ug/L** | 2 | 2.666667 | 2 | 1.666667 | 0.5 | 0 | 0 |
| **50ug/L** | 2 | 10 | 9.5 | 5.666667 | 2.333333 | 0.5 | 0 |
| **500ug/L** | 1 | 37.5 | 31.16667 | 17.33333 | 8 | 0.333333 | 0 |
| **500ug/L** | 1 | 0.333333 | 0.333333 | 0 | 0.333333 | 0 | 0 |
| **500ug/L** | 1 | 8.5 | 5.833333 | 2.333333 | 1.166667 | 0 | 0 |
| **500ug/L** | 1 | 73 | 57.16667 | 33.83333 | 16.66667 | 3.5 | 0.333333 |
| **500ug/L** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **500ug/L** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **500ug/L** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **500ug/L** | 2 | 25.83333 | 21.66667 | 13.5 | 5.5 | 1.666667 | 0 |

**April 12th and June 20th – 12th hour means**

| Treatment | Date | 0.5Hz | 1 | 1.5 | 2 | 2.5 | 3 |
|---|---|---|---|---|---|---|---|
| **Reference** | 1 | 2 | 1 | 0.833333 | 0 | 0 | 0 |
| **Reference** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Reference** | 1 | 21.5 | 17.66667 | 10.83333 | 7 | 1.833333 | 0.666667 |
| **Reference** | 1 | 5.833333 | 4.5 | 2.333333 | 0.833333 | 0 | 0 |
| **Reference** | 1 | 8.5 | 6.166667 | 3 | 1.166667 | 0 | 0 |
| **Reference** | 2 | 18.83333 | 14 | 6.333333 | 1.833333 | 0.5 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Reference** | 2 | 23.33333 | 16.66667 | 9.666667 | 4.666667 | 1 | 0 |
| **Reference** | 2 | 3.333333 | 3 | 1.833333 | 0.5 | 0 | 0 |
| **DMSO** | 1 | 12.83333 | 8.833333 | 6.166667 | 3.666667 | 0.666667 | 0 |
| **DMSO** | 1 | 17 | 12 | 8.5 | 1.666667 | 0 | 0 |
| **DMSO** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 1 | 15.33333 | 10 | 5.166667 | 1.833333 | 0 | 0 |
| **DMSO** | 1 | 6.166667 | 4.5 | 4 | 3.166667 | 0 | 0 |
| **DMSO** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DMSO** | 2 | 7.166667 | 4.666667 | 2.833333 | 0.333333 | 0 | 0 |
| **DMSO** | 2 | 23.83333 | 18.16667 | 12.33333 | 6.333333 | 0.666667 | 0 |
| **DMSO** | 2 | 20.83333 | 41.83333 | 45.66667 | 40.16667 | 29.5 | 24 |
| **5ug/L** | 1 | 25.83333 | 19.83333 | 13.33333 | 5.833333 | 1.5 | 0 |
| **5ug/L** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **5ug/L** | 1 | 1.666667 | 2.333333 | 2 | 2 | 0.5 | 0.666667 |
| **5ug/L** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **5ug/L** | 2 | 5.333333 | 4.166667 | 2 | 0 | 0 | 0 |
| **5ug/L** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **5ug/L** | 2 | 11.33333 | 6.666667 | 1.666667 | 0.333333 | 0 | 0 |
| **50ug/L** | 1 | 29.66667 | 27.16667 | 15.33333 | 6.333333 | 0.5 | 0 |
| **50ug/L** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **50ug/L** | 1 | 14.33333 | 13.33333 | 9.166667 | 2.666667 | 0.833333 | 0.333333 |
| **50ug/L** | 1 | 20.66667 | 18.66667 | 11.33333 | 7 | 2 | 0 |
| **50ug/L** | 2 | 46.83333 | 36.16667 | 15.5 | 5.166667 | 0 | 0 |
| **50ug/L** | 2 | 8.833333 | 6.666667 | 3.833333 | 1.666667 | 0.5 | 0 |
| **50ug/L** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **50ug/L** | 2 | 23.16667 | 16.33333 | 10.16667 | 3.333333 | 0.5 | 0 |
| **500ug/L** | 1 | 31.16667 | 23.83333 | 15.16667 | 7.5 | 2.333333 | 0 |
| **500ug/L** | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| **500ug/L** | 1 | 9 | 6.5 | 3.833333 | 1.166667 | 0 | 0 |
| **500ug/L** | 1 | 51.33333 | 41 | 21.16667 | 10.16667 | 3 | 0.333333 |
| **500ug/L** | 2 | 14.83333 | 11.66667 | 7.666667 | 3.333333 | 0.5 | 0 |
| **500ug/L** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **500ug/L** | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **500ug/L** | 2 | 36.66667 | 26.66667 | 12.5 | 5.666667 | 0.833333 | 0 |