THE UTILIZATION OF THE FRESHWATER INVERTEBRATES HYALELLA AZTECA AND DAPHNIA MAGNA FOR USE IN ASSESSING POTENTIAL ENDOCRINE DISRUPTION IN AQUATIC SYSTEMS

by

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ABSTRACT

The utilization of freshwater invertebrates Hyalella azteca and Daphnia magna to assess potential endocrine disruption in aquatic systems

Concern is growing over the number of anthropogenic compounds that find their way in to freshwater systems and end up in drinking water. Chemicals with acute toxic effects are screened for immediately, but some chemicals may have a more chronic, sinister, endocrine effect which can result not only in acute birth defects, but over a prolonged period may also result in altered gender ratios among children and change in population dynamics. The chronic physiological effects of the compounds Atrazine, Tributyltin and 17α-ethinylestradiol were tested on the freshwater aquatic organisms; *Hyalella azteca* and *Daphnia magna*.

For *Daphnia magna*, two experiments were conducted. The first involved a system which was developed to minimize the parthenogenetic cycle and maximize the sexual cycle, resulting in the formation of males and ephippia. The second was a reproduction assay looking at multiple parameters, including the number of offspring under exposure of the three contaminants.

For *Hyalella azteca*, two experiments were conducted as well. The first involved a static test where *Hyalella* were exposed to the three contaminants and left for a period of 42 days without renewal. The second was a renewal assay, where the *Hyalella* were offered renewed solutions of the contaminants on a weekly basis.

It was found that Atrazine and Tributyltin had no significant effects on the gender ratios or secondary sexual physiology of *Hyalella azteca*.

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LIST OF ABBREVIATIONS

DMSO = Dimethylsulfoxide

DRL = Dose-Response Lag

Ecotox = ecotoxicological

EE = 17α-ethinylestradiol

g = gram

L = Litre

mg = milligram

mL = millilitre

mm = millimetre

µm = micrometer

µS = microsiemens

TBT = Tributyltin

INTRODUCTION

There is no question that a wide variety of man-made chemicals with the capacity to disrupt the endocrine system are present in the aquatic environment. Observations publicized in Rachel Carson's Silent Spring were among the first to demonstrate the biological effects of persistent, bioaccumulative pollutants on wildlife (Bekoff and Nystrom, 2004) which acted as a major catalyst involved in subsequently increasing awareness of the long-term effects of many anthropogenic chemicals. It wasn't until 1993 that Colborn et al coined the phrase *endocrine disrupting chemical*, or compound (EDC) (Colborn et al., 1993). These compounds are able to enter the body through multiple pathways and are capable of altering sexual development, reproduction and in some situations, responses mediated through the endocrine system (Colborn et al., 1996; Solomon, 1998). There is also controversy and mystique with regards to these compounds because any observable effects of endocrine-modulating substances tend to appear at subsequent stages of development, not necessarily at the time of exposure (Colborn et al., 1996). It can take many years for an exposed human to show any physiological ill-effect after exposure. The effects may not appear until a child reaches puberty or afterward even though exposure may have taken place in the womb (Colborn et al., 1996), otherwise known as the dose-response lag (DRL). The dangers of these compounds is also rather pervasive, as by their very nature, they are difficult to detect, and can enact physiological responses at extremely low doses (Colborn et al., 1996).

As we become more dependent on cheap products for use in our daily lives, we also happen to be subjecting ourselves to potentially unknown dangers while using them and increasing our risk of exposure to potential hazards in the world around us. Some of us are realizing the danger in this and are taking additional steps to study the long-term safety of products we all consume, cook and store and serve food with. Chemicals may directly leach off of these products into our bodies, as in the case of Bisphenol-A (BPA), or they may biologically transform into more hazardous substances upon reaching the environment, as in the case of methylmercury being formed from inorganic mercury by anaerobic microorganisms in water

bodies (Ullrich et al., 2001), or androgens being released in pulp mill effluents from the breakdown of plant sterols (Howell et al., 1989; Hewitt et al., 2000; Ellis et al., 2003). These products may also be directly detrimental to the environment we depend upon. Unfortunately, these compounds are also quite ubiquitous in the environment, ending up in water bodies mainly through industrial, agricultural and pharmaceutical use (Rattner, 2009; Kurt-Karakus et al., 2010; Chang et al., 2011; Mita et al., 2011).

1.1 CASES OF ENDOCRINE DISRUPTION

Diethylstilbestrol

An early example of a dangerous endocrine disrupting compound, Diethylstilbestrol (DES); a synthetic, nonsteroidal estrogen synthesized in 1938 (Dodds et al., 1938). In the early 1940's DES was aggressively marketed and prescribed and became popular for a variety of purposes including the supplement of cattle feed, and treatment of prostate cancer, post menopausal symptoms, suppression of lactation, post-coital contraception, and prevention of spontaneous abortion (Dieckmann et al., 1953; Peña, 1954; Kogler, 1974). It was approved as estrogen replacement therapy for estrogen deficiency and was originally considered effective and safe for both pregnant women and the developing baby (Dieckmann et al., 1953). Although it was primarily given to women with high-risk pregnancies, it was also administered to normal pregnant women as well. Dieckmann et al., (1953) found that there was no benefit of taking DES during pregnancy, however until 1970 it was given to pregnant women under the belief it would reduce the risk of pregnancy complications and spontaneous abortions until 1971 when several studies showed that DES causes rare vaginal tumours in girls and young women who were exposed *in utero* (Herbst and Scully, 1970; Herbst et al., 1971; Greenwald et al., 1971). In essence, it took well over thirty years using human subjects to determine the hazards of using DES. If we are to learn from the past, it is that drugs must be tested on animals with short reproductive cycles for both acute and chronic toxicity, and perform multigenerational studies to determine if any population effects may occur.

Despite the copious amounts of research of DES on mammals, such as rats, mice and humans, there is little research to be found of its effects on invertebrates.

Bisphenol-A

Another example of such a compound that has made headlines in recent years is the chemical BPA. Once used as an antioxidant to plasticize baby bottles; it is now linked to several incidences of disease. For instance, excessive BPA exposure is associated with reduced fetal body weight and survival with a reduction in maternal weight (Ranjit et al., 2010). It is also associated with an increase in metabolic disorders (Lang et al., 2008; Vom Saal et al., 2008; Ranjit et al., 2010), with adverse effects on ovarian function and mammary gland development (Roy et al., 2009; Ranjit et al., 2010). Bonefeld-Jørgensen et al. (2007), found that BPA inhibits aromatase activity, and is an agonist of estrogen receptor and antagonist of androgen receptor. Perinatal exposure to environmentally relevant doses results in morphological and functional alterations of the male and female genital tract and mammary glands that may predispose the tissue to earlier onset of disease, reduced fertility and cancers (Maffini et al., 2006). In addition, and potentially the most frightening consequence is that it may be a cause of early onset of puberty and increased female gender ratio in animal and human populations (Williams et al., 2001; Nikaido et al., 2005; Maffini et al., 2006; Roy et al., 2009; Ranjit et al., 2010; Tena-Sampere, 2010).

Phthalates

Phthalates are used as plasticizers to increase flexibility, transparency and longevity to polyvinyl chloride (PVC) (Rakkestad et al., 2007). There are many types, but the most widely used phthalate is di-2-ethyl hexyl phthalate (DEHP) and is often used in medical tubing, catheters, blood bags (Rudel and Perovich, 2009). Exposure to DEHP and other phthalates have produced a range of adverse effects in laboratory animals, but of greatest concern are the effects on the development of the male reproductive system and sperm production in young animals, including humans (Mylchreest et al., 2000; Henley and Korach, 2006; European Union, 2007).

When exposed prenatally, phthalates may also play a role in disrupting masculine neurological development (Swan et al., 2009). Of added concern, phthalates are easily released into the environment as there is no covalent bond adhering them to the plastics in which they are mixed. As the plastics age, the release of phthalates accelerates causing them to be of environmental concern (Fromme et al., 2004; Heudorf et al., 2007; Rakkestad et al., 2007). However, they are subject to biodegradation and photodegradation, so their persistence in the environment is typically not prolonged (Stales et al., 1997; Xie et al., 2007). Despite this, In remote regions of the Norwegian Sea, where cold temperatures, low concentrations, and lack of nutrients can retard the degradation process, phthalates have been found, with atmospheric transport and deposition likely being the major source, with adsorption of phthalates by snow and ice both slowing down the degradation process and contributing to an underestimation of the total phthalate load (Xie et al., 2007).

According to the Centers for Disease Control and Prevention, most Americans have metabolites in their urine (Heudorf et al., 2007) and studies have shown that diet is the major route of exposure, especially DEHP (Fromme et al., 2007; Heudorf et al., 2007), so they are so ubiquitous in our environment that they are breaking down slower than they are being absorbed by humans and other organisms which is cause for concern. A number of the phthalates have been shown to interfere with androgen production, with the developing male fetus being the most sensitive to this effect. In animal studies, endpoints include effects on the developing male reproductive tract, including disrupted epididymal development, hypospadias, cryptorchidism, retained nipples, and reduced fertility (Henley and Korach, 2006; Mylchreest et al., 2000). **O**ne human study has shown an association between maternal levels of urinary phthalate metabolites and reproductive tract development in male offspring in the general population (Swan, 2006).

Glyphosate

Glyphosate is arguably the most used agrichemical of all time, sold even in hardware and garden stores by its more common name, Roundup. It is the most commonly used herbicide in

Ontario, with its use increasing over time due to the adoption of glyphosate resistant soybean technology (OMAFRA, 2004). It functions by inhibiting synthesis of tyrosine, tryptophan, and phenylalanine (Schonbrunn et al., 2001). However, it has also been shown to modulate plant cytochrome P450 (Lamb et al., 1998).

Normally produced by mature females, environmental estrogens are capable of inducing vitellogenin expression in male and juvenile fish. Xie et al., (2005) found that Glyphosate had no significant effect on juvenile rainbow trout vitellogenin expression, indicating that it is likely not an estrogenic compound.

It has been found that agricultural workers have pregnancy issues (Savitz et al., 1997). This could be for two reasons. Roundup has also been shown to inhibit steroidogenesis *in vitro* by disrupting the steroidogenic acute regulatory protein (StAR), which mediates a rate-limiting step in the process (Walsh et al., 2000). Walsh et al., (2000) also found that Roundup inhibited dibutyryl cAMP-stimulated progesterone production in MA-10 cells without causing cellular toxicity. Additionally, Richard et al., (2005), show that glyphosate is toxic to human placental JEG3 cells within 18hrs, with concentrations ten times lower than those found in agricultural use. Roundup, due to the added adjuvants, is always more toxic than its active ingredient, glyphosate. They also found that it disrupts aromatase activity and mRNA levels. Glyphosate interacts with the active site of the purified enzyme in a competitive manner and inhibits its gene expression as well. Adjuvants enhance glyphosate bioavailability and bioaccumulation.

Triclosan

Triclosan (TCS), a chlorophenol, is widely used as a preservative in different types of commercial preparations, from hand sanitizer to toothpaste. The reports on TCS-mediated endocrine disruption are controversial.

A study by Veldhoen, et al., (2006), found that exposure to low levels of triclosan disrupts thyroid hormone-associated gene expression and can alter the rate of thyroid hormone-

mediated postembryonic development in frogs, essentially hastening the transformation of tadpoles into adult frogs. A follow-up study by Zorrilla et al., (2009) indicated that triclosan exposure significantly impacts thyroid hormone concentrations in the male juvenile rat.

Kumar et al., (2009) found a reduced level of StAR protein in testicular Leydig cells after exposure to triclosan. Moreover, there was a significant decrease in the level of serum lutenizing hormone, follicle stimulating hormone, cholesterol, pregnenolone, and testosterone. Overall this study showed that TCS decreased the synthesis of androgens followed by reduced sperm production in treated male rats which could be mediated by a decreased synthesis of LH and FSH thus involving hypothalamo-pituitary-gonadal axis. Gee et al., (2008) found that triclosan has both estrogenic and androgenic activity, as it was able to displace each hormone from its respective receptor in cancer cell lines in vitro.

Pulp-Mill Effluent

Pulp-mill effluent (PME) has the potential to affect the endocrine systems of fish and invertebrates downstream of the effluent (Howell et al., 1980; Drysdale and Bortone, 1989; Ellis et al., 2003). Pulp and paper mill wastewaters contain chemicals such as wood extractives, additives, phytosterols, resin acids, polycyclic aromatic hydrocarbons, surfactants and organochlorines used or created in the processing of paper (Ellis et al., 2003). Despite the list of chemicals, the major source of steroids in pulp mill effluent is determined to originate from plant sterols (Rosa-Molinar et al., 1984). Plant sterols such as β-sitosterol, stigmasterol and stigmastanol can be broken down by microorganisms to produce androgenic steroids or androstane-like compounds (Howell and Denton, 1989).

Previous assays were performed on mosquitofish (*Gambusia affinis*). It was found that PMEs had the tendency to induce masculinisation of females. This includes the modification of the anal fin and the exhibition of male traits (Ellis et al., 2003), making mating difficult. Studies anaylzing the effluent did not detect androstenedione or testosterone, meaning the active

chemicals within the effluent are able to mimic those compounds during the masculinisation process (Ellis et al., 2003).

Many studies have been conducted looking at the reproductive effects of PMEs on fish models (Ellis et al., 2003; Munkittrick et al., 1997), but very few have looked at the reproductive effect on invertebrate reproduction and overall populations. Studies have looked at the acute effects of PMEs on survival (Tunstall and Solinas, 1976; McKean, 1980; Burtoletti et al., 1988), but the chronic and reproductive effects of PMEs on *Daphnia* populations were sparse prior to the early 1990s. A study by Kovacs et al., (1995) found no long-term effects of PMEs on the survival or growth of Ceriodaphnia; however, another study by Palva et al., (1998) found that bleaching effluent of Eucalyptus pulp had a significant effect on *Daphnia* reproduction at only 0.1% effluent, where reproduction was inhibited by half of the control value (Palva et al., 1998). It is more difficult to get a handle on PMEs, as they contain a cocktail of compounds which is individual to each plant. Regardless, PMEs, because of plant sterol degradation, have the potential to affect the endocrine systems of organisms downstream of the effluent.

Summary

These are just a few of the many, mostly anthropogenic compounds that wildlife and humans are bombarded with on a daily basis. There are also many other potential endocrine disrupting compounds that potentially disrupt various endocrine systems. Many were unaware that any of these compounds had caused any serious ill-effect while they were being utilized, and thus, their benefits had outweighed any potential deleterious impact. If in the past there was an easy way to detect the potential teratogenic and endocrine disrupting properties of these chemicals, they may have never made it on a shelf.

Most experiments testing endocrine effects have been performed on vertebrate systems. Therefore, there is still much to learn about the effects of environmental contaminants on invertebrate endocrine systems. Arguably, invertebrates have a more complicated endocrine system, so they are more vulnerable to a wider variety of contaminants.

Endocrine disruption is a serious issue. Because of the sensitivity, low dose requirement and the long dose-response lag (DRL) of these chemicals, the use of invertebrates to study these compounds is gaining interest, as their short reproductive cycles allow scientists to gain much information about the physiological nature of test compounds in a shorter period of time. The purpose of this thesis is to further investigate the utilization of the short reproductive cycles of invertebrate organisms to research the chronic toxicity and potential endocrine disrupting effects of test compounds: Atrazine, Tributyltin (TBT) and 17α-Ethinylestradiol (EE). In this thesis project, two test species are used which inhabit different compartments of the aquatic environment. These include *Daphnia magna,* a free-swimming crustacean which typically inhabits the water column and *Hyalella azteca*, an arthropod which lives at the sediment-water interface. Different chemicals are found in different regions of the water column, depending on their chemical properties, so if a detection system were to be developed from the results of this thesis, then it would be important to utilize diverse organisms to detect such chemicals.

Although analysis of industrial air and water effluents and various soil, plant and food toxicant tests may provide some predictability of environmental quality, the ultimate monitors are those organisms having metabolic activities that are comparable to man (Buck, 1979). The goal of this thesis is to follow methodology set out by Environment Canada to observe aquatic invertebrates, much like the coal miner observed his canary, in order for them to alert us to unseen and perhaps devastating environmental hazards with respect to potentially endocrine disrupting compounds.

All compounds of interest in this thesis are present and significant in North American waterways including drinking water sources (Graymore et al., 2001; USEPA, 2003; Konstantinou and Albanis, 2004; Coray and Bard, 2007; Ralston-Hooper et al., 2009; Kurt-Karakus et al., 2010; Lizotte et al., 2010). In addition to discussing the chemicals of interest and their hazards, it is important to discuss the systems that they can potentially impact as well as the organisms that will be used to assess their impact. Much has been gathered about these compounds and their

effects on individual tissues, for example, but there is much to learn about the effect of these compounds on the invertebrate system, especially those of *Hyalella azteca* and *Daphnia magna*.

1.2 THE ENDOCRINE SYSTEM

The endocrine system is a system of glands that involve the release of hormones; extracellular signaling molecules, which regulate metabolism, growth, development, life cycles, tissue function, circadian rhythm, and reproduction (Squires, 2004).

1.21 Vertebrates

In order to introduce the effects of vertebrate hormones on invertebrates, it is important to briefly discuss the vertebrate endocrine system. Across many species the molecular shape of most hormones has been conserved (Park et al., 2005; Fortin et al., 2009; Heimeier and Shi, 2010; Melamed, 2010). For example, cortisol, the chronic stress glucocorticoid found in humans with an–OH group at carbon 17, differs from the major stress glucocorticoid of poultry and many other vertebrates corticosterone only by the addition of this carbon group. However, when injected in a chicken, cortisol will elicit a similar response to corticosterone (Summers et al., 2005). The homology is close enough that one can use a cortisol ELISA kit to measure corticosterone and vice versa.

As this discussion is focused mainly on disruption of sexual hormones, there are many other endocrine glands outside the scope of this discussion. It is possible to disrupt the products of the islet of Langerhans, the adrenal glands, and even adipose cells, but this particular study is focused on compounds that have the potential to alter sex-hormones, or steroids. Other types of endocrine disruption would be difficult to characterize in invertebrate systems, as not all tissues are conserved across species.

Arguably, steroids are also the most profound classes of hormones. In vertebrates, steroids are secreted in a sequenced feedback pattern starting with the hypothalamo-neurohypophyseal complex, which has the role of releasing other hormones from the pituitary. Although this system specialized for vertebrates needs, it is homologous to the X-organ-sinus gland complex found in higher crustaceans, responsible for regulating molting, gonad development, water balance, blood glucose and pigmentation (Fingerman, 1997). The next step involves the release of gonadotropin releasing hormone (GnRH), which stimulates the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH), which are then released into the blood and act directly on the gonads (Senger, 2003). LH is responsible for causing ovulation and stimulating the production of progesterone in the female, while it causes testosterone production in the male (Senger, 2003). FSH causes the follicular growth in the ovary of the female, while in the male it stimulates Sertoli cells and is a key player in spermatogenesis. The release of the sex steroids are controlled in a feedback. As they increase in titer, GnRH secretion is reduced (Squires, 2004).

Androgens are the typical 'male' hormones produced by testis, while estrogens are considered 'female' hormones produced by the ovaries (Squires, 2004). There are exceptions to this, as androgens being a precursor to estrogens, can be present in females, just as estrogens can be present in males. The difference between androgens and estrogens are due to the enzyme aromatase, a member of the cytochrome P-450 (P450) superfamily. Aromatase is capable of catalyzing the reaction to change androgens into estrogens, and is therefore a potential target for several types of endocrine-disrupting compounds (Squires, 2004; Petkov et al., 2009; Hallgren and Olsén, 2010). Cytochrome P450 is conserved across multiple species from vertebrates to plants (Lamb et al., 1998; Goldstone et al., 2010), so EDCs that interfere with mammalian P450s have the potential to interfere with invertebrate P450s as well.

Endocrine disruption

Several types of endocrine disruption can occur. Chemicals, such as DES, can be estrogen mimicking and is therefore considered estrogenic. Chemicals such as testosterone or PMEs can

be androgen mimicking, and are considered androgenic. It gets complicated when chemicals are able to inhibit hormones by blocking the receptor, interfering with the carrier compound used to transport it through the circulatory system, or by directly interfering with the hormone itself. A chemical that interferes with the estrogenic receptor (ER) and inhibits binding of estrogen is considered an ER antagonist and is antiestrogenic; while a chemical that interferes with the androgenic receptor (AR) and inhibits the binding of androgen, is considered antiandrogenic. (Kolle et al., 2011). Not all EDCs will neatly fall into a category, and some will fall under multiple categories. For example, BPA is considered by Kolle et al. (2011) to be an ER agonist and an AR agonist, while DEHP is considered an AR agonist and an AR antagonist (Kolle et al., 2011). To further complicate the matter, if testosterone titers decrease, it does not necessarily mean that estrogen titers will increase, as testosterone is a precursor to estrogen.

The interest in vertebrate hormones with respect to this study is related to the fact that synthetic human estradiols end up in inland streams, rivers and lakes throughout the world potentially affecting countless species of wildlife.

1.2.2 Invertebrates

While much is known about vertebrate endocrinology, much less is known about the invertebrate endocrine system. It is known that invertebrates share several analogous glands and hormones to vertebrates (Lafont, 2000). However, they utilize several other invertebrate specific hormones important to their development, such as Juvenile hormones (JHs) and Ecdysones, (Jeng et al., 1978; Fairs et al., 1989; Novak et al., 1990) arguably making their endocrine systems much more complex to research and understand.

Juvenile Hormone

JHs are a group of hormones that regulate many aspects of insect physiology, such as development and reproduction (Riddiford, 1994; Maeno and Tanaka, 2009). They regulate diapauses, or periods of metabolic dormancy during adverse environmental conditions, much like hibernation (Sim and Denlinger, 2008; Schafellner et al., 2008; Ikeno et al., 2010), and they

also regulate polyphenisms, or multiple discrete phenotypes arising from a single genotype, which are usually phenotypes referring to caste differentiation (Tagu et al., 2005; Zhou et al., 2006; Verma, 2007; Maeno and Tanaka, 2009). For example, in termites, it appears that caste differentiation occurs as a result of the regulation of P450 genes by JH (Zhou et al., 2006). This relatesslightly to vertebrate endocrinology, as P450 appears to be conserved as an endocrinological staple among many species (Reitzel and Tarrant, 2010).

There are many types of JH, which tend to be analogues of four major classes, notably JH0, JHI, JHII and JHIII. They are acyclic sesquiterpeniod methyl-esters that have an epoxide bond at the 10,11 position (Dhadialla et al., 1998), also called sesquiterpinoid hormones. There are also a number of JH analogues which are more frequently studied, as they are more stable *in vitro*, and therefore more readily available. Whether or not they work in exactly the same way, is unknown, as they may bind to different receptors despite eliciting a similar response to the true compound (Soin et al., 2008). Many of the analogues are used as insecticides, preventing the target larvae from developing into adult insects. At high levels of JH, the larva will still molt, but the result will be a larger larva (Dhadialla et al., 1998). Methoprene, one such example, is approved by the World Health Organization for use in drinking water to control mosquito larvae (WHO, 2009).

JHs ensure growth of the larva while preventing metamorphosis (Dhadialla et al., 1998). The hormone is secreted by the corpora allata, a pair of glands located behind the brain, where it will disperse throughout the haemolymph and act on responsive tissues to control the developmental stage of the insect (Williams, 1959). JHs are degraded by the enzyme juvenile hormone esterase (JHE) or juvenile hormone epoxidehydrolase (JHEH), which leads to suppression of the signal and response (Anand et al., 2008). As the insect matures, the level of JH decreases, allowing it to proceed to successive instars with each molt (Williams, 1961), so when JH is completely absent, an adult is formed. The removal of the corpora allata from juveniles will result in a diminutive adult at the next moult, while additional levels of JH will produce an extra juvenile instar (Liu and Chen, 2001).

In honey bees JH is involved with inducing hive bees into field bees. An increasing JH titre is responsible for inducing the transformation (Fahrbach, 1997). It appears that there is also a complex interaction between JH, the molting hormone ecdysone, and the yolk-producing hormone vitellogenin (Barchuk et al., 2002). It also plays a relationship in the queen-worker caste relationship (Corona, 2007). Most importantly, during reproduction, JH stimulates the accessory glands of adult males, promoting growth and accessory gland secretion (Yamamoto et al., 1988). As mentioned earlier, JH also stimulates yolk production in female ovaries. Therefore, JH is involved with reproduction and potentially the resultant reproductive behaviour in both sexes.

Methyl Farnesoate (MF) is a precursor to Juvenile hormone that regulates male sex differentiation in some decapod crustaceans and regulates the formation of males in *Daphnia* (Sagi et al.,1993; Laufer et al., 1994, 2005; Olmstead and LeBlanc, 2002; Rider et al., 2005), and is therefore an important hormone involved in this assay.

Ecdysone

Ecdysone is a prohormone of 20-hydroxyecdysone, which is considered to be the major insect molting hormone from a class of hormones referred to as ecdysteroids (Mykles, 2010). Ecdysone is secreted from the prothoracic gland. In *Daphnia* and decapod crustaceans, the Y-organ is responsible for ecdysteroid synthesis, while the antennal gland is responsible for its excretion (Mykles, 2010). In arthropods it is strictly a molting hormone, but in other insects such as Drosophila, ecdysone causes the expression of certain genes required during development. In the European lobster, *Homarus gammarus*, ecdysone was found to be important in vitelline envelope secretion during embryonic development (Goudeau et al., 1990). The complexity of the invertebrate endocrine is exacerbated as it becomes difficult to generalize a system for invertebrates when hormones have different effects in different species.

Ecdysones can be used as insecticides to protect crops from insects. Insecticide RH-5992 is an insecticide which mimics ecdysone (Kreutzweiser et al., 1994). Acute toxicity tests were conducted on *Gammarus sp*. and the chemical was found to have no significant risk of adverse effects to macroinvertebrates (Kreutzweiser et al., 1994) The long-term effects are unknown, however. They also appear in plants as phytoecdysteroids, as protection from herbivorous insects (Dinan, 2001).

The molecular target of Ecdysone is the ecdysone receptor (EcR) and ultraspiracle in insects (USP), or its orthologous retinoid X receptor (RXR) in crustaceans (Hirano et al., 2010; Wang and LeBlanc, 2010). RXR forms heterodimers with various nuclear hormone receptors to initiate various transcription factors (Segars et al., 1993). In *Daphnia*, RXR binds to the EcR to form a heterodimer (RXR:EcR) which initiates transcription events induced by Ecdysone, such as molting (Hirano et al., 2010; Wang and LeBlanc, 2010)

As mentioned earlier, MF is involved in the formation of males in *Daphnia* (Sagi et al.,1993; Laufer et al., 1994, 2005; Olmstead and LeBlanc, 2002; Rider et al., 2005) However, an *in vivo* receptor for MF has not been identified (Wang and LeBlanc, 2010). MF and other sequesterpenoids can bind and activate RXR *in vitro* and they can also synergize with ecdysteroids to activate the RXR:EcR heterodimer transcription factor. However, these effects appear to be unrelated to the ability of MF to stimulate male sex determination (Wang and LeBlanc, 2010). Therefore, the biochemical reasoning for the shift in female to male production has yet to be elucidated. There is still much to learn about the intricacies involved in invertebrate endocrinology.

Vitellogenin

The induction of vitellogenin (VTG) in oviparous vertebrates has become the gold standard biomarker of estrogenic endocrine disruption (Hannas et al., 2010). It has also been used as a biomarker in arthropods, however little is known of the factors that regulate expression. What is known is that VTG is an egg yolk precursor protein expressed in insects, and many oviparous

vertebrates as well typically expressed only by females (Xie et al., 2005; Hannas et al., 2010). However, in the presence of EDCs, males can express the VTG gene in a dose dependent manner (Xie et al., 2005; Hannas et al., 2010). This is known to occur in many insect as well as fish species. Therefore, VTG is often used as a biomarker for exposure to estrogenic EDCs (Xie et al., 2005).

In honey bees, VTG is deposited in fat bodies in their abdomens and heads, which act as a food storage reservoir (Barchuk et al., 2002; Corona et al., 2007). It also appears to prolong queen bee and forager lifespan while affecting future foraging behaviour (Corona et al., 2007; Amdam et al., 2009). VTG is also involved in worker caste relationships (Barchuk et al., 2002; Corona et al., 2007). The higher the titer of VTG in a juvenile bee, the later in life they begin to forage for pollen, as pollen and VTG are the only protein sources available to honey bees (Corona et al., 2007).

JH stimulates transcription of the VTG genes and the downstream control of VTG production. Expression of VTG is part of a feedback loop which enables VTG and JH to suppress one another, working antagonistically to regulate development and behaviour (Corona et al., 2007). This relates to vertebrates and their control of sex steroids via GnRH.

Crustaceans

Crustaceans, including *Daphnia* and *Hyalella*, have several endocrine glands specific to crustaceans, such as the eyestalk (sinus gland and X-organ) and pericardial organ (Fingerman, 1997). However, the use of JHs has been conserved across many invertebrates, as they also utilize JHs like insects (Laufer et al., 1987; LeBlanc, 2006). The major JH in crustaceans is believed to be methyl famesoate (MF), which is JHIII lacking the epoxide group (Laufer et al., 1987; LeBlanc, 2006), although there is some controversy due to lack of scientific evidence. MF, as well as Farnesoic Acid are the immediate precursors of JHIII, the most ubiquitous of the JHs (Moshitzky and Applebaum, 2005).

The sinus gland was discovered in 1935 and was found to secrete pigment-controlling hormones in decapods (Fingerman, 1966). It is contained either within the eyestalk or the supraesophageal ganglion of crustaceans, where it is exposed to a large sinus (Fingerman, 1997). In amphipods, the glands are present in the head close to the optic centers (Hanstrom, 1939; Fingerman, 1997). The sinus gland is ultimately a storage-release center for hormones produced elsewhere. One of the ganglia in the eye stalks of higher crustaceans is the medulla terminalis X-organ. (Fingerman, 1997). It is estimated that approximately 90 percent of the axonal terminals that compose the sinus gland belong to neurons whose cell bodies lie in the medulla terminalis X-organ (Cooke and Sullivan, 1982) Therefore, the term medulla terminalis X-organ-sinus gland complex is used, which is analogous to the vertebrate hypothalamo-neurohypophyseal complex (Fingerman, 1997), responsible for releasing GnRH in vertebrates.

The specific details regarding *Daphnia* and *Hyalella* will be discussed later.

1.3 PARTHENOGENESIS

Parthenogenesis is the growth and development of an embryo or seed without fertilization. It has been known to occur in several species of plants, invertebrates and very few vertebrates. It can occur in a variety of forms, such as thelytoky, where only female offspring are produced and no mating is observed; pseudogamy, where mating occurs and the eggs require activation by entry of sperm, but only the maternal chromosomes are expressed; automixis, in which the eggs undergo meiosis, and apomixis, in which the eggs do not undergo meiosis. Plant parthenogenesis is outside of the range of this discussion, so it will not be mentioned. For animals capable of reproducing by parthenogenesis, the phenomenon appears to be a reproductive strategy optimized for their living conditions. This study outlines several organisms which employ this reproductive strategy. With properly designed experiments, these organisms can be used to study reproduction and the hormonal control of parthenogenesis, which is poorly understood.

In vertebrates, parthenogenesis is quite rare, but is known to take place in several species of reptiles, fish, birds and sharks. There are no known cases in mammals in the wild due to imprinted genes, but it can be induced in some laboratory animals when imprinting is circumvented (Lampert, 2009; Sritanaudomchai et al., 2010). It does however; occur naturally in some species of invertebrates.

1.31 Cyclical Parthenogenesis

Abiotic factors

Cyclical parthenogenesis is an adaptive reproductive strategy that occurs when organisms typically reproduce asexually producing clones. However, when a combination of several key abiotic factors is set, asexual reproduction halts and reproduction becomes sexual. This is a complicated process involving a sequence of hormonal releases that is currently poorly understood.

Synchronization to relative time of day and seasonal cycles is key to survival. Night-time and day-time environments differ in illumination, temperature, food supplies and predators. Organisms have, therefore, developed highly specialized temporal programmes to get better adapted to activity either during night or during the day (Kumar et al., 1997). Many species use annual cycle of changes in daylength as their calendar to synchronize their circadian rhythms to their daily and seasonal physiological and behavioural functions. This is described as photoperiodism (Kumar et al., 1997). Photoperiod is the length of daylight. As photoperiod decreases it indicates shortening of days, which is one of the factors involved in indicating to the organism that the season is changing and winter is approaching. A photoperiodic response system has three principal components: a photoreceptor that interprets photic input, a clock that measures photic signal, and a neurosecretory system that translates photic signal into endocrine secretions (Kumar, 1997). In insects, mollusks, crustaceans, fishes, amphibians, reptiles and birds, the photoreception occurs largely through extra-retinal photoreceptors

localized in the hypothalamus whereas in mammals, brain photoreceptors are apparently absent and light input is only through eyes (Kumar, 1997).

The Suprachiasmatic nuclei (SCN) of the hypothalamus in mammals functions as the clock, monitoring the photoperiodic message and decoding it by dictating the changes in rhythm via melatonin secretion in the pineal gland (Kumar, 1997; Maciel et al., 2008). The location of SCN homologues in non-mammalian vertebrates and invertebrates are still unclear. One study found that in the crab *Neohelice granulata*, melatonin is produced in the eyestalk (Maciel et al., 2008), but this may not be representative of all crustaceans. Another study was interested in genetic control of photoperiodism, and looked for genes that were up-regulated or down-regulated by short photoperiod conditions that lead to the sexual response in a species of aphid. They demonstrated the differential expression in relation with the photoperiod of 6 genes, 3 up-regulated and 3 down-regulated, by shortening the day length (Cortés et al., 2008). Among these, they identified expression of a tubulin gene, two cuticular proteins and a yet unidentified sequence along the day-night cycle (Cortés et al., 2008). There is still much to learn about control of circadian rhythms in invertebrates.

Temperature is another abiotic factor involved in control of circadian rhythms, but research focusing strictly on the effect of temperature while negating daylight, are limited. Typically when cyclically parthenogenetic organisms in the environment are exposed to decreasing daylight, they are also subject to a decreasing temperature.

Aphids

Many aphids (Superfamily Aphidoidea), reproduce by cyclical parthenogenesis. In the spring and summer, populations are typically all-female. The overwintering eggs that hatch in the spring result in females, called fundatrices. Reproduction is typically parthenogenetic and viviparous (Jahn, et al., 2005). Females undergo a modified meiosis that results in eggs genetically identical to their mother. In autumn, aphids undergo sexual, oviparous reproduction. A change in photoperiod and temperature, and perhaps lower food quantity or

quality is believed to cause females to parthenogenetically produce sexual females and males (Ramos, et al., 2003; Jahn et al., 2005). The males are identical to the female except for a missing sex chromosome. They may also lack wings or mouthparts. The sexual females and males mate and produce external overwintering eggs (Ramos, et al., 2003). Some aphids have telescoping generations, where the parthenogenetic viviparous female has a daughter within her who is parthenogenetically producing her own daughter (Ramos, et al., 2003). This behavior is homologous to the reproductive strategy of *Daphnia*. In the future, a land-based application assay may be used with aphids to determine the toxicity or endocrine-disrupting potential of land-applied biosolids much llike *Daphnia* are used to study aquatic contaminants.

Rotifers

Monogononts are a mostly freshwater class of rotifers which tend to have a reduced corona and a single gonad. Males are generally smaller, and are produced only during certain times of the year during seasonal duress, with females otherwise reproducing through parthenogenesis (Carmona et al., 2009). The males that occur develop without a functioning digestive tract, as they have the sole purpose of inseminating females to produce resting eggs (Schröder et al., 2007). At the start of the growing season, diploid asexual females hatch from thick-walled resting eggs deposited in the sediment during the previous season. Asexual females produce eggs mitotically, which develop into females. Much like aphids, upon receipt of particular environmental stimuli, asexual females produce sexual females via mixis (Wallace et. al., 1991). Sexual females subsequently produce eggs meiotically, which develop into haploid males or resting eggs if fertilized by males.

Brachionus are a genus of Monogonont rotifers commonly used in aquatic ecotoxicology because of their sensitivity to many contaminants (Yúfera, 2001). *Brachionus plicatilis* is commercially important as they are used in the aquaculture industry as food for fish larvae. They are a species complex of approximately ten different species distributed in salt lakes (Gomez et al., 2002). They typically have a low investment in sex as asexual reproduction tends to predominate (Carmona et al., 2009). *Brachionus calyciflorus*, are thought to be sensitive to

most toxicants, and because of this, are often favoured test animals in aquatic toxicology (Preston et al., 2000; Marcial et al., 2005; Xi et al., 2007). They were used by Preston et al., (2000) in a screening assay for potential endocrine disruptors that would disrupt asexual and sexual reproduction. They found that nonylphenol, flutamide and testosterone had inhibited fertilization of sexual females with no effects on asexual reproduction (Preston et al., 2000). This observation suggests that the reproductive effects observed for these three compounds may have resulted from an endocrine-mediated mechanism such as endocrine disruption rather than from another mechanism of toxicity such as narcosis, enzyme inhibition, or membrane disruption. Estradiol and methoprene were found to have no effect.

Summary

Parthenogenesis is an important reproductive strategy for many organisms. Cyclical parthenogenesis is especially important, and provides an interesting tool to study endocrine disruption. However, the endocrinology of parthenogenesis and cyclical parthenogenesis is poorly understood. More on parthenogenesis in the *Daphnia* Reproduction Section (1.412) below. One of the goals of this experiment is to manipulate the photoperiod and temperature and observe the sexual strategy utilized by *Daphnia*.

1.4 BIOASSAY ORGANISMS

1.41 *Daphnia magna*

The ideal invertebrates to study endocrine disrupting compounds, are the tiny creatures known as *Daphnia magna*. They are small freshwater crustaceans commonly used in ecotoxicology (ecotox) as their quick reproductive cycles and sensitivity to various compounds make them suitable organisms for determining acute chemical toxicity (Kieu et al*.,* 2001; Kiss et al*.,* 2003; Schmidt et al*.,*2005; Ren et al*.,* 2009). Their short, parthenogenetic reproductive cycles allow them to breed and mature quickly, making them easy to culture in a laboratory. Their body tissues, including gills are continually exposed to their aquatic environment, making them

exceptionally sensitive, especially to compounds which may interfere with those particular tissues (Green et al*.,* 2003). Because of their diet of algae and phytoplankton and placement in the trophic level in diets of higher organisms, including fish, amphibians and larger zooplankton, they are a vital organism in the freshwater ecosystem (Dodson and Hanazato, 1995; Fischer et al*.,* 2006). Their overall sensitivity, importance and relevance to the ecosystem, as well as their parthenogenetic reproductive strategy, make them ideal candidates as model organisms in this study.

***Daphnia* behaviour**

Daphnia are pelagic organisms. Variation in their swimming behaviour is an important endpoint in subacute toxicity bioassays (Dodson et al., 1995; Christensen et al., 2005; Ren et al., 2008, 2009; Marshall, 2009). They are commonly referred to as water fleas, as they can be seen 'jumping' throughout the water column like fleas in their saltatory swimming style due to the strokes of their powerful secondary antennae (See fig. 1) (Dodson and Hanazato, 1995; Marshall, 2009). Their normal swimming behaviour typically involves swimming to lower and darker water by day and moving up to graze at night (Reichwaldt and Stibor, 2005; Slusarczyk and Pinel-Alloul, 2010). However, in response to a predator, *Daphnia* will alter their migrational strategy and may display escape responses and swim in an irregular, short, circular burst to evade predators, commonly referred to as 'spinning' behaviour (Marshall, 2009; Slusarczyk and Pinel-Alloul, 2010). In the lab which typically consists of short water columns, they swimming is usually straight and vertical following beams of light, which allows them to move faster and synchronize their grazing of phytoplankton when in groups (Ryan and Dodson, 1998; Christensen et al*.,* 2005; Marshall, 2009).

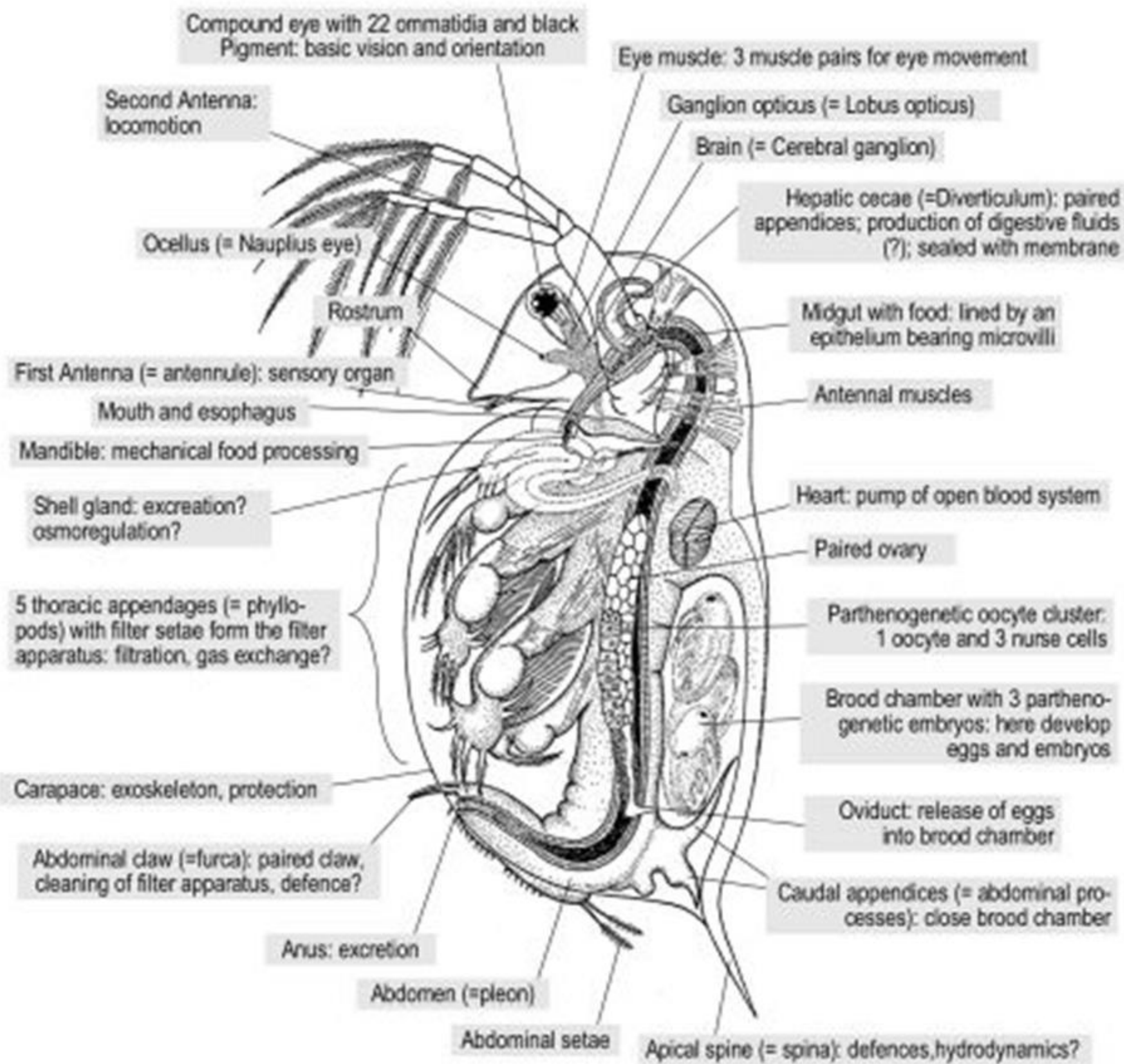

Figure 1: Anatomy of *Daphnia magna*. (Ebert, 2005a). Note: Second Antenna for locomotion and Brood chamber for reproduction.

Daphnia have been shown to be highly sensitive to a variety of common contaminants during subacute and acute bioassays (Ren et al., 2008, 2009; Marshall, 2009). All of the locomotory components are important responses when determining the behavioural effect of a chemical. In the presence of toxic compounds it has been shown that their swimming style can be altered (Dodson et al., 1995; Christensen et al., 2005; Ren et al., 2008, 2009; Marshall, 2009). Therefore swimming behaviour is a useful endpoint in ecotoxicological research.

Daphnia reproduction

Daphnia, like the aphids and rotifers mentioned earlier, also follow the cyclic parthenogenetic trend. Cladoceran populations are normally all female. Offispring are produced by ameiotic asexual parthenogenesis via thelytoky (Zaffagnini and Sabelli, 1972; Tatarazako & Oda, 2007) and have the same genotype as their mother. Females moult approximately every ten days, and shortly after each moult several eggs are laid into their brood pouch (See fig. 1), where they develop until released a couple of hours before the next moult. Parthenogenesis can continue indefinitely, but sexual reproduction is triggered if the environment deteriorates, through any combination of shortened day length, decreased temperature, food depletion, and high population density (Hobaek and Larsson, 1990; Kleiven et al., 1992; Tatarazako & Oda, 2007) which usually indicates seasonal change in wild conditions. Kleiven et al., (1992) argue that *Daphnia* require three key factors to be in place in order to make the switch to sexual reproduction, which are an inductive photopertiod less than 9 hours, food limitation and crowding, which can be chemically mediated (Kleiven et al., 1992). Once the sexual trigger is set, instead of producing solely females, *Daphnia* begin to produce haploid males (See fig. 2,3, 11) by parthenogenesis, and the females lay meiotically produced haploid eggs known as ephippia that require fertilization by males (Zaffagnini and Sabelli, 1972; Hebert and Ward, 1972). Females that feature this ephippia are known as Ephippial females, where the ephippia will appear as a dark pouch within her brood chamber (See fig. 1,2). Once fertilized, they are released at an early stage of development, and can diapause for weeks or years before resuming development into normal females (Stross and Hill, 1965). These diapausing embryos are resistant to desiccation and freezing, and therefore carry populations through inclement seasons and allow dispersal to other ponds. When performing ecotox tests on *Daphnia*, researchers can manipulate the abiotic factors involved in the sexual cycle in order to gain a better understanding not only of the effect of a toxicant on *Daphna magna*, but also gain a better understanding of cyclical parthenogenesis, as the endocrinology of it is poorly understood. It is one of the goals of this experiment to induce the conditions necessary to induce production of both ephippial females and males.

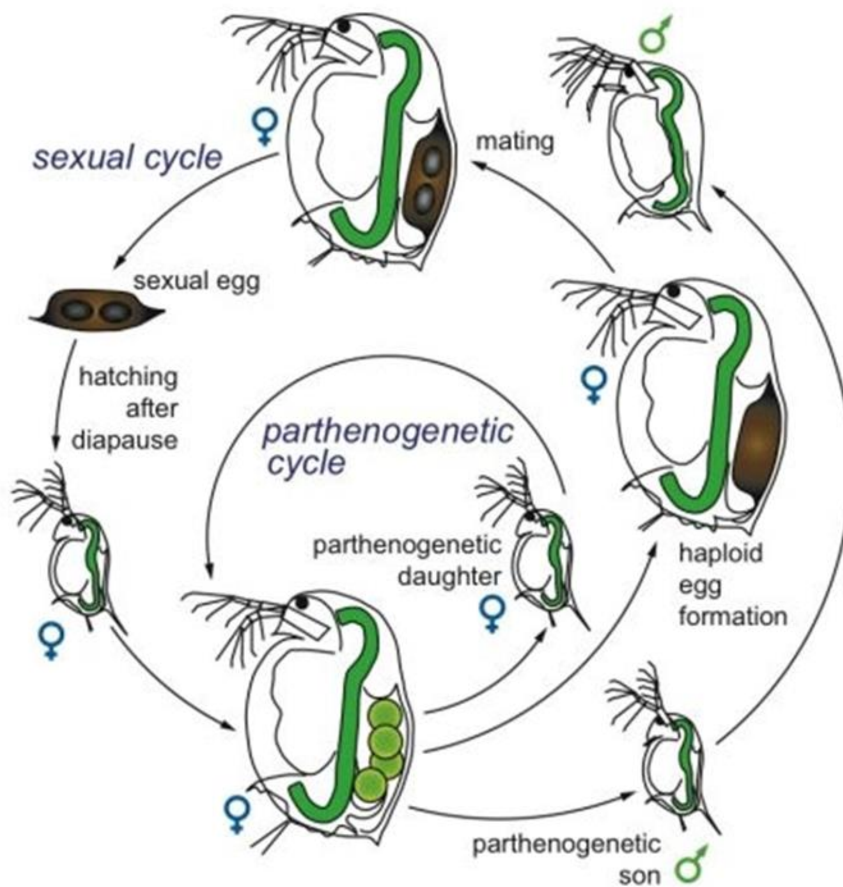

Figure 2: Life cycle of a cyclic parthenogenetic Daphnia. Note the ephippia in the brood chamber (Ebert, 2005b).

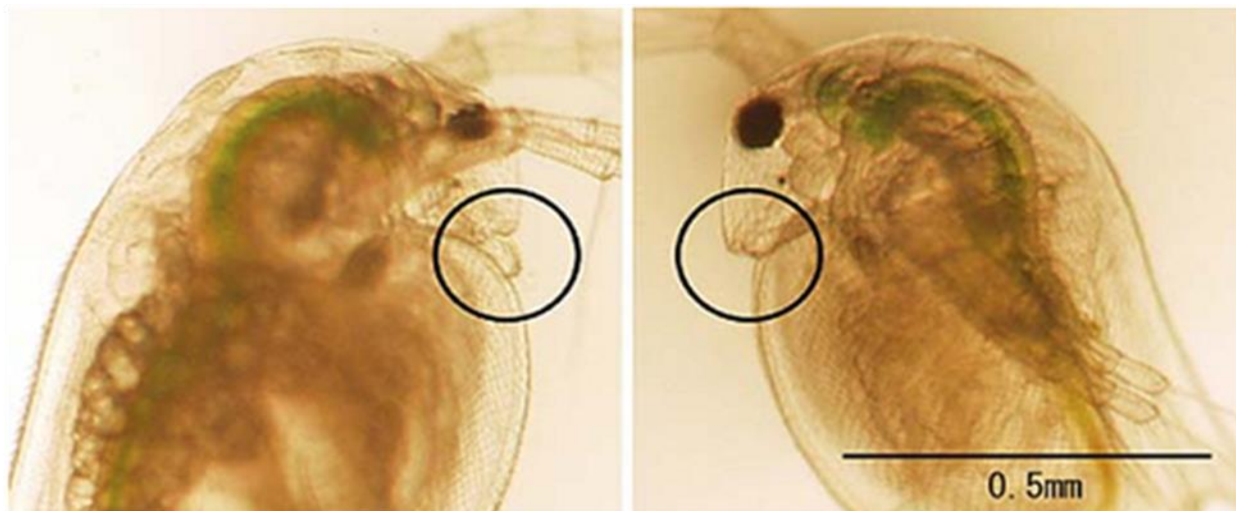

Figure 3: Differences between male (left) and female (right) Daphnia (Tatarazako and Oda, 2007). The circled areas feature the primary antenna, elongated in the male.

1.411 *Daphnia* Endocrinology

Juvenile Hormone

Studies have revealed that JHs may play an important role in the shift of reproductive mode from parthenogenesis to sexual reproduction (Olmstead and LeBlanc, 2002, 2003; Tatarazako et al., 2003; Oda et al., 2005), although the mechanisms involved are unclear. Using offspring sex ratio as a new endpoint has made it possible to identify chemicals with juvenile hormone-like effects on crustaceans. These results also show that parthenogenesis and the endocrine system are inextricably linked.

Vitellogenin

Daphnia are also known to express the VTG gene, but expression of which is not a common endpoint in toxicological testing. VTG mRNA was found to be downregulated in response to various EDC comounds, and found to be regulated by JHs (Hannas et al., 2010), indicating a similar feedback relationship in *Daphnia* to that of honeybees.

Exposure to diethylstilbestrol and bisphenol A have little effect on VTG levels on Daphnia, indicating that the gene is not induced by estrogenic exposure (Hannas et al., 2010). However it was found that exposure to piperonyl butoxide, chlordane, 4-nonylphenol (4-NP), cadmium and chloroform do induce VTG levels. Of those, only 4-nonylphenol is recognized to be estrogenic (Hannas et al., 2010).

Endocrine Disruption

Several studies (Baldwin et al., 1995; Zou and Fingerman, 1997; Kashian and Dodson, 2004; Brennan et al., 2006; Hannas et al., 2010) have also shown the effect of natural and synthetic vertebrate hormones and their analogs on *Daphnia magna*.

Kashian and Dodson, (2004) compared the effect of twelve hormones on the developmental and reproductive processes in *Daphnia magna*. Natural hormones tested included β-estradiol,

gonadotropin, hydrocortisone, insulin, melatonin, progesterone, somatostatin, testosterone, and thyroxine at concentrations ranging from 1 to 100 µg/L. Synthetic hormones tested included diethylstilbestrol (estrogenic), R-1881 (androgenic), and ICI-182,780 (antiestrogen). All chemicals were screened with a 6 day assay, while progesterone, insulin, testosterone and thyroxine were screened in an additional 25 day assay. They found that Diethylstilbestrol decreased Daphnia growth rate while thyroxine increased it. Short-term testosterone exposure reduced *Daphnia* fecundity; however, long-term exposure did not, potentially indicating testosterone hydroxylation with long-term exposure. Hormones commonly considered sex-hormones (estrogens and androgens) in vertebrates do not appear to control sexual differentiation in *Daphnia*; however, several vertebrate hormones do affect reproduction and development in *Daphnia* making Daphnia a potentially useful tool in monitoring for the presence of these hormones or compounds that mimic them. Despite the intensity of the assay, there is still much to learn regarding the endocrine system of *Daphnia*.

Brennan et al., (2006) tested the effects of 17β-estradiol (E2), DES, BPA and 4-NP on *Daphnia* and found that 4-NP decreased the number of offspring produced in first and second generation testing, DES had a slight effect on second generation daphnids, and E2 and BPA were found to have no effect. Zou and Fingerman, (1997) found that synthetic estrogens do not interfere with sexual differentiation, but they do inhibit moulting (Zou and Fingerman, 1997), potentially interfering with ecdysone or its receptor. However, Baldwin et al., 1995 found that DES exposed over multiple generations can result in reduced fecundity and altered steroid metabolic capabilities. Therefore, Daphnia can clearly be affected by vertebrate hormones, however, there is disparity as to what exactly the vertebrate hormones do in their invertebrate system. Barata et al., 2004, argue that some results may indeed be a true result of endocrine disruption; however, many results are falsely believed to be a result of endocrine disruption because they are likely due to other, more simple reasons, such as egg mortality and feeding inhibition (Barata et al., 2004). Therefore any studies studying endocrine disruption have to rule out any toxic effects from the treatment on egg and infant mortality as well as energy intake.

Daphnia are so small, that assays are used with whole organisms. To gain further physiological detail in the future, it may be possible to produce cell lines from *Daphnia* tissue, but for now all chemical effects have to be inferred mostly based on qualitative measurements. Because of their small size, it is difficult to directly manipulate their endocrine systems, for example, through means such as an injection. However, because they interact so intimately with the water column, it is believed that any compounds suspended or dissolved in the water column become internalized by the daphnids.

Chronic *Daphnia* bioassays

Chronic bioassays using *Daphnia* as the proprietary organism are lacking, as is the full understanding of their endocrine system. They are the quintessential tool when studying the chronic effects of contaminants on parthenogenetic organisms. With proper conditions, their reproductive cycle can be manipulated to induce production of ephippial females alongside males. It is one of the goals of this study to develop a chronic toxicity assay in which the sexual cycle of *Daphnia* is manipulated in order to produce males and ephippial females for analysis of potential endocrine disruption.

Dodson, et al., (1999b) patented a 6-day reproductive bioassay for testing the toxicity of aqueous samples for the presence of a potential endocrine disrupter. The bioassay is based upon the measurement of five endpoints that convey quantitative information about the biological activity of the substance: survivorship, numbers of female offspring, numbers of male offspring, number of resting eggs, and number of offspring that display developmental deformities. During the assay, a test sample is brought into contact with at least three adult, oviporous *Daphnia* of a single clone under conditions of crowding and suboptimal growth in order to cause stress and stimulate sexual reproduction. The preferred clone for use in the assay is *Daphnia galeata*-mendotae Wingra clone CDF-1. (Dodson et al., 1999b).

Baer et al., (2009) determined the influence of sewage plant effluents on sex ratios in *Daphnia magna.* Female daphnids were acclimated for several generations to effluents from a municipal

sewage treatment plant and a residential oxidation lagoon then placed under conditions to maximize male offspring production. Both effluents resulted in a statistically significant decrease in male production and a shift in production of male broods from earlier on, to near the end of the adult life cycle. Secondary sexual characteristics of both sexes were statistically significantly increased by the sewage lagoon effluent but not the municipal effluent. These results not only suggest that daphnia can display sex ratio differentiation with exposure to endocrine disrupting compounds, but they can also display shifts in timing, which can help indicate the nature of the chemical when used in an assay testing unknown chemicals.

This study will use methods inspired by Dodson et al. (1996b) and Baer et al. (2009) to perform a novel assay with knowledge provided by Stross and Hill (1965) and Kleiven et al. (1992) to provide information regarding the reproductive shift from parthenogenetic to sexual. This assay could be used as a method to detect endocrine-disrupting effects of a chemical within 6-10 days, without having to wait for the entire life cycle of *Daphnia magna* to complete.

1.42 *Hyalella azteca*

Hyalella azteca are freshwater amphipods extensively used to test acute and chronic toxicity of aquatic contaminants, especially in sediment toxicology testing due to their close association with sediments (Nebeker et al., 1986; Mackie, 1989; Borgmann et al., 1991; Phipps et al., 1995; Wang et al., 2004; Ingersoll et al., 2005). An added benefit to using *Hyalella* is that they are prolific and rather hardy; thus, they are easy to culture and their results also generate less false positives than *Daphnia*. Despite their hardiness, they are sensitive to aquatic contaminants and are able to survive in water with a wide range of dissolved oxygen, alkalinity, sediment size and organic content, making them easy to care for and culture (Wang et al., 2004).

Hyalella are widespread throughout the Americas and are present in the Great Lakes and inland lakes in Ontario (Blockwell et al., 1998; Wang et al., 2004). They are the most common freshwater amphipod in North America (Bousfield, 1958) and play an important role in the ecosystem in which they are present, as they are omnivorous detritivores feeding on algae,

dead leaves, animal carcasses, isopods, bacteria and aquatic plants (Borgmann et al., 1996). Therefore they are essential in the recycling of nutrients and the maintenance of water clarity in freshwater systems (Blockwell et al.,1998; Wang et al., 2004) Macroinvertebrate feeding is a major rate-limiting stepin the processing of stream detritus (Kunz et al., 2010). They are also a dominant food in the diet of many fish and waterfowl (El-Shamy, 1978; Brown and Fredrickson, 1986; Luecke, 1990; Ryder & Pesendorfer, 1992; Wellborn, 1994; Borgman et al., 1996). Therefore, they are rather vital to the aquatic system.

Life Cycle

Hyalella are born at a size of approximately 1 mm in length and reach a maximum size of 7 mm at an approximate age of 120 days (Othman and Pascoe, 2001). They have a maximum lifespan of approximately 180 days. Males are typically larger than females with a period of approximately 20 days in-between moults (intermoult period) , whereas females tend to have an intermoult period of approximately 11 days (Othman and Pascoe, 2001). Gender can be determined at 19-21 days of age, or after its 6th instar by examination of the secondary gnathopod. Males have an enlarged secondary gnathopod (See fig. 4, 15), while females possess a brood pouch or marsupium, which can be identified as a large mass beneath their thorax (See fig. 15) (Environment Canada, 1997; Othman and Pascoe, 2001). Amphipods engage in a phenomenon known as amplexing, precopulating or mate guarding behaviour. This occurs as early as 23 days of age, when a male can be seen attached atop a female using his large secondary gnathopods (See fig. 4) to manoeuvre the female while using his smaller first gnathopods to carry her. This occurs in anticipation of fertility prior to moulting and copulation and the time spent in this behaviour can range from 1 to 7 days (Othman and Pascoe, 2001). After mating, the fertilized eggs in the female marsupium develop directly and are released as juveniles during her next moult when they are ready to hatch (Othman and Pascoe, 2001; TAFI, 2008). A single female can release from 3 to 17 juveniles with each brood, depending mainly on her age and body length (Othman and Pascoe, 2001).

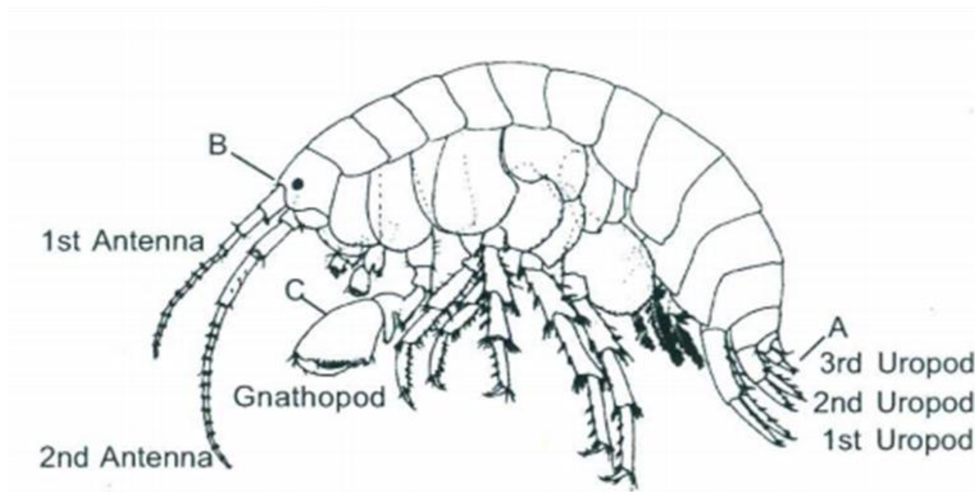

Figure 4: Hyalella azteca. (A) denotes the uropods; (B) denotes the base of the first antennae; (C) denotes the male secondary gnathopod used for grasping females. Measurement of length is made from the base of the 3rd uropod (A) to (B). Females are recognized by the presence of egg cases and the absence of an enlarged gnathopod. (USEPA, 2000; Reprinted from Cole and Watkins, 1977)

Culturing protocols

As *Hyalella* are benthic organisms, they prefer to have substrate to interact with during the course of bioassays (Wang et al., 2004). Some common substrates include silica sand (USEPA 2000) and Cotton gauze (DFO, 1989, 1992; USEPA 1991b, 1992; USFWS 1992, 1994a as cited in Environment Canada, 1997). For this assay, Hyalella will be cultured in a combination of silica and cotton gauze, but for the bioassays, only cotton gauze will be used, as neonates can be sifted out when sifting through silica to count *Hyalella*.

Behaviour studies

In subacute bioassays with *Hyalella azteca* different behavioural endpoints are used in sublethal toxicology studies, such as changes in burrowing, swimming, crawling, grouping and position within the test chamber (Hatch and Burton, 1999; Wang et al*.,* 2004; Marshall, 2009). Each behavioural endpoint is believed to have biological reasoning associated with it. *Hyalella* have the tendency to change swimming and crawling behaviour and form larger groups when stressed (Hatch and Burton, 1999; Wang et al*.,* 2004; Marshall, 2009).

Burrowing behaviour is believed to be associated with contaminants dissolved in the water column, as it is believed that *Hyalella* may attempt to avoid these contaminants by burrowing deep in the sediment to decrease their exposure to the aquatic compounds (Hatch and Burton, 1999; Moore et al., 2006). Wang et al., (2004) argue that the abundance of algae and oxygen at the sediment-water interface reduces the need for the organisms to continually burrow in order to collect food under normal conditions. They found that the organisms only burrowed as a response to being frightened, such as when the water was disturbed, and would re-emerge after varying periods of time (Wang et al*.,* 2004). Hatch and Burton (1999) showed that adult *Hyalella* spent significantly more time burrowed in sand and soil sediments when exposed to various concentrations of the polycyclic-aromatic hydrocarbon fluoranthene than organisms in control water. As concentrations of the contaminant increased from 6.25-25 µg/L, the amount of time and the number of organisms which had formed burrows increased as well (Hatch and Burton, 1999). Moore et al*.* (2006) found that *Hyalella* growth rates were significantly reduced in the presence of several pesticides when chronically exposed to the contaminants in the water column. However, the direct contamination of sediments with the equivalent levels of pesticides caused no change in the overall growth rates of the organisms (Moore et al., 2006), indicating that the contaminants may be sorbing to the sediment, making the contaminant unavailable to the *Hyalella*. It is therefore important to account for the chemical properties of the contaminants in toxicity testing. It is generally accepted that burrowing behaviour in *Hyalella* is a type of avoidance behaviour in an attempt to escape from contaminants or potential predators in the water column (Hatch and Burton, 1999; Wang et al*.,* 2004; Moore et al., 2006; Marshall, 2009).

Grouping behaviour occurs when two or more *Hyalella* aggregate together within the test vessel and is considered to be a stress response (Hatch and Burton, 1999). Hatch and Burton (1999) found that as concentrations of polycyclic-aromatic hydrocarbons increased, grouping behaviour of *Hyalella* increased significantly compared to the control treatments. Marshall (2009) found similar results when *Hyalella* were exposed to increasing concentrations of TBT; however, when exposed to increasing concentrations of Atrazine, there appeared to be no time

or concentration dependent relationship despite witnessing increased grouping behaviour during Atrazine exposure (Marshall, 2009). Despite the observations, there is little known as to the reason why *Hyalella* group as a stress response. What is known, is that the phenomenon is typically anomalous.

1.421 Endocrine Disruption in *Hyalella azteca*

Sexual differentiation within *Hyalella* poses a useful tool for use in studying endocrine effects. Several studies have used *Hyalella azteca* as an indicator for endocrinological studies. Their definitive genders, secondary sexual characteristics and amplexing behaviours are all worthy endpoints to look at when studying endocrine disrupting compounds on these invertebrates (Vandenbergh et al., 2003; Dussault et al., 2008). However, despite the copious amount of work with *Hyalella* as a subacute toxicity test organism, they are not as common a model organism when studying endocrine disrupting compounds. Notwithstanding, that may change in the near future.

A study by Flick et al., (2001) studied differential analysis of RNA between *Hyalella* exposed to 17α-ethinylestradiol (EE) and control organisms. This indicates that *Hyalella* show a genetic response to vertebrate hormones, indicating that there may be homology between *Hyalella* and vertebrates. A study by Segner et al., (2003) tested the effects of EE, BPA and octyphenol (OP) and found that these compounds had little effect on *Hyalella* at environmentally relevant concentrations during a partial life cycle test, but they found that low-dose concentrations had an effect in full life-cycle experiments, particularly in the second generation (Segner et al., 2003) Whether or not the effects were induced by disturbances in the endocrine system is unknown.

There is discrepancy between different experiments in both *Hyalella azteca*, and a related amphipod, *Gammarus pulex*. Watts et al., (2001) studied the effects of EE and BPA on the survival and reproductive behaviour of *Gammarus* in a series of bioassays. They observed

several aspects of the reproductive behaviour including the ability of males and females to detect each other, form precopulatory guarding pairs and to continue the guarding behaviour, during a 24h exposure period over a wide range of concentrations. They found reproductive behaviour was only disrupted at relatively high concentrations where it would be unrealistic to attribute the effects to an endocrine mediated process. Consequently, changes in precopulatory guarding resulting from acute exposure do not seem to be a suitable endpoint for detecting xenoestroges in the water column. In an additional experiment in 2002, Watts et al., found that when using amphipod *Gammarus pulex*, EE was found to increase female gender ratios after 100 days of exposure. A similar experiment performed a few years later by Schirling et al., (2006) studied the effects of BPA on *Gammarus.* They found that exposure to BPA resulted in accelerated maturation of oocytes in females and in a decline in the number and size of early vitellogenic oocytes. They also found that the level of hsp90, which plays a pivotal role in vertebrate sex steroid signal transduction, was significantly reduced by BPA. A follow-up study by the same group of researchers studied the effects of BPA on *Gammarus* over 103 days n a pulse-dose exposure scenario (weekly BPA application) and on day 103 measured the proportions of juveniles and of breeding females from the highest BPA treatment were in tendency reduced. They also found a concentration dependent decrease in brood size (Ladewig et al., 2008).

Vandenbergh et al*.,* (2003) developed a bioassay testing the effects of EE on sexual development of *Hyalella*. Organisms were exposed in a multigeneration experiment to EE concentrations ranging from 0.1 to 10µg/L and the development of both external and internal sexual characteristics was studied. Second-generation male *Hyalella* exposed from gametogenesis to adulthood to concentrations of EE from 0.1 and 0.32µg /L developed significantly smaller second gnathopods. In addition, they found a slight, but statistically insignificant shift in population in favour of females. They also found histological aberrations of the reproductive tract in post-F1-generation males in all EE exposures. For example, indications of hermaphroditism, disturbed maturation of the germ cells, and disturbed spermatogenesis. These findings provide evidence that sexual development of *Hyalella* is affected by exposure to

sublethal concentrations of EE .This experiment opened up a new avenue to test estrogenic effects on *Hyalella*. However, a follow-up study by Dussault et al*.,* (2008) found no significant effect of EE on secondary gnathopods and argued that what Vandenbergh discovered in 2003 was actually due to chromic toxicity of EE and not mediated by disruption of endocrine pathways. Therefore, further studies are needed to understand the actual affects of EE on *Hyalella*.

This study will follow the methods of Vandenbergh et al*.,* (2003) and expand upon it by looking not only at the endpoints of male gnathopod area, body length and gnathopod to length ratio as well as the ratio of males to females, but in addition, it will look at female brood pouch area, body length and brood pouch to length ratio and the body length of juveniles to determine if there is an effect on growth and gender differentiation via the contaminants. This study will also look at the acute toxicity factors such as number of amplexes observed over time, positioning in the test chamber, grouping, and death in order to gain a combined understanding of the effects of certain compounds on behaviour and reproduction.

Hyalella endocrinology

The endocrine system of *Hyalella* is poorly understood. It is usually assumed that their endocrine systems follow similar patterns to those of other sexual crustaceans, such as shrimp, mysids, crayfish, etc. Although reproduction can be attributed to endocrine effects, it is difficult to identify decreased reproduction as a direct result of endocrine effects rather than increased toxicity to a more sensitive neonate. The ability to distinguish sex using secondary rather than primary sexual characteristics allows the reduction of handling organisms. It is known that sexual differentiation in malacostracan crustaceans including amphipods is regulated by the androgenic gland (AG) (Vandenbergh et al., 2003; Ford et al., 2004). In males, it is known that the primordial AGs develop and synthesize androgenic gland hormone (AGH) which induces male sexual differentiation (Vandenbergh et al., 2003). In females it is known that primordial AGs do not develop and female sexual differentiation is induced spontaneously in the absence of AGH. This has homology to male development in vertebrates with formation of the Wolffian

ducts and respective production of testosterone (Hasegawa et al., 1993; Squires, 2004; Hannema et al., 2006). Köhler et al., (2007) argue that *Hyalella* possess specific binding sites for androgens, but do not appear to have any for estrogens (Lutz et al., 2006). This suggests the existence of an androgen receptor similar to vertebrates, and that estrogens may not play a significant physiological role in *Hyalella*. However, this has yet to be determined.
Secondary gnathopods are secondary sexual characteristics of Amphipods and regulation of their size is recognized to be under AG control (Vandenbergh et al., 2003; Ford et al., 2004). In males, these gnathopods grow proportionally larger and some researchers such as Vandenbergh et al., (2003) and Dussault et al., (2008), have used male secondary gnathopod growth as an endocrine endpoint. Although the mechanism is poorly understood, the growth of the secondary gnathopod in male *Hyalella* is believed to be under hormonal control (Vandenbergh et al., 2003 Dussault et al., 2008).

VTG has not thoroughly been studied in *Hyalella*, but it would be safe to assume that they follow similar patterns of hormone regulation as other invertebrates. Instead of VTG, *Hyalella* produce a homologous protein Vitellin (Croisille et al., 1974; Charniaux-cotton, 1985; Volz et al., 2002). Hormonal regulation of brood pouch development in *Hyalella* has not been thoroughly studied. It is possible that brood pouch development is under control of vitellin.

1.5 CHEMICALS OF INTEREST

Interestingly enough, reports of the effects of endocrine disrupting compounds (EDCs) on aquatic invertebrates are becoming increasingly common, despite the little that is known about the endocrine systems of most aquatic invertebrates (Preston, 2000). Endocrine disruption is often inferred through effects caused by the chemical of interest, which can often be caused by toxic factors; therefore, the effects should be observed in processes that are known to be under endocrine control, at life stages where the organism is responsive to the compounds being studied, and of course at concentrations below the acute and chronic effects (Preston, 2000).

The experimental design must incorporate these factors in a way to efficiently isolate the cause of the problem, rather than the symptoms to make the research truly worthwhile.

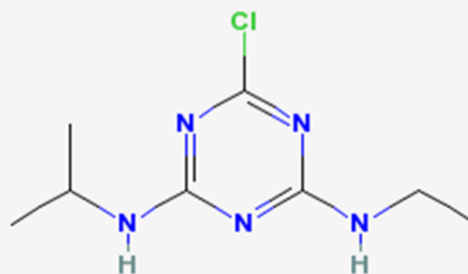

Figure 5: Chemical structure of atrazine (NCBI, 2011a).

Most research has focused on estrogen-inducing or mimicking compounds, such as atrazine (Dodson et al., 1999), or synthetic estradiols (Vandenbergh et al., 2003; Dussault et al., 2008). Others have focused on the androgenic effects of pulp mill waste (Ellis et al., 2003; Bandelj et al., 2006), which contains potential cocktails of EDCs. Androgenic effects are often a partial result of microbial degradation of sterols to progesterone and androgens, while anti-androgenic effects are often directly due to anthropogenic chemicals (Barbosa et al., 2008). The effluents of aquaculture, dairy, waste-water facilities can all be sources of EDCs in the environment.

The following discussion will elaborate on the compounds of interest to this study, elucidate any known endocrine effects and detail the organisms used to determine that information.

1.51 Atrazine

Atrazine is one of the most common herbicides used worldwide, the most heavily used pesticide in North America, and the second-most common herbicide used in Ontario; used extensively on corn (OMAFRA, 2004). As a result, it is found in many aquatic systems ranging from agricultural streams to remote lakes.

Physical properties

More specifically, Atrazine is also known as 2-chloro-4-ethylamino-6-isopropylamino-s-triazine. It is a chloro-N-diakyl substituted triazine compound with a chemical formula $C_8H_{14}ClN_5$ (See fig.

5) (Health Canada, 1993; Detenbeck et al*.,* 1996). It has a molecular weight of 215.7, a melting range of 175-177 degrees Celsius and a boiling point of 279 degrees Celsius (USEPA, 2003; Health Canada, 1993). Atrazine has a low water solubility of approximately 33 mg/L at 25 degrees Celsius, an octanol-water partition co-efficient of 2.82 and a hydrolysis half-life of over 1000 days (USEPA, 2003). Its chemical properties make it a persistent and toxic contaminant in aquatic environments, as it is able to associate and partition to sediments.

Atrazine catabolism

The breakdown of atrazine in the water column is accomplished through a combination of biological and chemical mechanisms (Winkelman and Klaine, 1991; Graymore et al*.,* 2001; USEPA, 2003). Bacteria, especially *Pseudomonas*, and some fungi in the water column initiate the degradation by splitting the ethyl groups from the triazine ring to use as an energy source (Winkelman and Klaine, 1991; Wackett et al., 2002). The compound is degraded by one pathway into metabolites: deethylatrazine, deisopropylatrazine and dealkylatrazine (Winkelman and Klaine, 1991; Wackett et al., 2002). In the other pathway it is degraded into Hydroxyatrazine then N-Isopropylammelide and finally into Cyanuric Acid (Wackett et al., 2002). Atrazine can also be broken down through photochemical processes; however, this mechanism is of lesser concern as it takes a much longer period of time to occur than biological breakdown (Graymore et al*.,*2001).

Atrazine use

Atrazine functions as an herbicide by inhibiting photosystem II of the of plant chloroplast by binding to the quinine-binding protein (Wackett et al., 2002), preventing the energy transfer required for photosynthesis (DeNoyelles et al*.,* 1982; Anderson and Lydy, 2002). Since its introduction during the 1950s, it has been applied to fruit orchards, citrus groves, vines, sugar cane, vegetable and grain fields to control weeds as a pre- and post-emergence control agent (Detenbeck et al*.,* 1996; Anderson and Zhu, 2004). Since then, it has been used extensively with 70 to 90 thousand tonnes applied to croplands each year (Graymore et al*.,* 2001). Over 20

thousand tonnes are sold in Canada, with approximately 70 percent used in Ontario (Health Canada, 1993); used mostly to spray corn and rapeseed fields.

Atrazine in the environment

After application, atrazine enters aquatic ecosystems via leaching and run-off from rain or irrigation (DeNoyelles et al*.,* 1982; Waring and Moore, 2004). It can adsorb to soil particles which then erode into running water. Despite having a relatively low volatility it can drift via atmospheric transport into nearby water immediately following spraying (Health Canada, 1993). It is also persistent with a half-life of approximately 12 weeks in acidic water conditions, and up to 2 years or more in neutral or basic waters (Health Canada, 1993; Detenbeck et al*.,* 1996). It is for these reasons that it is the most frequently detected pesticide in surface and well water in Canada and the United States (Health Canada, 1993). Well and surface water contamination by atrazine has been reported in British Columbia, Nova Scotia, Quebec, Saskatchewan, Ontario, and Prince Edward Island; with higher concentrations reported in spring due to increased run-off from fields (Health Canada, 1993). Health Canada has set the maximum acceptable concentration in drinking water at 5 µg/L, representing a sum total of atrazine and its metabolites (Health Canada, 1993), whereas the USEPA recommends that concentrations in drinking water not exceed 3 µg/L (USEPA, 2003). The Canadian Drinking Water Quality Guidelines stipulate that concentrations of Atrazine not be over 2 µg/L for the protection of aquatic life (CDWQG, 2008). However, studies have shown that concentrations in both drinking water and surface water frequently exceed recommended values. Concentrations in drinking water have been reported up to 81 µg/L in Canada, while surface water concentrations can reach as high as 108 µg/L in the United States following spring application (Graymore et al*.,* 2001; USEPA, 2003). Concentrations as much as 1000 µg/L have been recorded in streams and rivers next to fields where atrazine has been applied (DeNoyelles et al*.,* 1982). Many coastal and estuarine areas have reported detectable levels of atrazine with concentrations often lower than freshwater bodies located near farm areas due to dilution (Graymore et al*.,* 2001). Concentrations of atrazine in freshwater can vary depending on season, with spring and summer months showing increased levels from applications during the

growing season and high runoff during summer storms (Graymore et al., 2001; Anderson and Lydy, 2002; USEPA, 2003). Ambient concentrations in many lakes and rivers can vary between 1 and 10 µg/L, depending on the time of year and the size of the water body (USEPA, 2003). Aquatic environments are thus chronically exposed to low levels of Atrazine, as well as short, acute pulses with high concentrations of the pesticide (Detenbeck et al.,1996; USEPA, 2003).

Atrazine concerns

Aquatic populations first affected by atrazine are often algae and aquatic macrophytes (Graymore et al., 2001; USEPA, 2003). Reduction in algal biomass have been seen with exposure to concentrations as low as 20 µg/L (Graymore et al., 2001). A decrease in photosynthesis in phytoplankton and periphyton communities have been seen In concentrations less than 10 µg/L (Graymore et al., 2001). At concentrations over 500 µg/L, photosynthesis, carbon uptake and biomass are reduced by 95 percent in under two days (Graymore et al.,2001). These results are alarming as concentrations nearing these are often found in aquatic systems located near fields sprayed with atrazine. Decreases in photosynthesis in these areas can affect the entire food web, as animals within the aquatic system rely on primary producers in their diet (DeNoyelles et al., 1982).

Humans are most likely to be exposed to atrazine through consumption of contaminated drinking water (Health Canada, 1993). Contact through air is unlikely, except during or immediately after application due to the low volatility of the compound (Health Canada, 1993). Contact through food is also unlikely as food products sprayed with the chemical tend to have low or non-existent residues (IARC, 1999). When it enters the body through drinking water consumption, 93-100 percent of atrazine is absorbed across the gastro-intestinal system and taken into cells where it is broken down into metabolites by P450 (Health Canada, 1993), which is also the enzyme responsible for aromatizing testosterone into estradiol (Squires, 2004). Exposure via drinking water has been linked to a number of health issues in humans. Acute problems include nausea and dizziness (Health Canada, 1993); however chronic exposure to low concentrations of atrazine can lead to severe problems. Atrazine has been demonstrated to

act on the pituitary-gonadal system which is responsible for the regulation of several hormones (Health Canada, 1993). Exposure has been shown to increase levels of FSH and LH and interfere with the metabolism of testosterone (Health Canada, 1993).

Atrazine is possibly carcinogenic

There have been no conclusive findings regarding atrazine as a carcinogen and it is therefore classified as a Group 3 Carcinogen by Health Canada, as possibly carcinogenic to humans (Health Canada, 1993). Despite this, an increased risk of ovarian, uterine and breast malignancies, as well as non-Hodgkin's lymphoma have been associated with chronic atrazine exposure in workplace and rural settings (Donna et al*.,* 1984; Hoar et al*.,* 1988; Health Canada, 1993). The International Agency for Research on Cancer (IARC) also classifies atrazine as a possible human carcinogen (IARC, 1999). Both Health Canada and the World Health Organization (WHO) recommend that human intake not exceed 0.5 mg atrazine per kilogram body weight per day in order to reduce the risks associated with atrazine intake (Health Canada, 1993). Many studies have also looked at the health effects of atrazine on aquatic organisms and larger land mammals. Several studies have been conducted using rats as models for the effects of atrazine in humans. In rat studies, dose-related increases in mammary gland and lymph system tumours were reported (IARC, 1999), as well as increased embryonic and foetal deaths, decreased foetal weights, and retarded skeletal development in young following two years of chronic low level exposure to 20-40 mg/kg atrazine in food (Health Canada, 1993). In an environmental context, this is an extremely high level of exposure, unlikely to ever be seen at an equivalent level in an aquatic setting.

Atrazine bioassays

Studies of uptake in aquatic organisms exposed to the contaminant have also been performed. When exposed to atrazine concentrations of 230 µg/L and higher, the aquatic insect *Chironomus tentans* had reduced hatching success, abnormal larvae development, and a reduction in the number of organisms which reached the pupae life stage (Dewey, 1986). Fish and larval tadpoles are also common test species used during atrazine exposures. After

exposure to 120 µg/L atrazine, brook trout experienced a significant reduction in growth rate while both zebrafish and rainbow trout experienced changes in swimming behaviour and motility when exposed to concentrations of 6 and 80 µg/L respectively (Dewey, 1986; Steinberg et al.*,*1995). *Rana catesbiana* tadpoles exposed to 20 µg/L of atrazine for 80 days showed a significant decrease in biomass compared to controls, and have an LC_{50} of 410 µg/L (Detenbeck et al.,1996).

Several bioassays have been conducted in the past examining the acute effects of atrazine on the survival of *Daphnia magna, Hyalella azteca,* and *Lumbriculus variegatus*. LC_{50} values for various periods of time have been reported for all three organisms. The USEPA (2003) reports that *Daphnia magna* have a 48-hour LC_{50} value of 49 mg/L atrazine, that *Hyalella azteca* have 48-hour LC_{50} value of 14.7 mg/L and *Lumbriculus variegatus* have a 48-hour LC_{50} value of 37.1 mg/L. An 18-hour LC_{50} for *Hyalella* has also been determined to be 2 mg/L (USEPA, 2003) and a 72-hour LC_{50} for *Daphnia* has been reported to be 72 mg/L (Wan et al.*,* 2006).

Atrazine endocrine disruption

Abnormal gonadal development such as feminization, hermaphroditism, and reduced laryngeal muscle size in *Xenopus laevis* tadpoles have been reported following prolonged exposure to concentrations as low as 1 µg/L (USEPA, 2003). This is likely connected to endocrine disruption caused by the compound (USEPA, 2003). Hayes, et al., (2002) found that atrazine causes male frogs to develop ovaries, indicating either aromatase activation, estrogen-mimicking, testosterone inhibition, or all of the above. Friedmann (2002) found that atrazine inhibits testosterone production in rat males following peripubertal exposure to atrazine. He found that atrazine reduced serum and intratesticular levels of testosterone by 50 percent *in vivo*, and reduced testosterone production in leydig cells *in vitro*. Keller and McClellan-Green (2004) found that atrazine induces aromatase activity in an immortal green sea turtle cell line (GST-TS). So not only does atrazine reduce the initial amount of testosterone being produced, but it also helps to convert whatever remains into estradiol, making it a very dangerous chemical. The

increased risk of reproductive tumours mentioned above may be linked to the effect atrazine has on hormone regulatory systems through its interaction with the P450 enzyme.

Several researchers have found that Atrazine causes the production of male *Daphnia* (Macek et al., 1976; Dodson et al., 1999; Stoeckel et al., 2008), which are essentially a dead-end to the Daphnid population, as they cannot self-propagate like the females. Although it was observed that atrazine caused an increase in male production, there was no apparent observation regarding the production of ephippial females. This leads to the conclusion that production of males and production of ephippia within females are mutually exclusive. Whether or not this response to atrazine was under endocrine control is unknown, as daphnids are quite sensitive to stress, and it is possible that atrazine exhibited stress on the *Daphnia*. This however, does not weaken the argument against atrazine being an EDC as there is evidence that it has a direct effect on endocrine tissues. Other reproductive bioassays have also been conducted using *Daphnia magna*. At concentrations of 250 µg/L and higher, a significant reduction in the number of offspring produced was observed (Dewey, 1986). Regardless, there is room for scientific advancement regarding the effect of chronic exposures of Atrazine on *Daphnia magna* and *Hyalella azteca*.

Summary

It is the goal of this experiment to provide a controlled situation to determine whether male production by atrazine is a function of stress, or a function of endocrine disruption. In this experiment, environmentally relevant concentrations of 100, 50 and 5 µg Atrazine/L were used. These are also conservative concentrations, as levels as high as 1000 µg/L have been reported near areas of application (Denoyelles et al., 1982). That is however, an extreme case. Typically, concentrations in drinking water have been reported up to 81 µg/L in Canada and have been known to reach as high as 108 µg/L in the United States following spring application (Graymore et al., 2001; USEPA, 2003). The lowest concentration, 5 µg/L, represents the maximum recommended concentration according to Canada's drinking water guidelines. Therefore the concentrations used in this study are both environmentally relevant and conservative.

1.52 Tributyltin

Tributyltin is arguably the most toxic chemical ever deliberately introduced into the aquatic environment (Chau et al., 1997). Tributyltin (TBT) (Fig. 6) is a tri-substituted organotin that is highly persistent in the aquatic environment and toxic at the nanogram per litre level to many organisms (Alzieu, 1998; Horry et al., 2004). Tributyltin is hydrophobic with an octonol-water partitioning co-efficient ranging from 3.21 to 3.85 depending on the species of the compound and temperature and pH of the water (Alzieu, 1998). The compound comes in many forms including oxides, chlorides, fluorides, and acetates, each of which are slightly soluble in both freshwater and seawater (Alzieu, 1998). Solubility values for tributyltin oxide range from 1-10 mg/L depending on the composition of the water, while the solubilities for other types of the compound are under 20 mg/L (Alzieu, 1998).

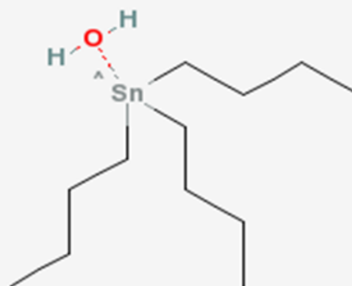

Figure 6: Chemical structure of TBT hydroxide (NCBI, 2011b)

Sources of Tributyltin

The major source of TBT in the aquatic environment is through leaching from anti-fouling paints on boats and cargo ships, which been in use since the 1960s (Fent and Looser, 1995; Borgmann et al., 1996; Chau et al., 1997; Alzieu, 1998; Konstantinou and Albanis, 2004). This represents over 70 percent of the usage of TBT (Alzieu, 1998). TBT is applied as a paint additive to the submerged section of boats and other floating structures such as oil rigs, buoys, and fish cages (Alzieu, 1998; Konstantinou and Albanis, 2004). The compound acts as a biocide which prevents the growth of living organisms on surfaces that may cause corrosion, or accumulate thereby slowing ships down, increasing the weight of floating structures and increasing fuel usage (Alzieu, 1998). Ship paints often include solvents which aid in application and make TBT compounds more soluble in water (Alzieu, 1998). It is estimated that the daily leaching rate of TBT is between 1 and 10 µg/L TBT per cm^2 of application area (Alzieu, 1998). This results in a

release of TBT of 0.2 to 2 grams of TBT per day from a small sailboat and between 50 and 500 grams of TBT per day from a large commercial vessel (Alzieu, 1998). Concentrations of TBT are highest in areas of high shipping and boating traffic, but the compound has also been detected in freshwater bodies remote from harbours and shipping areas (Borgmann et al., 1996).

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

Regulation of TBT

Many countries began to restrict its use due to toxic effects observed in several aquatic species (Alzieu and Heral, 1984; Alzieu et al., 1986; Fent, 1996; Chau et al., 1997). In 1982, France was the first country to restrict its use on boats less than 25 metres in length (Chau et al., 1997; Alzieu, 1998). Bans in the United Kingdom (1987), the United States (1988), Australia (1989), the Netherlands, Hong Kong and Japan (1992) soon followed (Fent and Looser, 1995; Chau et al., 1997). Canada first introduced restrictions in 1989 under the Pest Control Products Act which restricted the use of TBT as an anti-fouling agent on boats under 25 m in length (Borgmann et al.,1996; Chau et al., 1997; Alzieu, 1998). In 2003, the ban was extended to include vessels of any length (Coray and Bard, 2007). However, TBT is still found in measureable quantities in many ecosystems causing concern about the impacts it may have on aquatic life (Borgmann et al., 1996; Konstantinou and Albanis, 2004). Pulse introductions of TBT into the environment are also of concern as large ships move through waterways (Fent, 1996). Worldwide chronic concentrations exceeding 100 ng/L have been found in both fresh and marine environments following the ban (Fent and Looser, 1995). Prior to the TBT ban in North

America concentrations in freshwater averaged between 50 and 500 ng/L, with a highest recorded chronic value of 1 µg/L in several heavily-travelled harbours (Alzieu, 1998).

In 2001, the European Council included TBT as a priority substance in its policy on water quality and all countries within the European Union (EU) were required to restrict the use of the substance (Alzieu, 1998; Horry et al*.,* 2004). An international ban on the use of TBT was introduced in 2003 by the International Maritime Organization (IMO) and the Marine Environment Protection Committee; however, many countries which export goods are not signatories to the convention and large boats still may use it (Konstantinou and Albanis, 2004). Environment Canada has established interim water quality guidelines of 3.3 ng tin/L of water in order to protect aquatic organisms (Chau et al*.,* 1997). This value was derived by determining the lowest reported chronic exposure effect in literature and applying a safety factor of 10 (Chau et al*.,* 1997). Health Canada has no drinking water quality guidelines for either TBT or tin in general (Canadian Drinking Water Quality Guidelines, 2008).

Environmental Fate of TBT

TBT is very persistent in the aquatic environment and its chemistry and fate in the environment are not completely understood (Fent and Looser, 1995; Fent, 1996; Alzieu, 1998; Horry et al*.,* 2004). The compound is present in all components of the aquatic environment, including water, sediments, bound particles, and in living organisms (Alzieu, 1998). In the water column, the half-life of the compound will vary depending on temperature, pH, turbidity and light conditions, but generally ranges from a few days to several weeks (Fent, 1996; Alzieu, 1998). TBT degrades much more slowly in sediment, with a half-life of several years (Borgmann et al*.,*1996; Alzieu, 1998). TBT can be broken down by pH-dependent hydrolysis, UV photolysis and by the action of some micro-organisms by breaking the bond between the tin and carbon molecules to form the less toxic metabolites dibutyltin (DBT) and monobutyltin (MBT) (Fent and Looser, 1995; Alzieu, 1998).

TBT in sediments can be resuspended in the water column and thus pose a continual threat to water quality (Fent and Looser, 1995; Fent, 1996; Chau et al.,1997; Alzieu, 1998). Approximately 5 percent of TBT introduced to the aquatic ecosystem is found adsorbed to suspended particles within the water column, available to filter-feeding organisms (Alzieu, 1998).

Presence in the environment

Concentrations in industrial effluent have been recorded as high as 61.8 μg/L in Germany, indicating that industrial emissions may be a significant source of pulses of TBT into the environment (Schmidt et al., 2005). In 1994, a study following the TBT ban in Canada found that several freshwater areas still had concentrations of TBT exceeding the interim limit to prevent damage to aquatic life (Chau et al., 1997). In this study, 12 of 89 tested sites had detectable levels of TBT in freshwater, with concentrations up to 17.8 ng/L (Chau et al., 1997). Of these 12 sites, 9 had concentrations which exceeded the guidelines to protect freshwater aquatic life (Chau et al., 1997). Within the sediment, 42 of 89 samples had detectable levels of TBT, with the maximum recorded concentration being 975 ng tin/g sediment (Chau et al., 1997). Despite this, In Canada, TBT, DBT and MBT are found in freshwater much less frequently, in lower concentrations and with lower mean ranges than before the restrictions were set in the 1980s (Chau et al.,1997). Reductions in concentrations of TBT in freshwater systems and the subsequent recovery of organisms affected by TBT have been observed since the bans were enacted in Canada and throughout the world (Chau et al., 1997). Although concentrations have generally declined since the ban, its presence is still seen in heavily-travelled shipping areas exposed to large ships not subject to the restrictions, or in areas where boats are present which may have been painted prior to 1989 (Fent, 1996; Chau et al., 1997). In some areas, TBT concentrations still exceed the Environment Canada interim water quality guidelines for the protection of aquatic life (Chau et al., 1997; Coray and Bard, 2007). Because of the persistence of the contaminant, the introduction of more TBT from boats not restricted by international bans and because of its potential to be resuspended in the water column, TBT still remains an important environmental contaminant.

TBT Toxicity

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

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

TBT as an endocrine disruptor

Several experiments have been conducted looking at TBT as an endocrine disrupting compound. In the dog whelk (*N. lapillus*), it was found that TBT caused imposex in females, thereby causing death or infertility. In several cases, it was not uncommon for males to develop egg sacs as well (Bryan et al.,1986; Minchin et al., 1996). Santos et al., (2005) found that TBT induces an elevation of free-testosterone, while free-estradiol biosynthesis in TBT-exposed females does not seem to be affected. A selective aromatase inhibitor can induce imposex in *N. lapillus* but not to a similar extent of TBT, which may suggest the involvement of other mechanism in imposex induction, besides aromatase inhibition. Additionally, the study points to the involvement of the angrogen receptor (AR) in imposex induction, so the mechanism of action of TBT is still poorly understood.

TBT is an example of an environmental endocrine disruptor that promotes adverse effects from snails, to mammals through common signalling (Iguchi and Katsu, 2008). Yamabe et al., (2000) found that trialkyltin compounds have an ability to activate AR-mediated transcription in mammalian cells. They suggest that a novel target site other than the ligand-binding site of AR is involved in this activation (Yamabe et al., 2000). TBT also induces adipogenesis in *X. Laevis* and in mice, possibly by endocrine disruption processes (Iguchi and Katsu, 2008). Other organisms appear to be less sensitive to the presence of TBT in their environment, but still show a reaction in low concentrations of the compound. Concentrations between 1 and 10 µg/L affect the reproduction of most species of fish studied during chronic assays, whereas concentrations between 1-1000 µg/L affect the swimming behaviour of several species of fish during acute assays (Alzieu, 1998). Several species of crustaceans have also demonstrated reduced reproduction, as well as reduced neonate and juvenile growth rates when exposed to sub-lethal concentrations of TBT (Schmidt et al*.,* 2005). LC_{50} values for organisms are very low as well, with 10-day LC_{50} values in amphipods ranging from 1.5-32 µg/L depending on species and 4-day LC_{50} values for rainbow trout and lake trout of 1.4 and 5.2 µg/L respectively (Borgmann et al*.,* 1998).

TBT Bioassays

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

Bioassays examining the changes in swimming behaviour of *Daphnia magna* in response to TBT have also been performed. Schmidt et al., (2005) monitored changes in swimming speed, depth and secondary antennae use after 21 days of exposure to 6.6 µg/L of TBT in adult organisms. A significant decrease in mean velocity was noted after 19 days of exposure and a significant decrease in swimming depth and antennae movement was observed after 10 days of exposure (Schmidt et al*.,* 2005). Mortality bioassays for *Daphnia magna* have determined a range of 48-hour LC_{50} values based on the species of TBT examined and are generally between 2.3 and 70 µg/L (Schmidt et al*.,* 2005). A 21-day LC_{50} value of 2.5 µg/L was also found for organisms used in this experiment (Schmidt et al*.,* 2005). NOEC values for mortality were determined to be 1.2 µg/L and 5.5 µg/L after 96 and 24 hours respectively (Schmidt et al*.,* 1995). During the course of the 21-day experiment, a 35 percent decrease in reproduction was found with an NOEC concentration of 0.16 µg/L (Schmidt et al*.,*2005), implying either toxicity to neonates, or reproductive effects of TBT.

Borgmann et al*.,* (1996) conducted 1 week and 4 week exposure bioassays to determine LC_{50} values and to examine the relationship between body size and accumulation of TBT within the tissues of *Hyalella*. TBT concentrations of 0.56, 1.0, 1.8, 3.2, 5.6 and 10 µg/L TBT were all shown to cause bioaccumulation in the organisms and the concentrations in tissues increased rapidly

in the first 3-4 days of exposure before reaching a peak concentration after 1 week (Borgmann et al*.,* 1996). Rapid equilibration with TBT concentrations in water occurred at all levels and final concentrations in tissue did not vary among treatments (Borgmann et al*.,* 1996). Accumulation was not dependent on body size, indicating adult or juvenile organisms were suitable for bioassays (Borgmann et al*.,* 1996). A one-week LC_{50} of 2.3 µg/L and a 4-week LC_{50} value of 0.58 µg/L was found for *Hyalella* (Borgmann et al*.,* 1996).

Summary

Despite the wealth of knowledge of acute responses of *Daphnia* and *Hyalella* to TBT, there is little known about the long-term reproductive effects of the compound at chronic low exposure. For this experiment, environmentally relevant concentrations of 10, 1 and 0.1 µg of TBT/L will be used with 0.1% Dimethylsulfoxide as a carrier compound. Initial concentrations for the bioassays in this experiment were set at 100, 50 and 10 µg/L, but it was found that 100 and 50 µg/L were so acutely toxic, that 1 and 0.1 µg /L concentrations were used instead to reach the full test duration of 42 days for *Hyalella azteca*.

1.53 17α-Ethinylestradiol

17α-Ethinylestradiol (EE) (Fig. 7) is an orally bio-active synthetic human estradiol commonly used as a method of birth control in oral contraceptive pills (Sneader, 2000). It is also released into the environment as a xenoestrogen from the metabolic waste of individuals that take it (Hannah et al., 2009). So with respect to this study, it is both a control as an estrogenic compound, and an environmentally relevant contaminant. Depending on the water body, it can be reported in concentrations below detection limits of 0.01 ng/L, or as high as 273 ng/L (Hannah et al., 2009). Studies have shown

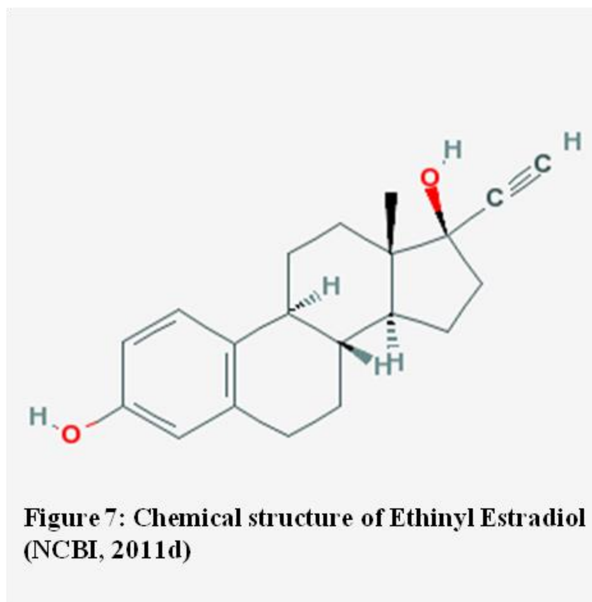

Figure 7: Chemical structure of Ethinyl Estradiol (NCBI, 2011d)

that synthetic estradiol designed for human use in birth control, has had an effect on fish populations downstream of wastewater facilities when runoff has entered local water bodies (Purdom et al., 1994; Jobling et al., 1998; Lange et al., 2001; Nash et al., 2004).

Lange et al., (2001) studied the effect of EE on the fathead minnow and found that males exposed to concentrations of 4.0 ng/L or greater failed to develop secondary sexual characteristics. Testicular tissue also failed to develop and vitellogenin levels were significantly higher in all fish studied at that concentration. However, this phenomenon caused by EE is not necessarily permanent, assuming fish can be removed from contaminated water, or that contaminated water can be cleaned. Larsen et al., (2009) studied the effect of EE on the development of male zebrafish (*Danio rerio*). They studied courtship behavior following estrogenic disruption of sexual differentiation. They exposed sixty zebrafish at 28°C to 5 ng/L (nominal concentration) of 17α- ethinylestradiol (EE_2) from the egg stage until adulthood at four months of age, resulting in a female-biased sex ratio. Afterwards, removed 25 EE-exposed phenotypic female zebrafish and held them in clean water for eight months. During this period, eight phenotypic males developed. These phenotypic males demonstrated significant behavioral aberrations and a low fertilization rate compared to control males. Therefore, although not entirely permanent, EE has a significant effect on Zebrafish, and likely other fish as well.

Despite all of the research of synthetic human estradiols on fish, the effect of these compounds on invertebrates have been studied to a much lesser extent. Knowledge of the endocrine system of invertebrates is limited. The presence of both vertebrate-type and invertebrate-specific hormones in invertebrates has been demonstrated (Lafont, 2000). Vertebrate-type steroid hormones have been found in several crustacean species , but the physiological role and metabolism are not fully understood (Fairs et al., 1989; Jeng et al., 1978; Novak et al., 1990). Vandenbergh et al., (2002) have studied the effect of 17α-ethinylestradiol (EE) on the sexual development of the amphipod *Hyalella azteca*, and found that it has an effect on gender ratios, favouring females; also affecting the size of male secondary gnathopods. However, a follow-up

study by Dussault et al., (2008) has found no effect due to EE on *Hyalella*. With only two major publications regarding the effect of EE on Hyalella, there is much room for advancement in the field of knowledge, especially since both studies are contradicting.

Dietrich et al., (2010) studied the effect of EE on *Daphnia magna* as part of a chemical mixture and alone at environmentally relevant concentrations on life-history and morphological parameters on six generations of *Daphnia magna* and found that *Daphnia* had reduced brood sizes when exposed to ethinylestradiol.

Brennan et al., (2006) studied the multigenerational effects of estrogens on *Daphnia* and found no significant effect. Despite being an obvious endocrine compound in vertebrates, there is much conflicting data regarding the effect of EE on invertebrates, especially *Daphnia* and *Hyalella*.

Kashian and Dodson, (2004) found that estrogenic hormones and mimics do not appear to control sexual differentiation in *Daphnia*, at least under the conditions of a 6 day assay. Therefore, a long-term assay studying the effect of EE on *Daphnia* would be useful in understanding more about the endocrine system of Daphnia.

In this experiment a concentration of 0.1 µg/L was used, as it was the lowest concentration that Vandenbergh et al. (2002) had success with. For the experiment with *Hyalella azteca*, EE will serve as a control. However, with the *Daphnia magna* experiment, EE was to serve as a test compound.

1.54 Dimethyl Sulfoxide

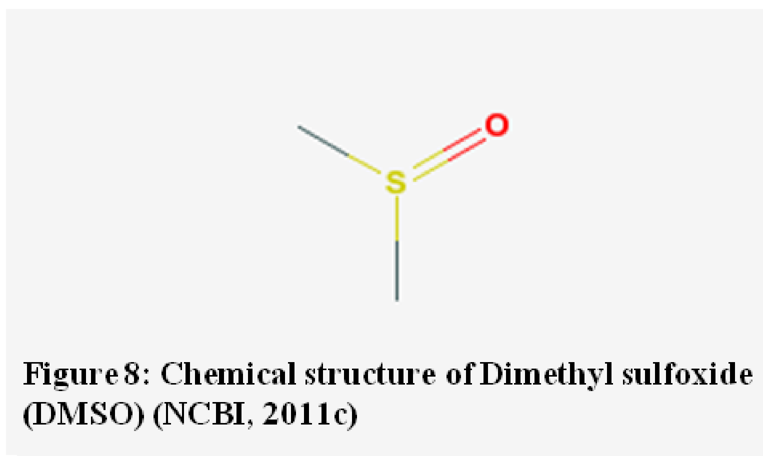

Figure 8: Chemical structure of Dimethyl sulfoxide (DMSO) (NCBI, 2011c)

Toxicity bioassays involving water-antisoluble chemicals hinge geavily on the use of carrier solvents as delivery systems (Hallare et al., 2006). Dimethysulfoxide (DMSO) (Fig. 8) is a commonly used organic solvent in toxicity testing. DMSO was used in the following study to make stock solutions of Atrazine, TBT and EE, which have low solubilities in water. DMSO is an amphiphilic carrier compound which helps to solubilise the organic contaminants and distribute them evenly throughout the water column, rather than having them settle in the sediment and partition to the glass in the assay vessels (House and Ou, 1992; Hallare et al., 2006). There is some concern in using carrier solvents, as they may have toxic effects of their own toward organisms, and their use may influence the results of toxicity tests. However, at low concentrations on a per volume basis, DMSO is less toxic than other commonly used solvents such as methanol, ethanol and acetone (Hallare et al., 2006). In this study, an overall concentration of 0.1 percent v/v DMSO was used for all bioassays. This concentration has been shown to be a safe working concentration that has no impact on behaviour or mortality in test organisms (Hallare et al., 2006; Martins et al*.,* 2007; Ren et al*.,* 2008; Ren et al*.,* 2009).

1.6 PROBLEMS STUDYING ENDOCRINE DISRUPTION

According to Hutchinson (2002), the toxicity of EDCs to crustaceans such as copepods is in most cases due to other undefined modes of actions rather than endocrine disruption. Thus, it is important to develop bioassay designs which can be used to differentiate between endocrine disruption and other causes of reproductive and developmental impairment.

Confounding factors, such as stress can have an effect in a chronic toxicity assay. It was noted by Stoeckel et al., (2008), that when atrazine was added to a particular field for crops, which entered the runoff in the spring, it was noted that *Daphnia* downstream of the runoff produced males. Dodson et al., (1999) states that low exposure concentrations *in vitro* (5 µg/L to 500 µg/L of atrazine significantly increases male production in *Daphnia pulicaria*. However, it is likely that it is not Atrazine alone causing these particular effects in these experiments. *Daphnia* happen to enter a 'survival mode', where they produce ephippia and males when stressed (Tatarazako & Oda, 2007). Unfortunately, It cannot be determined whether atrazine itself causes the physiological change from female to male production, unless the proteomic effect of atrazine was studied as well, which were not included in these studies. It could be that doses of atrazine causes the formation of males in daphnia by means other than endocrinological. They are exceptionally sensitive to stress, and any stress, especially from ephemeral ponds may cause the formation of males.

The majority of studies of endocrine disruption in wildlife have focused on mechanisms by which chemicals interact with endogenous endocrine receptors (Ankley et al., 1998), although alternative mechanisms of endocrine disruption have been described (Parks and Leblanc, 1996). Unfortunately, little is known about the endocrine systems of many aquatic invertebrates, making it difficult to demonstrate receptor-mediated mechanisms of toxicity. As a result, inference of endocrine disruption in aquatic invertebrates is often based on the effects on reproductive or developmental endpoints such as fertility, growth and differentiation, or sex determination. When relying on such effects, it may be difficult to distinguish endocrine disruption from other mechanisms of toxic action such as narcosis, immune- or genotoxicity, enzyme inhibition, and disruption of membranes or metabolism.

Several criteria should be met to ensure that observed effects are consistent with an endocrine disruption mechanism. First, the observed effects should occur in processes that are known, or at least suspected, to be under endocrine control. Second, exposure should impact life stages where endocrine signals are known to be active (e.g., sexual development and reproduction).

Third, the effective concentration at which endocrine disruption is observed should be below that of known acute and chronic toxicity. Last, if molecular assays are available, the compound should bind to endogenous endocrine receptors at a concentration sufficient to account for the observed effects.

Endocrine toxicity is difficult to study, especially when a disease requires time to manifest, such during pregnancy. Such infinitesimally small quantities of a toxin at particular life stages can have an astronomical effect. There are also multiple sources of endocrine disrupting compounds, and multiple types, to add more confusion. It may also take weeks to years to see any effect of the initial treatment, which can leave room for confounding problems. At this point it becomes increasingly difficult to determine the exact source of the disease, and toxicity testing becomes even more confusing and frustrating. For this reason, it is important to determine endpoints rapidly, to reduce as many confounding factors as possible. Also, because toxicity tests that include the entire life cycle of an organism are also difficult and costly to be applied. Several short-term tests would be ideal to estimate, in order to measure chronic toxicity, representing speed, sensitivity and cost. The benefit to such tests would be that if a product was tested as it was being released to the environment, or even the public, and a problem was found with the chemical, its release could be limited or aborted with minimal exposure.

An example of the complications involved in whole organism endocrine disruption testing; a genetically male human fetus takes at least 52 days to start producing Wolffian ducts (Hannema, 2006). Before this time, if the mother is to consume quantities of either: estrogen; an estrogen-mimicking compound, such as Bisphenol A; an androgen-inhibiting compound, such as Atrazine; phytoestrogens for soy and other legumes, or any combination of the above; there is a very real possibility that these compounds can inhibit the formation of the Wolffian ducts, and produce a phenotypically female baby (Turner et al., 2003; Bowman et al., 2005), as the Wolffian duct formation responds to a hormone concentration gradient within the uterus. Unfortunately, there is no way to detect the gender of the fetus until at least day 72, or for

some parents who refuse to know the gender until birth, potentially up to 9 months after the initial dose. While often considered more accurate, vertebrate whole-organism assays are difficult to perform due to costs, and length of time required for results. Hence the need for understanding more of the invertebrate endocrine system.

In conclusion, it may be in our best interests to begin analysis of some of the compounds we expel into our environment, which we happen to re-ingest. The induction of male offspring in *D. magna* has proven to be a highly specific endpoint for the detection of juvenile hormone like activity of chemicals, which are as such endocrine disruptors for arthropods. However, for the detection of (anti-)ecdysteroid effects of chemicals, *Daphnia* might be less suitable as a test organism compared to other crustaceans, such as mysids or copepods. The endocrine systems of many invertebrate groups are still not fully characterized, possibly even unknown. The detection of an endocrine disruptor is best conducted with more than one species, so that the results may complement each other.

1.7 OBJECTIVES

There is a great deal of understanding left to learn about the endocrinology of invertebrates, especially those that reproduce by cyclic parthenogenesis. There is also a need to validate test methods to determine the reproductive effects on organisms *Daphnia magna* and *Hyalella azteca*.

The objectives of this study are as follows:

- To gain understanding of invertebrate endocrinology and potential endocrine disruption
- To gain understanding of the potential endocrine effects of Tributyltin, Atrazine and Ethinylestradiol
- To perform a chronic toxicity assay with *Daphnia magna* under conditions which are known to induce formation of males and female ephippia .
- To perform a multigenerational reproduction assay with *Daphnia magna* to determine the toxicity and reproductive effects of potential endocrine disruptors Tributyltin and Atrazine
- To perform a chronic toxicity and reproductive assay with *Hyalella azteca* to determine the toxicity and potential reproductive effects of contaminants Atrazine and Tributyltin
- To utilize a novel parameter in chronic assays with *Hyalella*; analysis of the female brood pouch in assessment of potential endocrine effects.

2.0 MATERIALS AND METHODS

There are two major purposes to this study. The first is to gain a deeper understanding of invertebrate endocrinology, as there is still a dark void of information left to uncover. The second purpose of these experiments is to expand upon current protocols in order to account for the occurrence of endocrine disruption in freshwater systems in addition to chronic and acute toxicity, as well, expand upon the experimental endpoints utilized in the current protocols for *Hyalella azteca*.

2.1 WASHING PROCEDURES

Prior to use, all glassware, aquaria, and other reusable pieces of lab equipment were washed thoroughly to ensure that any traces of chemicals from prior use were removed and did not affect test organisms. Washing procedures were based on those described by Environment Canada (1998). All glassware were first washed by soaking in an Extran detergent solution for 15 minutes and scrubbed afterward to remove any residue. The Environment Canada (1998) protocol does not specify or recommend a detergent. Extran was used for this particular experiment as it contains no phosphorous, is biodegradable, leaves no residue and is efficient at removing organic debris. The glassware was then rinsed twice with dechlorinated municipal drinking water (DMDW) and then washed in 10% v/v hydrochloric acid to remove any traces of heavy metals, or any calcium build-up, and then rinsed three times with deionised water. The glassware was then rinsed with acetone three times as a redundant method to remove any potential organic debris that may still have adhered to the glassware. This was a necessary step as unaccounted residue may potentially affect the results. Finally, the glassware was rinsed with deionised water three times before being placed in an inverted position to dry.

2.2 CHEMICAL CONCENTRATIONS AND DILUTIONS

All concentrations detailed below are based upon environmentally relevant concentrations and previous methodologies. The concentration used for EE is based on results from Vandenbergh et al. (2003) and test solutions all had a final concentration of 0.1% DMSO, a value which has been used in past bioassays and is not considered to have an impact on aquatic organisms (Hallare et al*.,* 2006; Martins et al*.,* 2007; Ren et al*.,* 2008; Ren et al*.,* 2009).

All dilutions were made from stock solutions of 100 mg/L tributyltin (TBT) in Dimethylsulfoxide (DMSO), 100 mg/L atrazine in DMSO and 100 mg/L ethinylestradiol in DMSO. In all bioassays, reference and 0.1% DMSO control treatments were performed at the same time as the TBT and atrazine treatments to examine normal behaviour and to ensure that the DMSO was not contributing any toxicity. Concentrations used for initial bioassays were 100, 50 and 10 µg/L TBT; 100, 50 and 5 µg/L Atrazine; 0.1 µg/L 17α-ethinylestradiol and 0.1% DMSO as an amphiphilic carrier control. After some preliminary experiments with *Hyalella azteca*, it was determined that concentrations of TBT at 100 and 50 µg/L were too acutely toxic to be considered for chronic assays, so concentrations were changed to 10, 1 and 0.1 µg/L in order to reach the full test duration of 42 days. See appendix A for dilution charts.

2.3 *DAPHNIA MAGNA*

2.31 *Daphnia magna* culturing

Culturing procedures were developed based on Environment Canada (1998) protocols. Starting cultures were obtained from previous laboratory stock cultures utilized by Marshall (2009). Starting cultures were maintained in two 20L glass aquaria with an overlay of 18L DMDW. The overlying water was continuously aerated with an air pump to maintain oxygen saturation of at least 80%, or 8.5 mg/L O_2. Oxygen concentration was measured by use of an O_2 probe. Water temperature in the aquaria was maintained at room temperature, 18-22 degrees Celsius (post-air conditioner installation – more on this later). Aquaria were kept under a customized light

bank where light intensity was maintained between 600 to 800 lux, or approx. 11 to 15 PAR (µmol photons/m^2/s) and a photoperiod of 16hr light: 8hr dark, which represents ideal summer conditions. Glass sheets were placed above the aquaria with a hole drilled into the glass to allow entrance of an airtube attached to a Pasteur pipette submersed several inches below the water surface to allow airflow, while keeping dust, debris, and potential contamination out of the aquarium. *Daphnia* were fed 3x a week using cultures of *Selenastrum* and fed 20 mL at approx. $1.5x10^6$ cells/mL during each feeding period. Approximately one quarter of the overlying water was changed weekly to prevent build-up of these carbonates as well as nitrites which can cause stress and affect the pH. A 200 L carboy at the same room temperature was aerated continuously for such a task.

2.32 *Daphnia magna* sexual bioassay

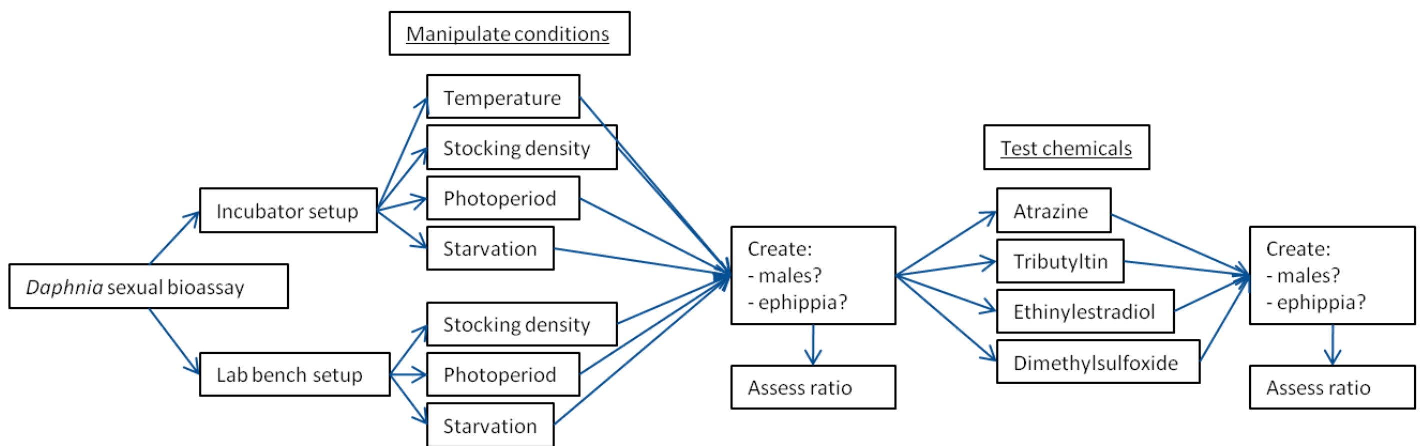

Figure 9: Outline of Daphnia sexual bioassay to determine ratios of male/ephippial female Daphnia between controls and chemical treatments to determine if the chemicals of interest disrupt endocrine pathways.

In order to maximize the output of males and ephippial females from the sexual phase of the *Daphnia* parthenogenetic cycle, a large number of daphnids were placed in a small culturing vessel with a lower-intensity light during a short photoperiod. This was to artificially stimulate the onset of a winter season, a time traditionally observed to induce ephippial females and males, as emphasized in the introduction. There are no established protocols for this particular

type of test as the primary purpose is to induce stress rather than alleviate it. Methods were inspired by Stross and Hill (1965), Dodson et al (1996) and Baer et al (2009). Other stressors attempted to induce sexuality were overcrowding and starvation. See figure 9 for an outline of this experiment. The goal of this experiment was to have the sexual cycle induced within 7 test days. The tests were allowed to run until successful or until the organisms died.

When chemicals were to be added, observations of the number of female adults, number of male adults and number of neonates were to be recorded along with behavioural observations of swimming behaviour.

Environmental parameters

Two experimental setups were arranged. One was in a Sanyo MIR-153 incubator in order to control for any potential variations in temperature. The temperature was set to 19 degrees Celcius. A black plastic plate was placed on the window of the incubator to block out ambient light. Light intensity was maintained approx. 600 lux (approx. 11 PAR) and light:dark ratio was reduced to 8hrs light: 16hrs dark per day to mimic winter photoperiods.

The second experimental setup was on a lab bench at laboratory room temperature which fluctuated from 18 to 22 degrees Celcius. A fluorescent light fixture was mounted overhead to produce light intensity of approx. 600 lux (approx. 11 PAR), attached to a timer to produce a light:dark ratio of 8hrs light: 16hrs dark per day to mimic winter photoperiods. A cardboard box was placed over top to reduce ambient light. The important difference between the two setups was in the second setup, temperature was not controlled as precisely as in the first setup. It was subject to perturbations as windows and doors were opened in the lab.

Feeding and vessel setup

Ten 400 mL beakers with 300mL of overlying solution were prepared. At the commencement of the assay, 0-2 day old neonates of *Daphnia magna* were collected from stock cultures. In each beaker a set number of neonates, ranging from five to fifty in increments of five were introduced in each experimental setup. This system was set up in order to determine at which point daphnid overcrowding would produce males and ephippial females. An ideal number of daphnids would then lead to a 'crowding factor' that would be used in subsequent experiments (See Fig. 10).

After a series of deaths within the beakers, Dissolved Oxygen (DO) was measured with the O_2 probe and it was determined that cultures needed to be aerated, despite attempting to induce stressful conditions. Within each beaker a borosilicate glass Pasteur pipette with an airline tube inserted within it and sealed tightly with parafilm™ and then connected through a network of gang valves to an air pump set to bubble air in each vessel air at a rate of 2-3 bubbles per second which maintained a dissolved oxygen concentration of approximately 80 percent, which is the O_2 saturation level recommended for bioassays by Environment Canada (1998). One round of testing attempted starvation of the organisms. Organisms were fed on day one and left to starve for the duration of the experiment. After another series of deaths, it was determined that starvation would not prove useful, so organisms were In both setups were fed in weekly intervals of approx. $7.5x10^5$ cells of *Selenastrum* per vessel per feeding session.

Figure 10: Experimental setup. Numbers represent numbers of *Daphnia* in each vessel

2.4 *HYALELLA AZTECA*

2.4.1 *Hyalella azteca* culturing

Culturing procedures were developed based on Environment Canada (1997) protocols. Starting cultures were obtained from previous laboratory stock cultures from G. Marshall (2009). Starting cultures were maintained in a 20L glass aquarium with an overlay of 16L DMDW. The overlying water was continuously aerated with an air pump to maintain oxygen saturation of at least 80%, or 8.5 mg/L O_2. Oxygen saturation was monitored with the MPP. Two litres of silica sand with four 4”x4” gauze sheets over top the silica were used as substrate for the *Hyalella*. Silica was utilized because it increases the surface area for the *Hyalella* to graze, as well as for the growth of nitrifying bacteria. Water temperature in the aquaria was maintained at room temperature, 19-21 degrees Celsius. Aquaria were kept under a customized light bank where light intensity was maintained between 600-800 lux (Approx. 11 to 15 PAR) as recommended in Environment Canada (1997) and a photoperiod of 16hr light: 8hr dark. Glass sheets were placed above the aquaria in such a way to keep dust and debris out of the aquarium, but allowing enough air to enter. *Hyalella* were fed 3x a week on non-consecutive days using 8-12 milligrams of Tetramin™ flakes ground and sieved through 500 micron nitex™ screen. Tetramin flakes, as utilized by DFO (1989, 1992), USFWS (1990, 1992), USEPA (1991a, c, 1994) and documented in Environment Canada (1997), were determined to be the most suitable feed for *Hyalella*, as it did not cause a significant increase in ammonia (as measured by API pharmaceuticals ammonia test). Approximately one quarter of the overlying water was changed weekly to prevent build-up of carbonates which can affect the pH. A 200 L carboy at the same room temperature was aerated continuously for use as a source of DMDW.

2.4.2 *Hyalella azteca* chronic toxicity and secondary sexual characteristics bioassay

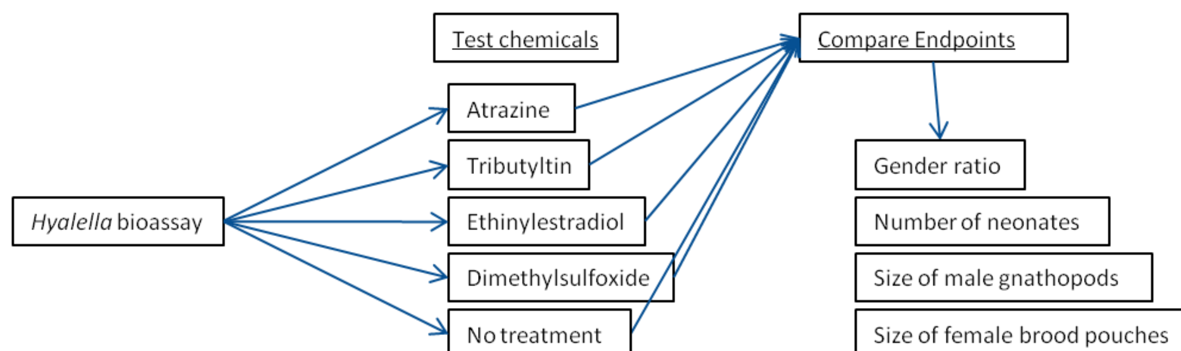

Figure 12: Outline of *Hyalella* bioassay highlighting the major endocrine endpoints

This bioassay was conducted to determine if there were potentially endocrine disruptive effects of atrazine and tributyltin on *Hyalella azteca*. Protocols for this experiment were based on USEPA (2000) protocols for *Hyalella azteca* 42-d Test for Measuring the Effects of Sedimentassociated Contaminants on Survival, Growth, and Reproduction (USEPA, 2000), as the Environment Canada (1997) protocols are for acute toxicity testing. Prior to the commencement of the bioassay, it was determined that at least five replicates were required for the nine treatments requiring the need for 45 bioassay vessels. As this was a 42 day assay, it would be difficult to repeat several times, so it was important to commence the experiment with a large number of vessels. It was also determined that treatments had to be staggered because it would be impossible to count all organisms at the same time if all treatments were started at the same time. Five was found to be the ideal number of vessels per day in order to maximize the number of experiments per day while not being overwhelmed with counting individuals at the end of the experiment. A block randomization procedure was developed for seven replicates in order to have five beakers managed per day out of a total of 63, with 3 therefore managed on the last day. The block randomization technique utilized the random number generator in Excel. Each random number was associated with a beaker, randomized, and then sorted. The order of sorting determined the order of treatments.

Culture vessels

Each beaker was fitted with two 4-ply 2”x2” cotton gauze pads as substrate for the Hyalella, with 300 mL of overlying solution. Silica was not used for the experiment as it was for the culturing, because neonates at the end of the experiment smaller than the grains of silica would be imposible to count. Solutions used were Atrazine at 100, 50 and 5 µg/L with 0.1% DMSO in DMDW, Tributyltin at 10, 1 and 0.1 µg/L with 0.1% DMSO in DMDW, 17α-ethinylestradiol at 0.1 µg/L with 0.1% DMSO in DMDW, a carrier control of 0.1% DMSO in DMDW, and a negative control of just DMDW. Preliminary tests determined that original concentrations of 100 and 50 µg/L concentrations of TBT were too acutely toxic (100% lethality >72 Hrs) for use in a chronic toxicity bioassay. Within each beaker there was also a borosilicate glass Pasteur pipette according to USEPA, (2004). Attached to each Pasteur pipette was an airline tube sealed with parafilm and then connected through a network of gang valves to an air pump, bubbling through each pipette at a rate of 2-3 bubbles per second. Solutions were allowed to aerate for 24 hours prior to the commencement of the assay. Twenty juvenile amphipods were sifted between two sizes of nitex screens. Individuals larger than 500 µm and smaller than 350 µm were returned to the main culture tank. Individuals between 350 to 500 µm were used for the experiments. Individuals between these sizes are between 3-9 days of age (See fig. 13) (USEPA 2000; Othman and Pascoe, 2004). Organisms were kept in a holding tank for 24hrs prior to their use in the bioassay so that individuals would not be stressed upon entrance to the test chamber.

Beginning of assay

At the commencement of the bioassay, twenty amphipods were distributed throughout the five beakers assigned for that particular day. During the first three hours of their addition to the bioassay vessels, observations were taken as to various parameters. Chemical parameters were also logged with a MPP. Organisms were to remain in their chambers for the duration of the 42-day chronic toxicity bioassay, as set out in USEPA 2000.

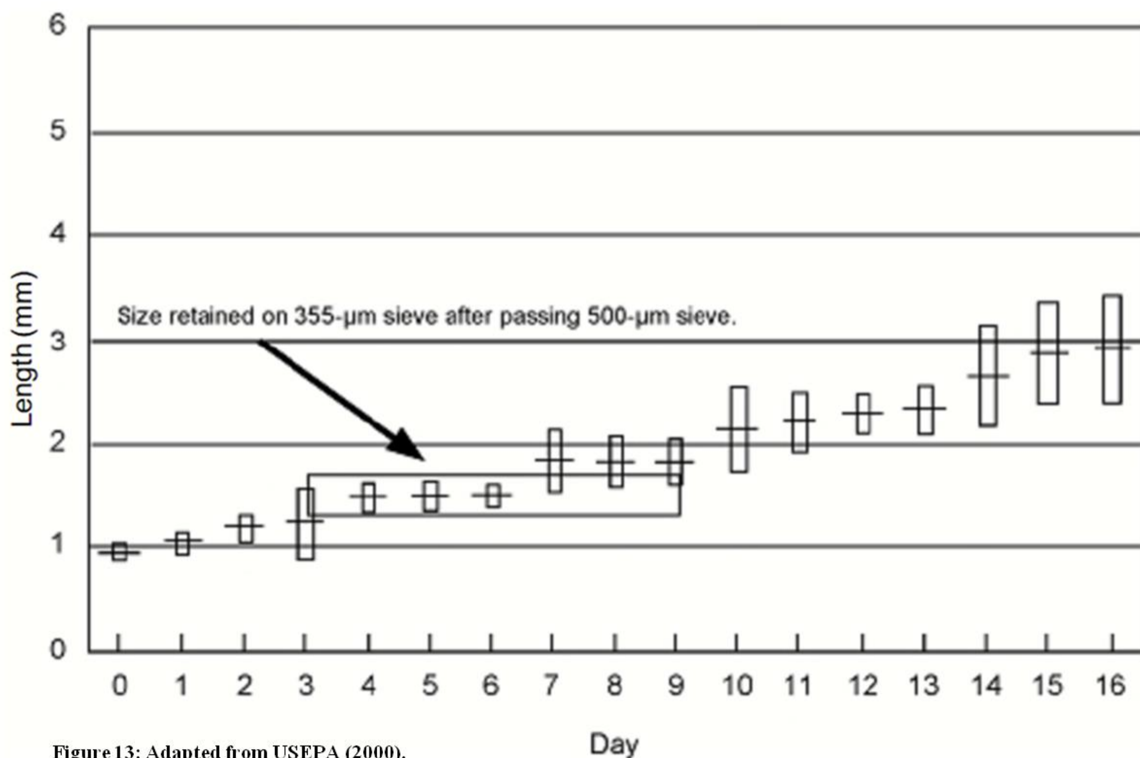

Figure 13: Adapted from USEPA (2000).

Mean length (+/- 2SD) and relative age of *Hyalella azteca* collected by sieving in comparison with length of known-age organisms. P.V. Winger, USGS, Athens, GA, unpublished data.

Throughout the assay, organisms were fed approximately 8-12 mg of ground Tetramin™ flakes three times per week as suggested in Environment Canada 1997; USEPA 2000. At intervals throughout the assay (1, 2, 3, 24, 48, 72Hrs, 7, 10, 14, 21, 28, 35, 42 days), individuals were counted and several parameters were noted as to their location in the vessels. These included presence in the water column, or presence within the sediment. Chemical parameters were also logged with a MPP. Any dead amphipods, indicated by the pink colour they formed when dead, were removed. Cultures were maintained as described in section 2.41 with weekly renewal of the toxicants.

End of assay

In order to capture digital images of the entire organisms, a dissection scope was needed, as light microscopes have more magnification than required. A dissection microscope set at 1x magnification affixed with a Big Catch™ EM-C560 Eyepiece digital Camera connected to a PC running minisee image capture software was utilized for this purpose.

At the termination of the assay, organisms were removed, sieved over a 750 µm nitex screen to determine the amount of adult organisms that have experienced growth. They were then placed on a slide on their side at approx. 4 per slide. Slides of *Hyalella* were observed under the microscope and oriented facing left for consistency and ease of analysis. If necessary, a small stream of water was gently flowed under their coxae (see fig. 14) with a needle and syringe in order for them to relax and show their gnathopod structure. Images were taken with minisee and saved for image analysis. Images were organized during capture into folders with respective titles. For example, vessels commenced on April 20th were placed in a folder called "APR 20" and then placed in folders according to their treatment. A block randomization

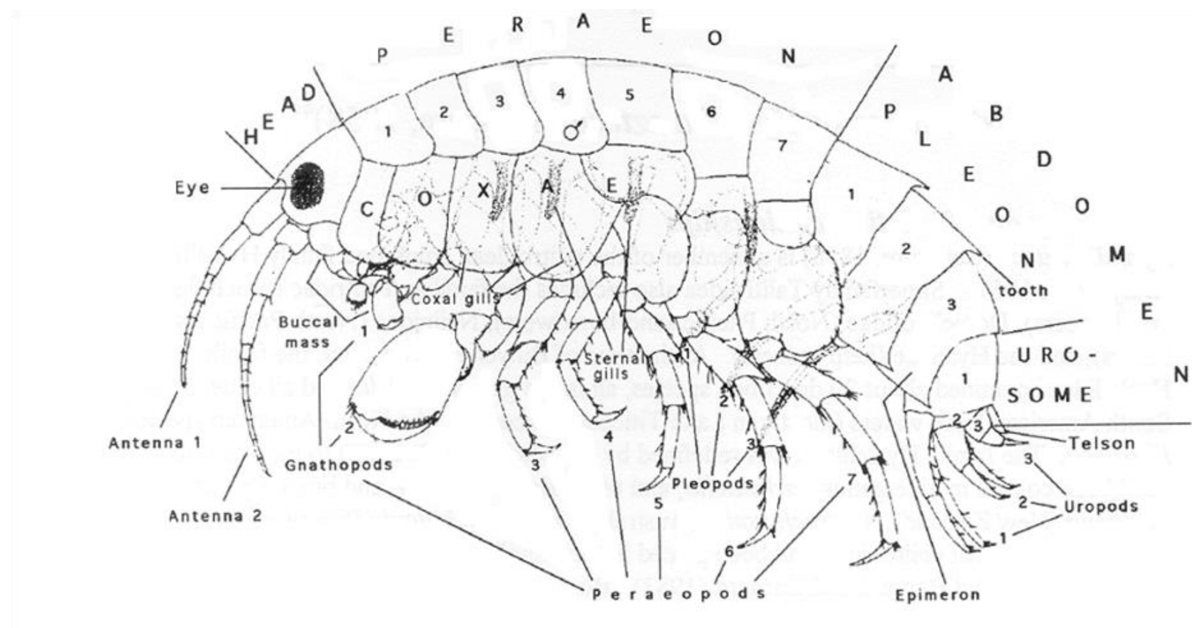

Figure 14: Anatomy of Hyalella azteca (Environment Canada, 1997; Updated from Bousfield, 1973)

technique similar to the one used above was used to randomize folder names. Before analysis, folders were arbitrarily renamed after recording folder information in a spreadsheet. This helped to negate any researcher bias during analysis.

Measurement

Each organism had its own image file, and each image is measured by pixel size. In order to get an actual size of each digitally captured organism, a scale was needed. A micron to pixel ratio was determined via calibration with a micrometer. For the sake of consistency, all organisms were digitally orientated to face the left side of the screen. Males were differentiated from females based upon the size of the presence of the characteristic male secondary gnathopod (See fig. 15). Females were determined by absence of the male secondary gnathopod plus the dark cloudy brood pouch (See fig. 15). Juveniles were categorized as such if their gender could not be determined visually. If an individual *Hyalella* did not appear to have male secondary gnathopods or a brood pouch and were less than 3mm in length, they were classified as a juvenile.

Length was measured digitally using the segmented line function in imageJ, from the base of the first antennae (See fig. 4 B) to the base of the 3rd uropod (See fig. 4 A). Areas of the gnathopods and of the brood pouch were measured using the polygon selection function within the imageJ image analysis program, following the shape of the gnathopod or brood pouch. Example images are shown in figure 15.

Endpoints

After image analysis was complete, data was tabulated into Excel for statistical analysis. Endpoints measured were gender ratio, number of neonates, size of male gnathopods, and size of female brood pouches.

Figure 15 : Image of a) male and b) female *Hyalella azteca*. "L" is the length from the base of the 3rd uropod to the base of the first antenna measured in mm, highlighted in white (see fig 4. for anatomy). "A" is the area of the secondary gnathopod in the male, and the brood pouch in the female. Both measured in mm^2 and highlighted in white.

2.5 Statistical analysis

All data were entered in Excel and organized for use in Systat 12. Excel was utilized for its simple user interface in sorting and managing data. Systat 12 was utilized for its reliability, simplicity, accuracy and ease-of-use in analyzing statistics. The values for all organisms in each beaker were averaged as a single replicate, as each beaker was considered a single treatment. Control values were entered alongside area and the ratio was calculated as body length / area, creating the value "L/A".

Basic statistics, including means and standard deviations, were used for the bar graphs of averages vs. standard deviations. More intensive statistical analyses were utilized to gain a full appreciation of the data. Values were tested for normality using Kolmogorov-Smirnov One Sample Lilliefors test, selecting for Normal Distribution in Systat 12. Length was found to be normally distributed. Male area had to be transformed using a power transformation of X^2, while female area had to be transformed with a power transformation of $X^{1.5}$. Variances were found to be equal using Levene's test.

Measurements were then analyzed using One-way Analysis of Variance (ANOVA) setting length and area vs. the treatments setting α=0.05. For the data taken during the test, including location within the water column, number of dead individuals, etc., non-parametric tests were

required as the data were not discrete. The Kruskal Wallace test was used, as it is the non-parametric equivalent of a one-way ANOVA analysis.

3.0 Results and Discussion

3.1 *Daphnia magna* sexual cycle assay

Attempts were made to culture *Daphnia magna* under conditions which allowed for development of males and ephippial females. Two separate laboratory setups were used in the process: one used an incubator with the ability to control temperature; the other used a laboratory bench with temperature controlled by a room thermostat.

Incubator setup

As discussed earlier, *Daphnia* switch their reproductive cycle when they receive environmental signals that winter is approaching. These signals include a decrease in temperature and a decrease in daylight hours. This was the rationale behind the use of an incubator. This experiment ran for sixteen weeks, and each attempt lasted approximately one week. In the Sanyo MIR-153 Incubator setup, it was found that despite the low temperatures (12-19 degrees Celsius) induced by the incubator and relatively high volumes of test solutions in the beakers (approx. 300 mL), water would evaporate at exceptionally high levels in all ten test vessels. For example, during week two of testing the experiment was left unattended for three to four days and upon return it was found that approximately 150 of the 300 mL of overlying solution had evaporated resulting in many deaths, especially in vessels 9 and 10 where there were 45 and 50 juvenile daphnids, respectively. During weeks 3 and 4 of testing, the temperature was reduced to 12 degrees Celsius, yet the evaporation phenomenon still occurred. Thus it was difficult to maintain constant water parameters such as hardness, pH and conductivity during the test as laid out. Upon this discovery, test vessels were checked daily. This included daily dead organism removal and daily test water top-up with distilled water. Yet In all ten beakers it was found that daphnids would die with 100 percent lethality within 72 hours under these conditions without developing ephippia. The reasons for death are unknown. Vessels were then covered with petri dishes from week 5 to 16 in attempt to reduce evaporation, but this did not appear to have any effect on livelihood either. When it was suspected that death was caused by the increased pH of test water as water evaporated from the vessels, bi-daily distilled water top-ups initiated on

week 6 did not appear to have any effect on livelihood. Therefore, the experiment had to be terminated in the trial phase after sixteen weeks before chemicals could be tested. The placement of the beakers within the incubator, and the black plate placed across the window made it difficult to observe the organisms for behavioural changes during these conditions. Stringent observations regarding number of organisms, numbers of deaths, time of death, etc., were not recorded because the organisms were dying in control water. The purpose of this experiment was not to gain information about the sensitivity of *Daphnia* to control water, but to test the sensitivity of *Daphnia* to test compounds Atrazine, TBT and EE. Therefore, this experiment did not provide any significant findings.

Lab-bench setup

The laboratory bench-top setup experiment ran for twenty-four weeks. In this setup a series of beakers were covered by a cardboard box to reduce ambient light during dark hours. This experiment ran from mid-June to mid-December. In this experiment it was found that the temperature had the tendency to fluctuate during summer months. In a single day the temperature could vary by as much as ten degrees Celsius. Unfortunately, it wasn't until all *Daphnia* in both test vessels and stock cultures were found dead that an air conditioner could be installed in the lab space.

During the test, in all ten culture vessels, *Daphnia* would die in high numbers, either due to the temperature fluctuations or from an unforeseen circumstance. When observed daily, many appeared to be spinning and swimming erratically along the bottom of the test vessels. According to Marshall (2009), these swimming behaviours are seen as a sign of stressful conditions. Unlike the incubator setup, this experiment had some success in producing female daphnids with ephippia during August. However, starting cultures in this lab had crashed due to the severe temperature fluctuation.

This experiment was re-attempted from September to mid-December after a new stock culture was started and an air conditioner was installed. Experiments ran for up to 14 days, or when 100% lethality was observed. In vessels that contained 15 *Daphnia* or less, they appeared to

reproduce successfully and behave normally under test conditions for the duration of the test. Numbers of individuals in these vessels would not exceed 60, including juveniles. Vessels with daphnid numbers 20 or greater had 100% lethality after 72 hours. It appears that Daphnia can produce high numbers in a vessel with no significant loss, but when high numbers are introduced into a vessel, they experience loss. This could be due to any number of reasons, which may include ammonia levels.

Unfortunately, despite lighting conditions and crowding effects, there was no success in producing females with ephippia. Stringent observations regarding number of organisms, numbers of deaths, time of death, etc., were not recorded because the organisms were dying in control water. The purpose of this experiment was not to gain information about the sensitivity of *Daphnia* to control water, but to test the sensitivity of *Daphnia* to test compounds. Therefore, this experiment did not appear to provide any significant findings.

Summary

In both conditions, water parameters were monitored. Dissolved oxygen was measured with a USB probe attached to a PC. Ammonia and hardness (GH; Calcium and Magnesium) were monitored with Aquarium Pharmaceuticals (API) test kits. All conditions tested consistently within the normal range (0 ppm, Ammonia; 0 dGH, GH; 0 mg calcium oxide) for cultivation of *Daphnia magna*. Since it appeared that there was no consistent way of having *Daphnia* produce ephippia and males without a massive die-off, both experiments had to be terminated in the trial phases before chemicals were added.

Explanations in the literature regarding explanations for die-offs within populations of *Daphnia magna* are lacking. It is a poorly understood phenomenon, why *Daphnia* would simply die without producing any ephippia, perhaps leaving room for future studies. Postulations as to reasons behind the deaths could be due to starvation or ammonia buildup (Hülsmann, 2003; Xiang et al., 2010). It is also possible that there is little information in the literature regarding die-offs, as they are seen as accidental occurrences during bioassays, when there could be definitive reasons that are overlooked. Individuals that have had success in the past

manipulating the reproductive strategy of *Daphnia* in the past have either used a different species, such as *Daphnia galeata* (Dodson et al., 1999) or a different culture medium, such as COMBO medium (Baer et al., 2009). So perhaps one of these factors is absolutely necessary for success with this type of study.

If future studies were to attempt similar methods, it would be recommended to use COMBO medium, as the use of DMDW may have had unknown effects on *Daphnia*. Water parameters, including changes in pH and hardness, would have to be monitored stringently over time, as these factors may have contributed to stress and/or death. It is unfortunate that the Hanna probe was not available for use during this phase of the experiment, so analysis of these parameters was impossible at the time. These experiments have provided insight on experimental setups with *Daphnia magna* within the literature. Literature regarding chronic or reproductive effects of chemicals on *Daphnia*, must be taken with a 'grain of salt', as *Daphnia* are obviously quite difficult to work with during reference conditions. There is future research potential in the investigation of the sensitivity of Daphnia to various temperature, lighting and water parameter conditions during chronic and multigenerational studies. This would provide validation of test parameters and help create standardized protocols for initiating the sexual reproductive cycle in *Daphnia*.

3.2 *Hyalella azteca* assay

Several preliminary experiments determined various problems that were originally unforeseen. During the preliminary experiments, static assays was utilized, where test water added on day one of the experiment, would be the same test water on the last day of the experiment, with bi-weekly top-ups with distilled water. Instead of using neonates to start the experimental cultures, three precopulatory pairs were added to each of the experimental vessels, a method utilized by Vandenbergh et al. (2003). Adult *Hyalella* for this experiment were therefore not age synchronized. *Hyalella* were also fed Roti-rich™ invertebrate food. During counting it was noted that cultures were contaminated with Ostracods and Copepods, genus and species undetermined. Analysis of the water with the Hanna instruments multi-parameter probe indicated an increase in Total Dissolved Solids (TDS) and pH over time, indicating a build-up of

carbonates. All vessels were also started on a single day; however, it was impossible to count all vessels at the end of the experiment within a single day. It was determined at the end of the experiment that the problems with this method are:

1. It could not be determined that any adults that died at the end of the experiment died as a result of chemical exposure, or due simply to old age.
    a. Organisms used in the bioassay should be within the same life-stage, or age-synchronized
2. Food must be made from monocultures, sterilized, or at least come from a reliable source.
3. If treatments are all started on a single day, all the resulting organisms must therefore be counted within a single day. Therefore for a multiple-vessel bioassay, the commencement of the various replicates must be staggered throughout multiple days, or have one complete set of replicates on each day.
    a. If replicates are to be staggered, they must be randomized.
4. Test water must not only be topped up, but changed frequently, as carbonates can build up over time due to evaporation which can lead to an increase in pH, which can affect results. Ammonia, nitrite and nitrate levels could also build up over time
5. Test water should be aerated so that substrates could promote the growth of aerobic nitrifying bacteria to reduce ammonia build-up.

For the major experiment, cultures commenced on April 20th, counting and image capture began June 1st after the 42-day interval based on USEPA, (2000). At the predetermined intervals prior to the 42 days, measurements were taken and recorded and can be found in Appendix B. Additionally, on the same table at the end of the 42 day assay, the most accurate count of the number of organisms found and the number of organisms found greater than 750 microns were also recorded. All surviving organisms used in the experiment were then placed in a retirement tank.

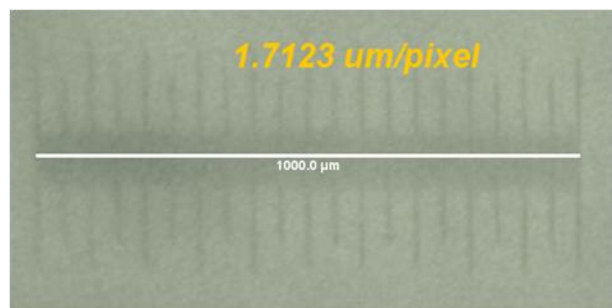

Figure 16: Calibration for image analysis

ImageJ was calibrated using a stage micrometer which determined that each pixel for an image taken with that particular microscope setup at 1x magnification measured 1.7123 µm (Shown in fig. 16). All images were taken with those exact settings to maintain consistency.

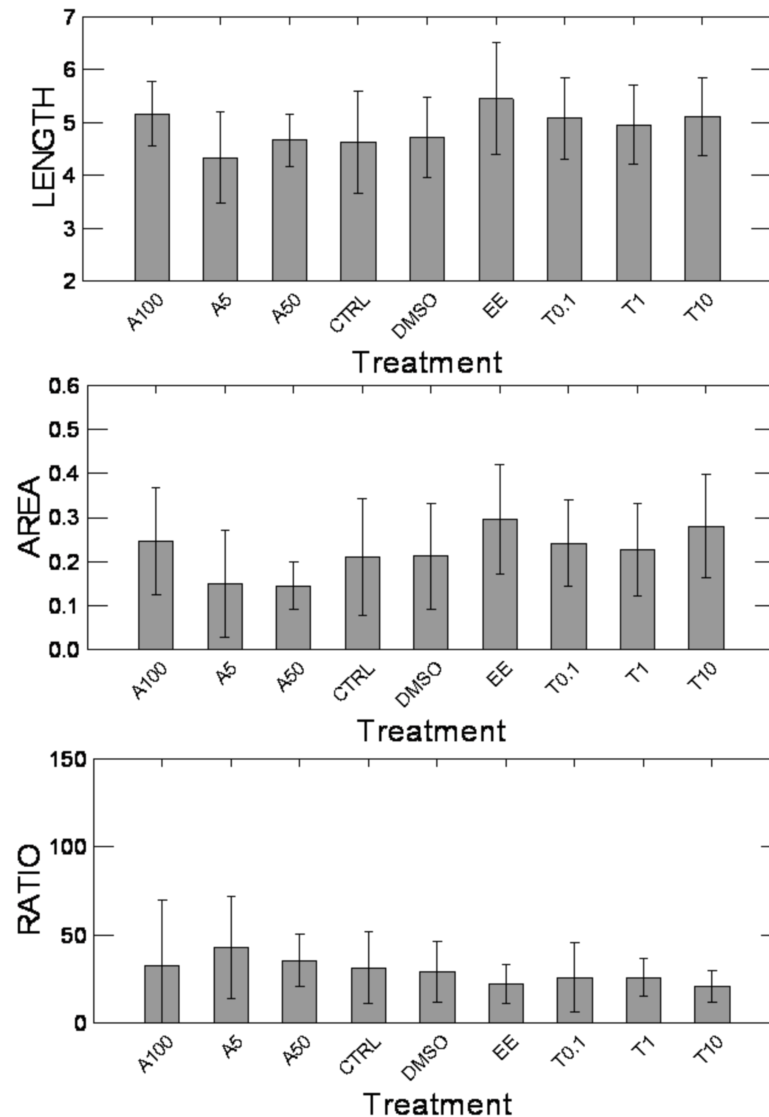

Figure 17: Male values. Error bars represent Standard Devation. No significant differences between treatments

Body lengths, gnathopod and brood-pouch size were measured individually, carefully, by hand after folder randomization. After values were noted on each image, folders were renamed back to their original name (example: "CTRL, APR 20", instead of "86") Results were recorded and can be found in Appendix C.

3.21 Male data

Basic statistics

Figure 17 shows the average values for body length, secondary gnathopod area and the ratio of body length to secondary gnathopod area according to the various treatments with standard deviations ($p= 0.6827$). When the data is analyzed by this method there appears to be no significant difference between treatments, as the error bars are quite large.

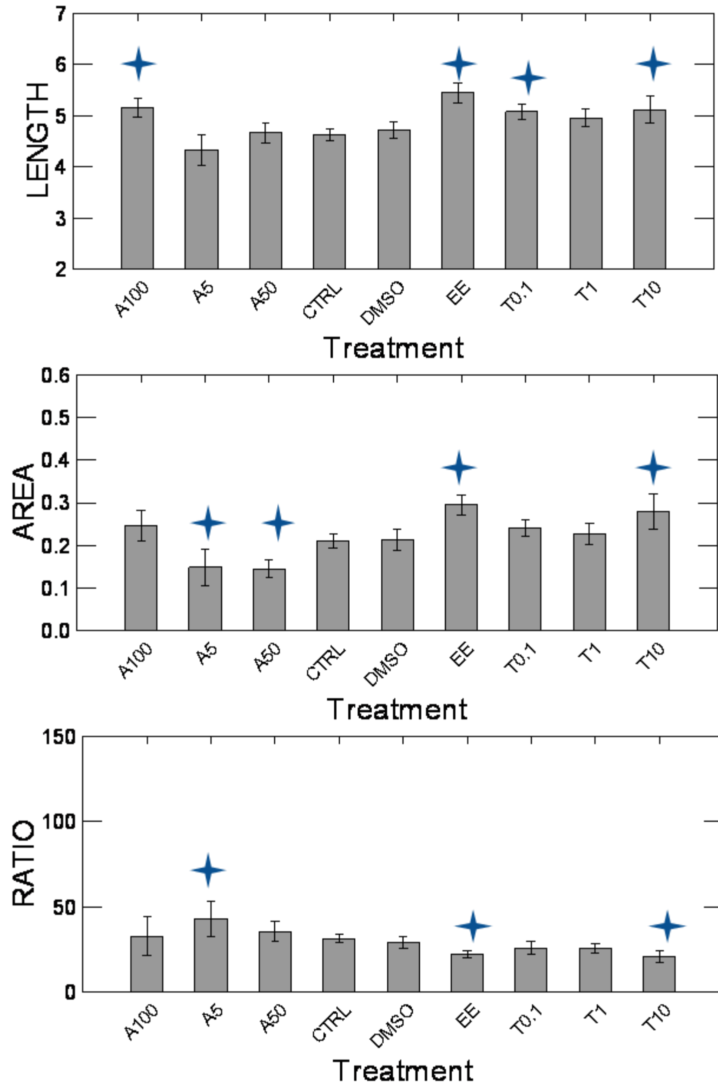

Figure 18: Male values. p-value = 0.6827. Error bars represent Standard Error. Stars represent values significantly different than control.

This would indicate that atrazine, and tributyltin are no different than the no treatment control, and therefore appear to have no effect on secondary gnathopod development in a 42-day assay.

These results also indicate that the positive control, ethinylestradiol also appears to have no effect on secondary gnathopod development in a 42-day assay, contradicting results found in Vandenbergh et al. (2003) and agreeing with results found in Dussault et al. (2008).

Figure 18 shows the average values for body length, secondary gnathopod area and the ratio of body length to secondary gnathopod area according to the various treatments with standard error (p= 0.6827). When the data is analyzed by this method there appears to be a significant difference between controls in all three categories.

Concentrations of atrazine at 100 µg/L, EE at 0.1 µg/L, TBT at 0.1 µg/L and 10 µg/L appear to have an effect on body length, indicating that these chemicals cause *Hyalella* to be longer, on average. However, body length is not known to be under endocrine control, so the true reason for these results is unknown.

Concentrations of atrazine at 5 and 50 µg/L, EE at 0.1 µg/L, TBT at 10 µg/L appear to have an effect on overall secondary gnathopod area, indicating that these chemicals may indeed have an effect on the growth and development on tissues known to be under endocrine control (Vandenbergh et al., 2003). When factoring body length into the equation, atrazine at 5 µg/L, EE at 0.1 µg/L, TBT at 10 µg/L still appear to have a significant effect on the growth and development of secondary gnathopods. The results with respect to atrazine, do not appear to be dose-dependent either, indicating that *Hyalella* are more sensitive to atrazine at 5 µg/L than any other concentration. The reason for this is unknown, but it could be due to a hormesis response. A larger value for the length to gnathopod ratio indicates that gnathopods are proportionally smaller, indicating an inhibitive effect on the growth of the gnathopod tissues. Smaller values indicate a stimulatory effect. The results in Figure 18 indicate that EE and TBT at 10 µg/L have a stimulatory effect on gnathopod development. These results contradict that of Vandenbergh et al. (2003), as they found the opposite was true with EE at concentrations of 0.1 µg/L.

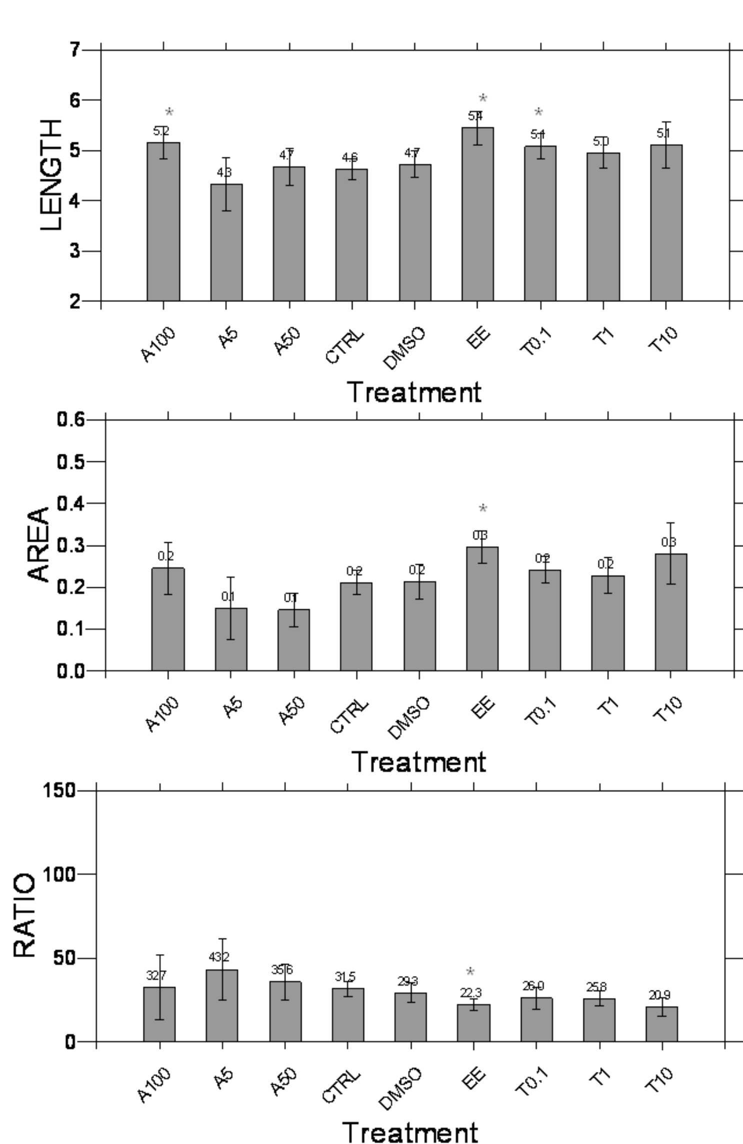

Figure 19: Male values. Error bars represent Standard Error, p-value = 0.9. Stars represent values significantly different than control.

This would indicate that atrazine, and tributyltin are no different than the no treatment control, and therefore appear to have no effect on secondary gnathopod

development in a 42-day assay. These results also indicate that the positive control, ethinylestradiol also appears to have no effect on secondary gnathopod development in a 42-day assay, contradicting results found in Vandenbergh et al. (2003) and agreeing with results found in Dussault et al. (2008). When the p-value is increased, as shown in Figure 19, results show that only EE at concentrations of 0.1 µg/L have a stimulatory effect on secondary gnathopod development in *Hyalella azteca*, while atrazine at 100 µg/L and TBT at 0.1 µg/L appear to have a stimulatory effect on body length.

Advanced statistics

More advanced statistics were used to determine whether there was more to be learned from the data. Before testing the data using an ANOVA, the data were checked for normality with One-sample KS Lilliefors tests. Length and Area were found to be normally distributed (p-values of 0.811, 0.696; respectively). Ratio, however was not found to be normally distributed, but was found to be normally distributed (p-value = 0.393) when log-transformed. The data were also checked for equality of variance with Levene's test to check for equality of variance between day and treatment for length, area and the log-transformed ratio. Day was found to have equal variances within length (0.264), area (0.181) and log-transformed ratio (0.422). Treatment was found to have equal variances within length (0.448), area (0.578) and log-transformed ratio (0.820). Therefore, Length, area and log-transformed ratio followed the assumptions for parametric testing. However, it was revealed using ANOVA that there was no significant difference between male lengths within the various treatments (0.870). There were also no significant differences between area or log-transformed ratio between treatments (0.882 and 0.917, respectively).

Summary

Although atrazine at 5 µg/L appeared to have a small effect on length and gnathopod size with a slight decrease, it was not found to be significant. Atrazine at 50 µg/L was found to have a slight, but insignificant decreasing effect on the size of the gnathopods. TBT at 10 µg/L appears to have a slight increase in the average length and gnathopod size, although insignificant.

Although determined to be insignificant, body length and gnathopod area appear to decrease with decreasing concentrations of Atrazine. This could be due to hormesis, or an effect of Atrazine that is currently poorly understood. Body length and gnathopod area tend to slightly increase under exposure to TBT. It is believed that TBT is an androgen inducing compound (Yamabe et al., 2000; Santos et al.,2005), which would indicate that body length and gnathopod size are possibly under androgen control.

Follow-up experiments could be conducted where testosterone, or other androgen analogs are used as a positive control for androgen-disrupting compounds. However, using the most conservative methods of statistical analysis, it appears that there is no significant difference between treatments, indicating that the compounds atrazine and tributyltin have no effect on the growth and development on male secondary gnathopods.

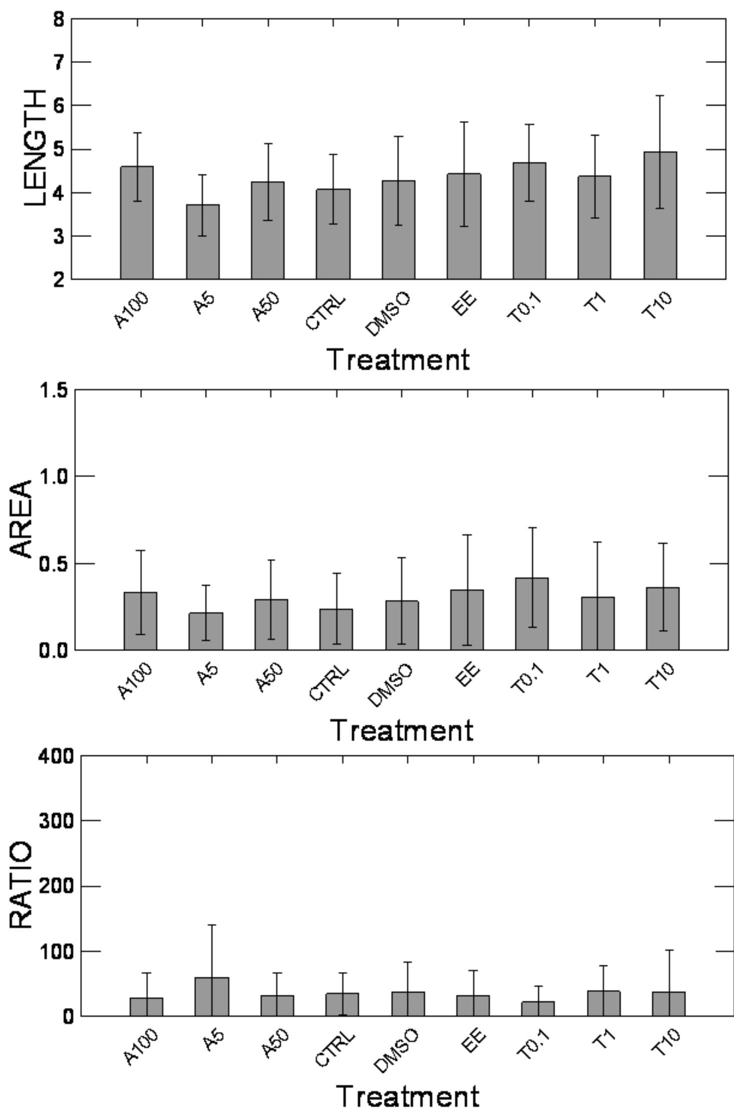

Figure 20: Female values. Error bars represent Standard Deviation, p-value = 0.6827. No significant difference between treatments.

3.22 Female data

Basic statistics

Figure 20 shows the average values for body length, brood pouch area and the ratio of body length to brood pouch area according to the various treatments with standard deviations (p= 0.6827). When the data is analyzed by this method

there appears to be no significant difference between treatments, as the error bars are quite large.

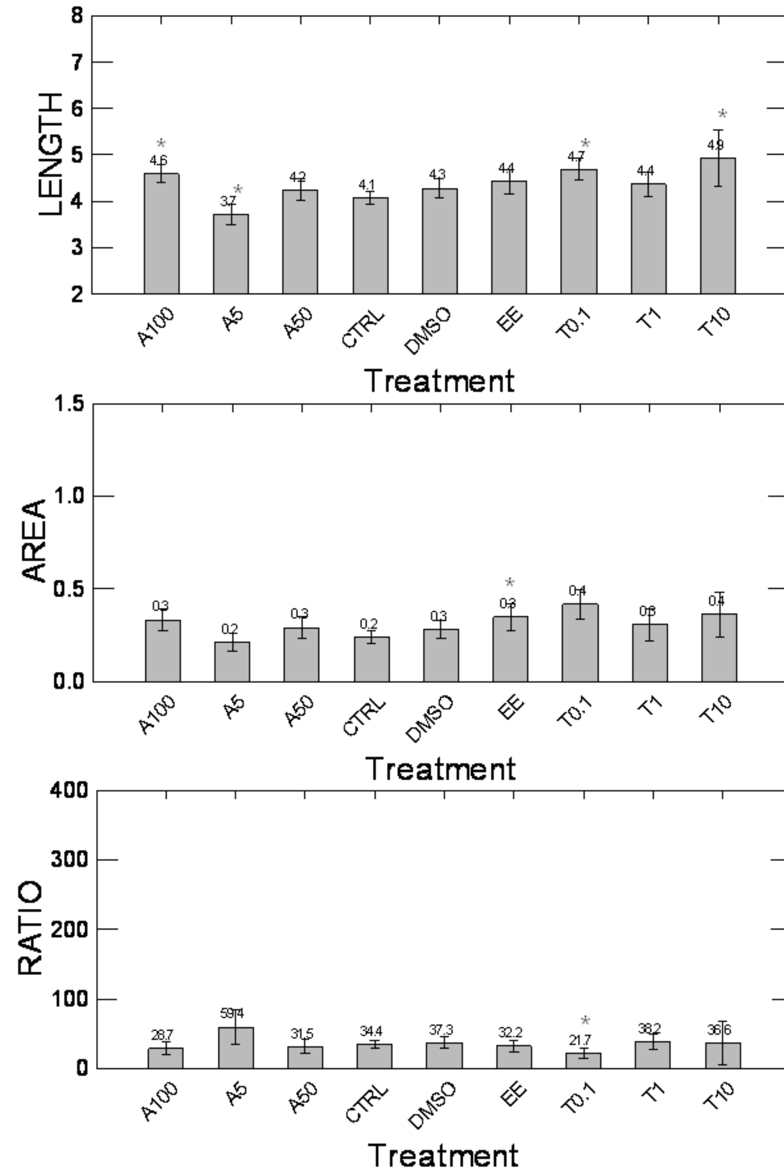

Figure 21: Female values. Error bars represent Standard Error, p-value = 0.9. Stars represent values significantly different than control.

Figure 21 shows the average values for body length, brood pouch area and the ratio of body length to brood pouch area according to the various treatments with standard error (p= 0.9).

When data is analyzed with this method it appears that there are some significant differences between treatments. With respect to body length, atrazine at 100 µg/L and 5 µg/L, and tributyltin at 0.1 and 10 µg/L are significantly different compared to control solutions. Atrazine at 5 µg/L appears to inhibit growth, while at 100 µg/L it appears to stimulate growth. The reason for this is unknown. TBT at 0.1 and 10 µg/L appear to slightly stimulate growth of female *Hyalella*, which could be due to a stimulatory hormonal effect, a toxic effect on smaller organisms, or an alternate, unknown reason.

Brood pouch area appears to be significantly affected by concentrations of EE at 0.1 µg/L, but not when body length is factored in. This could be due to a slight stimulatory response of estrogen on the egg development within the brood pouch. Or, because EE does not appear to significantly affect brood pouch development when length is factored in, these results could be due to an error. When body length is factored in, TBT at concentrations of 0.1 µg/L appears to have a significantly smaller body length to brood pouch ratio, indicating that there is a stimulatory effect of TBT on the development of the brood pouch. TBT is thought to raise androgen levels (Yamabe et al., 2000; Santos et al.,2005, and it is possible that the androgens were aromatized and stimulated development of the brood pouch. However, there is so little known about the hormonal control of brood pouch development in *Hyalella azteca*, that it is impossible to dictate a cause. What can be determined from these results, is that the utilization of this novel endpoint may be a useful tool in detecting endocrine disruption, if endocrine disruption were to occur.

Advanced Statistics

Before proceeding to the ANOVA analysis, the data were checked for normality with One-sample KS Lilliefors tests. Length, Area and Ratio were found to be normally distributed (p-values of 0.667, 0.178, 0.182; respectively). The data were also checked for equality of variance with Levene's test to check for equality of variance between day and treatment for length, area and ratio. Day was found to have equal variances within length (0.100), area (0.013) and ratio (0.509). Treatment was found to have equal variances within length (0.862), area (0.996) and ratio (0.695). Therefore, Length, area and ratio followed the assumptions for parametric testing. It was revealed using ANOVA that there was no significant difference between female lengths, areas or ratios within the various treatments (0.854, 0.435 and 0.495, respectively).

Summary

Atrazine at 5 µg/L appeared to have a slight decrease on length, although insignificant. Atrazine at 50 µg/L L had a very slight decrease in the average brood pouch area, but also insignificant.

TBT at 0.1 µg/L was found to have a moderately large increase on length and brood pouch area, although insignificant.

Although determined to be insignificant, body length and brood pouch area appear to decrease with decreasing concentrations of atrazine. This could be due to hormesis, or an effect of atrazine that is currently poorly understood.

TBT and Estradiol have similar average values, which could be due to an increase in free testosterone (CITE) due to the TBT, which becomes aromatized in the females. Since TBT appears to have no effect on gender ratios, however, it is unlikely that this is the case, as this would likely cause an increase in male ratios, unless all of the testosterone released by TBT becomes aromatized.

Follow-up experiments could be conducted where testosterone, or other androgen analogs are used as a positive control for androgen-disrupting compounds. Multigenerational assays may provide more accuracy as to the true effect a chemical may have. However, using the most conservative statistical methods, the results show that the compounds atrazine and tributyltin, as well as the compound ethinylestradiol, appear to have no effect on brood pouch development on female

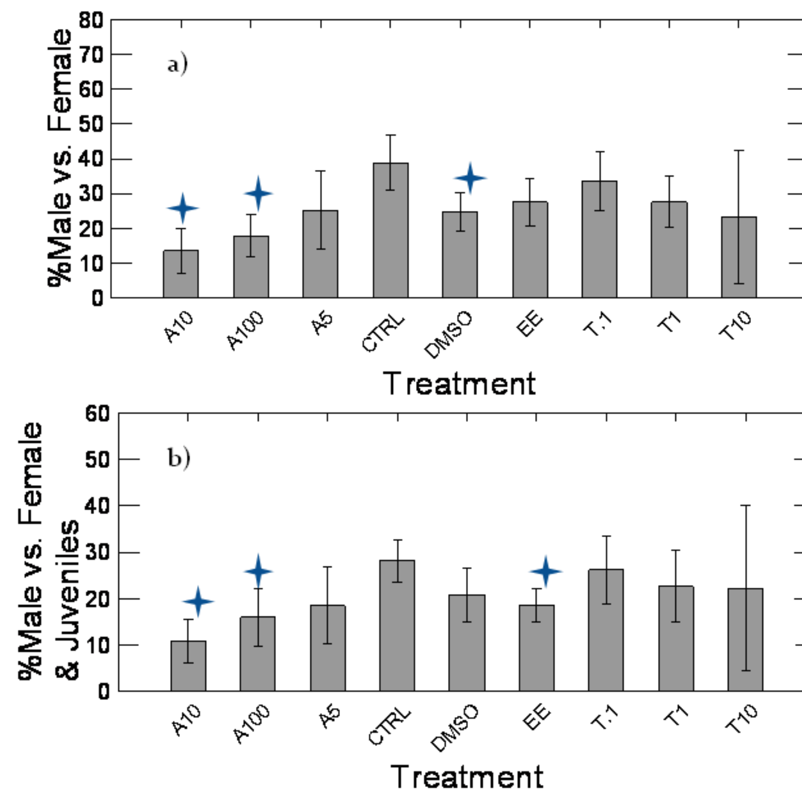

Figure 22: Gender ratios as %Male vs. a) females b) females and juveniles. Error bars represent Standard Error, p-value = 0.6827. Stars represent values significantly different than control.

Hyalella azteca during a 42-day chronic assay.

3.23 Gender ratio

Basic Statistics

When basic statistics finding standard deviations between means of the varying treatments, there appears to be no difference (p=0.6827; Graph not shown). However, when standard error with a p-value of 0.6827 is used as shown in Figure 22, there appears to be some significant differences. When comparing the ratio adult males to adult females as shown in a), atrazine at 100 and 10 µg/L and by a thin margin as well, the carrier control DMSO; tend to favour a larger female population. Because the carrier control is not to have any effect on gender ratios, these results may come across as erroneous. When juveniles are factored into the equation as shown in Figure 22 b), atrazine at 100 and 10 µg/L and EE at 0.1 µg/L tend to favour an increased female population. When standard error is used with a higher p-value (0.9) (Figure not shown), it is found that atrazine at 10 µg/L significantly causes a decrease in the male population vs. Females and juveniles. There does not appear to be a dose-response trend with these results, as the organisms appear to be most sensitive to the concentration of 10 µg/L. The reason for this is unknown.

Advanced Statistics

Data were checked for normality using a One-sample KS Lilliefors test and was found to be normally distributed (p-value of 0.709). The data was also checked for equality of variance using Levene's test. Day and Treatment were found to have equal variances (p = 0.3 and 0.551, respectively). Therefore, the data followed the assumptions for parametric testing. However, after running an ANOVA, and Kruskal-Wallis for additional statistical satisfaction, it was found that there was no difference between treatments (p=0.392, 0.380 for ANOVA and KW, respectively).

Summary

Using the most conservative statistical analysis, there appears to be no significant difference between treatments. Despite the slight population effects, it can be stated that atrazine and tributyltin after a 42 day bioassay, appear to cause no significant gender bias in populations of *Hyalella azteca*.

3.24 Juvenile data

Basic statistics

The rationale for studying juvenile length is that it can gauge the developmental effects of a chemical on an organism. For example, if a chemical acts as a growth inhibitor, there would be more organisms in a treatment classified as juveniles than adults. It could also be the case that a compound increases fertility. The amount of juveniles in conjunction with the brood pouch data could give information on the ability of a compound to induce fertility. Juveniles were also

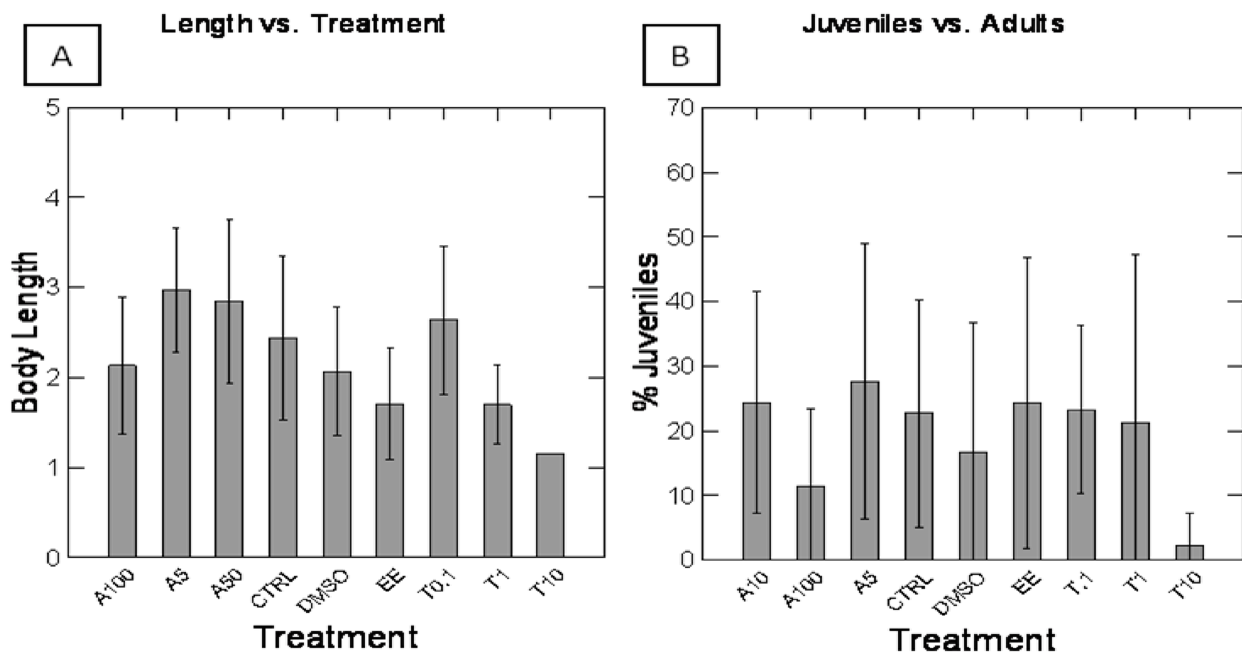

Figure 23: Physiological effects of the various treatments on juvenile *Hyalella azteca* by treatment. **Figure 3.3A:** The effect of each treatment on average juvenile length. **Figure 3.3B:** The effect of each treatment on the percentage of juveniles at the end of the 42d assay.

classified as such when they could not be differentiated as male or female. Length of juveniles also helps determine whether or not the chemical has an effect on sexual differentiation. If a chemical had an effect where it was able to delay the onset of sexual differentiation, while growth remained constant, juveniles would be larger than average in a population. An undifferentiated organism could also be a form of imposex in *Hyalella*. Lastly, if a compound had the ability to accelerate growth rates, there would be less juveniles overall in that population, as they would have grown and differentiated.

Figure 23 a) shows the average juvenile lengths and their respective standard deviations ($p=0.6827$). There appears to be no significant difference between treatments when data is analyzed with this method. TBT at 10 µg/L only had one juvenile in the whole experiment, so there is not enough information to make a conclusion based on that result. Atrazine at 5 µg/L was found to have a slight increase, while atrazine at 100 µg/L and EE were found to have a slight decrease on the average length of juveniles. This would indicate that sexual differentiation becomes prolonged when juveniles are found to be longer on average. Although EE, T1 and T10 showed a decrease in average juvenile length, these were found to be insignificant. A decrease in average length would indicate enhanced sexual differentiation at a younger age. Figure 23 b) shows the average percentage of juveniles in the population with their respective standard deviations ($p=0.6827$). TBT at 10 µg/L has a significantly decreased proportion of juveniles, no doubt due to its toxicity, not necessarily for any endocrine disruption events.

When standard error with a p-value of 0.9 (data not shown) is used to analyze the results, EE, TBT at 10 and 1 µg/L have a significant decrease in the average juvenile lengths. But because these chemicals were not found to have any significant effect on gnathopod or brood pouch development, it is not conclusive that these effects are due to any endocrinological reason.

Summary

There appears to be no endocrinological reasoning for differences in juvenile length, however, TBT is known to be toxic, so it is very likely that the reduced percentage of juveniles is due to the toxic effects of TBT during a more sensitive life stage.

3.25 Acute data

Amplexes

Figure 24 shows the number of amplexes, or precopulation events that were counted over the course of the 42 day bioassay. Naturally, the number of occurrences of amplexus increase as the organisms get older. The largest numbers of amplexes go to DMSO on day 21, atrazine at 100 µg/L on day 28, EE on day 35 and DMSO on day 42. However, these values do not reflect overall values. Figure 25 shows the number of amplexes as a percentage of the sum. Interestingly enough, atrazine at 100 µg/L has a higher rate of amplexus than lower concentrations of atrazine for reasons unknown. The highest three concentrations of TBT have the fewest rate of amplexing. When you combine the amplex data with the data for death, you can see that TBT is also the most toxic compound in this study.

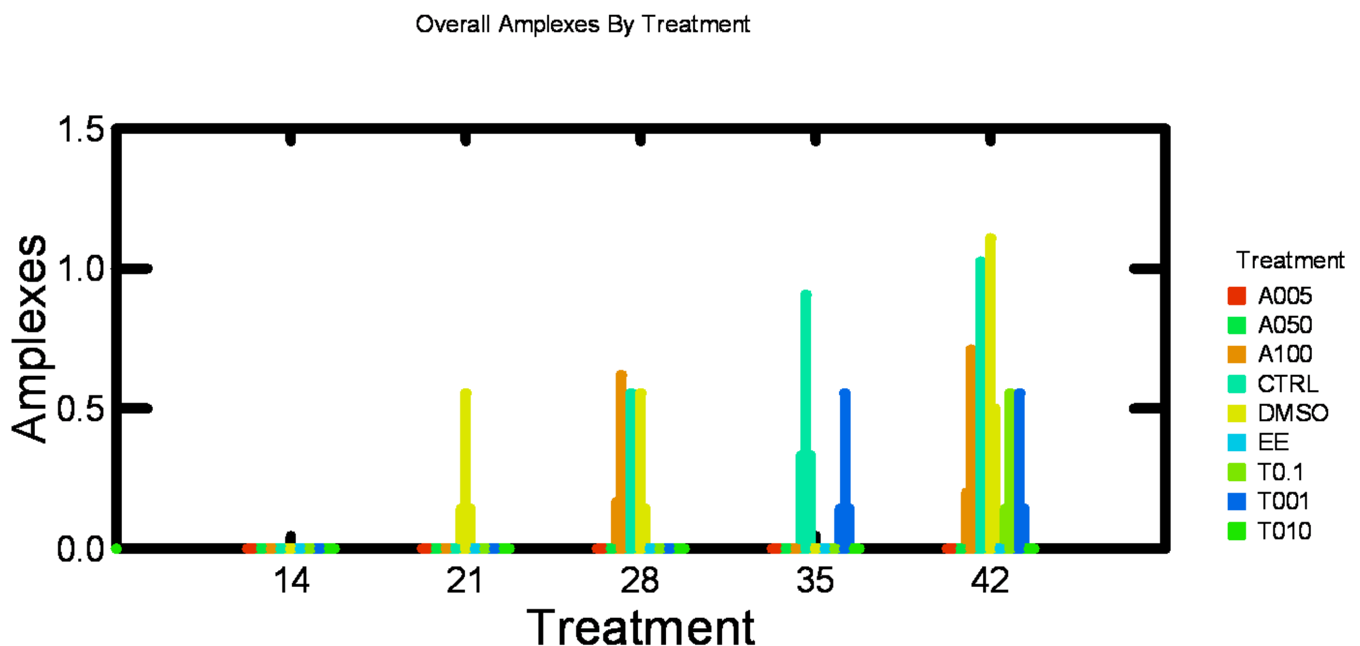

Figure 24: Number of amplexes by day of treatment

Figure 25 shows that the control treatments had the highest number of amplexes throughout the experiment, followed by DMSO at 0.1%, then atrazine at 100 µg/L, EE, TBT at 1 µg/L then TBT at 0.1 µg/L. The other concentrations had zero observed amplexes throughout the 42day assay. Also curious is that despite being more toxic, TBT at 1 µg/L has more observed amplexing than TBT at 0.1 µg/L for reasons unknown.

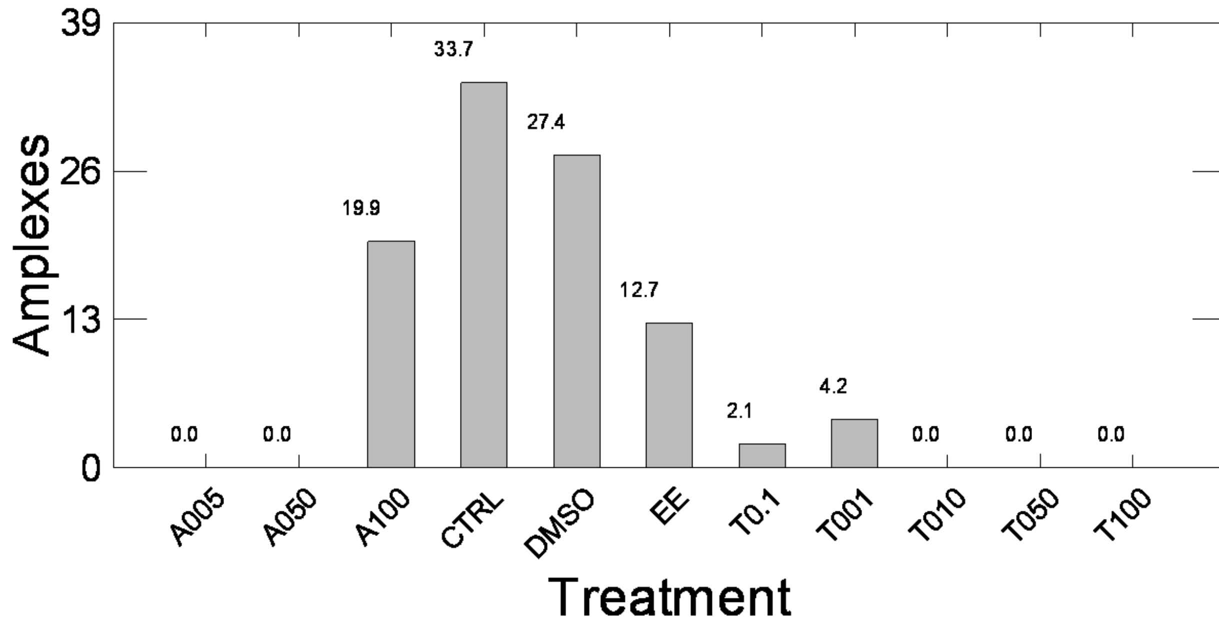

Figure 25: Number of amplexes as a percentage of the sum

Death

Figure 26 shows the overall figures for death throughout the 42 day bioassay. TBT is the most toxic substance in the bioassay in a seemingly dose-dependent manner. The reason the value for TBT at 50 µg/L is much higher than TBT at 100 µg/L, is due to the fact that an additional trial of TBT at 50 µg/L was run compared to TBT at 100 µg/L.

Naturally, the number of deaths increase over time (Fig. 26b). The bioassay likely becomes more stressful as time progresses. The number of individuals increases, while the amount of space stays the same, so the competition for grazing space increases throughout the experiment.

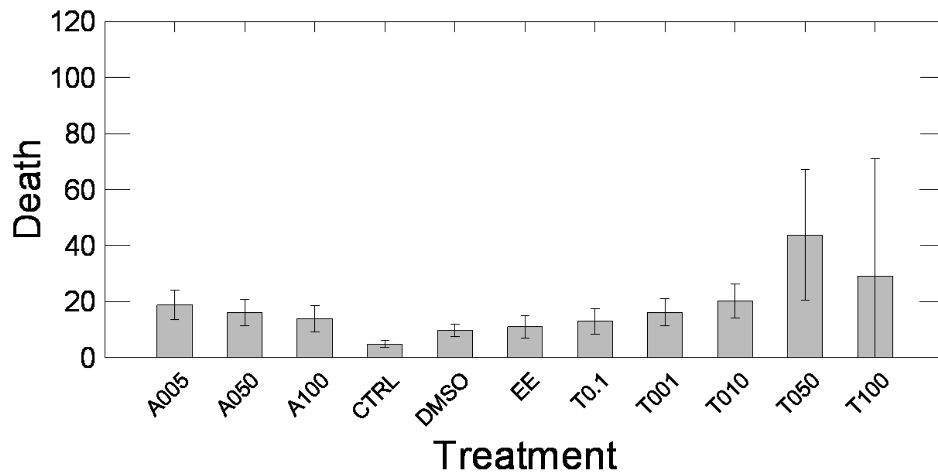

Figure 26: Overall occurrence of death. Error bars represent Standard Error (p=0.9)

Figure 26b: Death as a function of time by treatment

Grouping behaviour and sediment association

Figure 27 shows grouping behaviour over time and sediment association over time. The reason they are both together is because they share an interesting peak and valley pattern that may or may not be related. On day zero, time 0.12 (3Hr) there is a peak in the proportion of Hyalella in the sediment. The following day, there is a peak in the number found in groups. On day 14, there is a dip in the proportion of *Hyalella* found in the sediment, and the following week there is a dip in the proportion of *Hyalella* found in groups. The only logical reasoning behind this is that Hyalella within the sediment are more likely to group together than those swimming about the water column. The curious thing however, is the temporal pattern that emerges. There is no explanation for why there are less Hyalella in the sediment on day 14 than any other day of the bioassay.

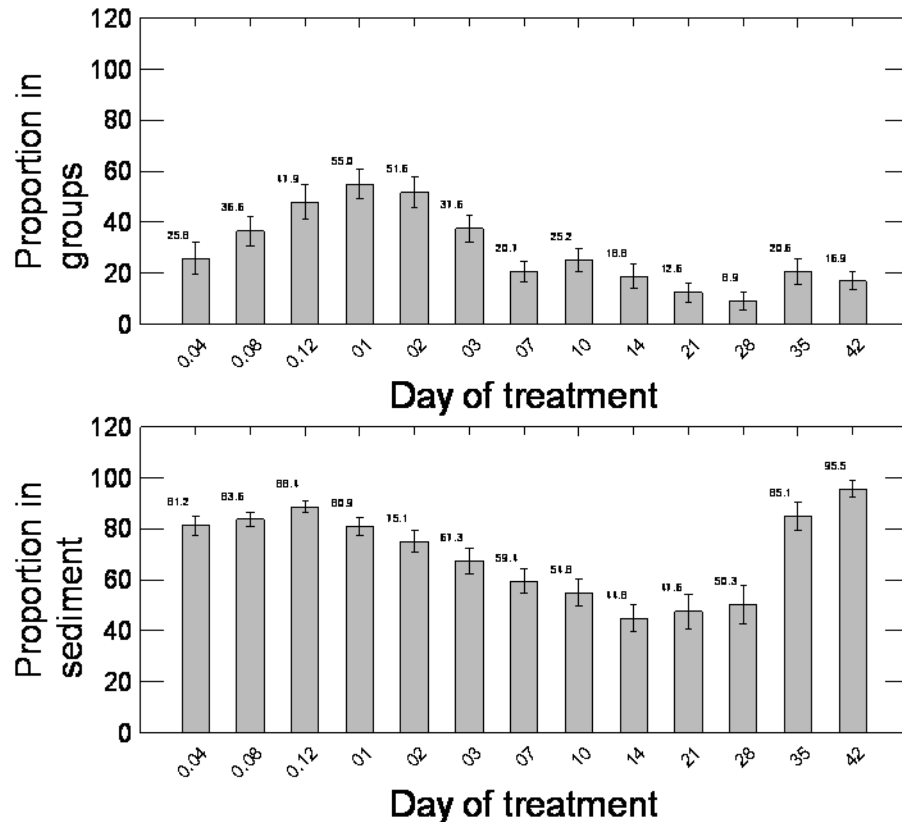

Figure 27: Proportion of Hyalella in groups and in sediment over time. Note that both graphs dip between day 14 and 28.

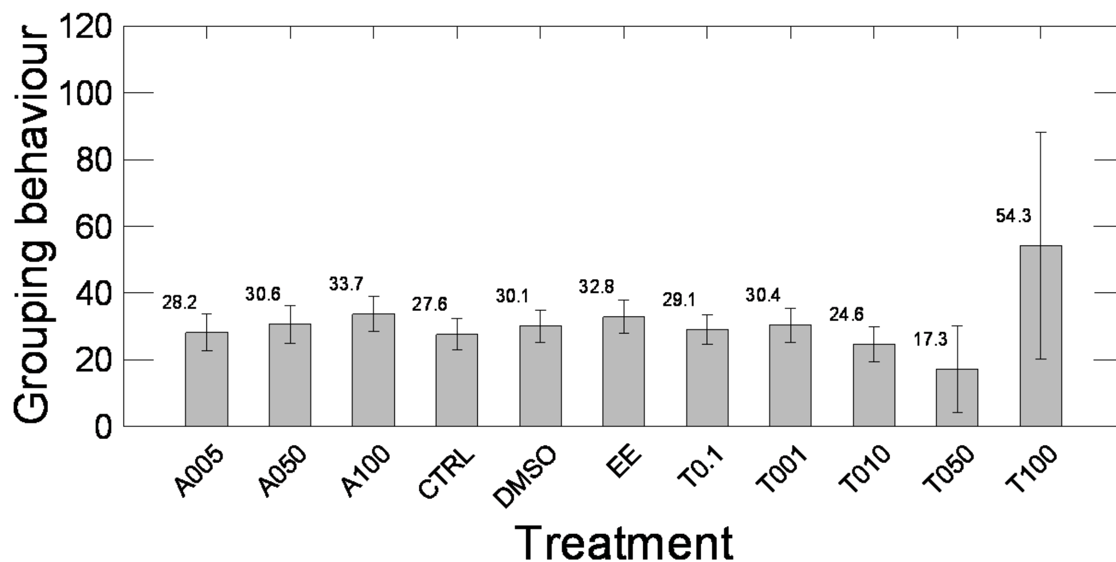

Figure 28: Grouping behaviour as a function of treatment. Standard Error (p=0.9).

Figure 28 shows grouping behaviour as a function of treatment, and it appears that there is no significant difference between any treatments and an increased tendency to group together, except for TBT at 100 µg/L which has a higher, yet insignificant proportion of *Hyalella* in groupings. This particular concentration of TBT is the most toxic used in this bioassay, so grouping behaviour could be toxic stress induced. Hatch and Burton (1999) claim that Hyalella tend to form larger groups when stressed. Being subject to the most toxic compound at the highest concentration in the bioassay could be considered stressful.

Figure 29 shows sediment association in the bioassay vessel as a function of treatment. There appears to be no significant correlation between any particular treatment and an increased likelihood of being found in the sediment, except for a slight, yet insignificant dip in TBT at 50 and 100 µg/L. TBT has a tendency to sorb to substrates rather than disperse throughout the water column, so it is possible that there is a slight decrease in sediment association in attempt to avoid the TBT.

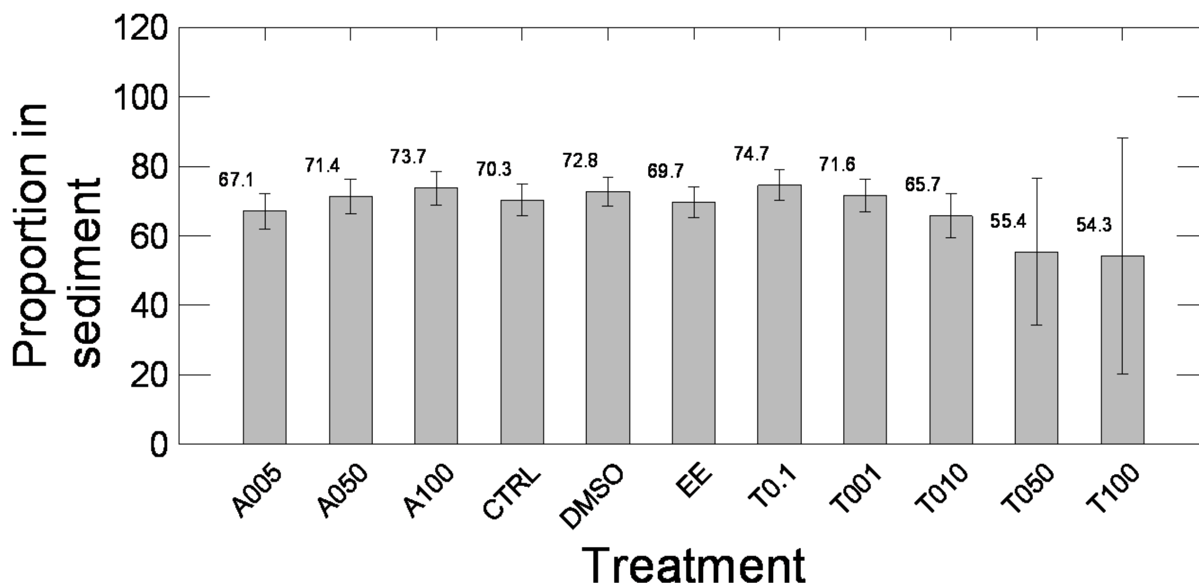

Figure 29: Sediment association as a function of treatment. Standard Error (p=0.9).

Figure 30 shows a weak, inverse association between death and the probability of finding *Hyalella* in the sediment. Toxic compounds appear to show a correlation with increased grouping behaviours and decreased sediment association. It appears that

Summary

Daphnia magna have the ability to create males and ephippial females under certain environmental conditions, but to manipulate these factors in a laboratory setting has to take many factors into consideration. The sheer complexity of the setup makes it difficult to run a relatively quick endocrine screening assay.

Atrazine and TBT appear to have no significant effect on the secondary sex characteristics of *Hyalella azteca*. Despite some slight hints at significance, the most conservative methods of statistical analysis show no connection. The acute results taken during the course of the assay show that TBT is both acutely and chronically toxic (See fig 26b). However, there appears to be no concrete evidence that it affects the brood pouch or secondary gnathopod of female and male Hyalella, respectively.

4.0 SUMMARY AND FUTURE DIRECTIONS

Summary

Two assays were attempted for the screening of potentially endocrine disruptive compounds. One assay was developed to utilize the water flea, *Daphnia magna*, the other developed to utilize the amphipod, *Hyalella azteca*. Only one assay was successful.

The *Daphnia magna* assay was quite complicated and intricate. It is always difficult working with live organisms, because their responses can be unpredictable, especially when systems are being manipulated that are poorly understood. Thus, much was learned about *Daphnia magna*, but unfortunately, not enough to find an adequate method to produce a supply of females with ephippial brood pouches and males to fertilize them. It could have proven to be a useful tool to detect various endocrine-acting compounds, but it came prematurely to an end.

A 42 day bioassay was used to determine the potentially endocrine disruptive effects of atrazine and tributyltin on *Hyalella azteca* and gain some insight into their reproductive and endocrine systems. The image analysis of female *Hyalella* brood pouches is a novel endpoint used in endocrine screening assays. Overall it was found that atrazine and tributyltin had no significant impact on the growth and development of secondary sexual characteristics in *Hyalella azteca*. It was found; however that TBT was quite toxic to *Hyalella* in a dose-response manner irrespective of any endocrine disruption.

Future Directions

This study opened a gateway into learning more about the endocrine systems of *Daphnia* and *Hyalella*. Currently, these systems are very poorly understood.

The underlying mechanisms behind cyclic parthenogenesis have yet to be elucidated. The chemical signals involved, the messenger systems, and the hormones involved all play a huge role, but it can be quite difficult working with such a small, sensitive organism. There is much

potential research that can be done with cladocerans as well as aphids to determine these underlying causes of the shift from female production to male production, and get a better understanding. When these systems are better understood, they could provide an invaluable tool to screen *in situ* drinking water systems for potential hazards to humans. It is possible to detect individual compounds, but when a fully-understood organism is placed *in situ*, it will be able to respond to any endocrine disrupting compound in a nonspecific way, so that if someone were to drink from that water source, they would at least know the potential dangers of drinking that water.

The 42-day assay is a staple within USEPAs arsenal for detecting chronic toxicity of certain compounds. However, it can be built upon and modified to allow for more endpoints so that it may become an even better tool, not just for studying compounds with long-term toxicity risks, but also for studying compounds with potential endocrine disrupting effects. Hopefully the analysis of gnathopods and brood pouches could be standardized and added to those and other protocols.

5.0 APPENDICES

Appendix A: Dilution Calculations

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

A 100 mL volume of 100 mg/L TBT in DMSO stock solution was made for use in teh bioassays
D = m/v
V = (100 mg TBT) / (1.103 g/cm^3)
= 0.09066 cm^3
= 0.09066 mL TBT in 1L DMSO
= 90.66 µL TBT in 1L DMSO
= 9.066 µL TBT in 100 mL DMSO
The stock solution was made by adding 9.066 µL TBT in 100 mL DMSO.

Preparation of 50 mg/L (TBT in DMSO) Substock
A 100 mL substock of 50 mg/L TBT in DMSO was also made for use in the bioassays.
$C_1V_1 = C_2V_2$
100 mg/L (V_1) = 50 mg/L (0.1 L)
V_1 = 0.05 L = 50 mL
The substock was made by adding 50 mL of the 100 mg/L substock to 50 mL of DMSO.

Preparation of 10 mg/L (TBT in DMSO) Substock
A 100 mL substock of 10 mg/L TBT in DMSO was also made for use in the bioassays.
$C_1V_1 = C_2V_2$
100 mg/L (V_1) = 10 mg/L (0.1 L)
V_1 = 0.01 L = 10 mL
The substock was made by adding 10 mL of the 100 mg/L substock to 90 mL of DMSO.

Preparation of 1 mg/L (TBT in DMSO) Substock
A 100 mL substock of 1 mg/L TBT in DMSO was also made for use in the bioassays.
$C_1V_1 = C_2V_2$
10 mg/L (V_1) = 1 mg/L (0.1 L)
V_1 = 0.01 L = 10 mL
The substock was made by adding 10 mL of the 100 mg/L substock to 90 mL of DMSO.

Preparation of 0.1 mg/L (TBT in DMSO) Substock
A 100 mL substock of 0.1 mg/L TBT in DMSO was also made for use in the bioassays.
$C_1V_1 = C_2V_2$
1 mg/L (V_1) = 0.1 mg/L (0.1 L)
V_1 = 0.01 L = 10 mL
The substock was made by adding 10 mL of the 100 mg/L substock to 90 mL of DMSO.

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

A 100 mL volume of 100 mg/L atrazine in DMSO stock solution was made for use in the bioassays.

10 mg of atrazine (powder) was added to 100 mL DMSO to make 100 mg/L atrazine in DMSO.

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

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

$C_1V_1 = C_2V_2$

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

V_1 = 0.05 L = 50 mL

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

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

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

$C_1V_1 = C_2V_2$

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

V_1 = 0.005 L = 5 mL

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

Preparation of 0.1 mg/L (EE in DMSO) Stock

A 100 mL volume of 0.1 mg/L EE in DMSO stock solution was made for use in the bioassays.

0.1 mg of EE (powder) was added to 100 mL DMSO to make 0.1 mg/L EE in DMSO.

Dilution calculations for Hyalella azteca bioasays

Test Concentration	Total Volume	TBT Substock Used	Volume Substock Added
0.1% DMSO	300 mL	DMSO	300 µL
100 µg/L	300 mL	100 mg/L	300 µL
50 µg/L	300 mL	50 mg/L	300 µL
10 µg/L	300 mL	10 mg/L	300 µL
1.0 µg/L	300 mL	1.0 mg/L	300 µL
0.1 µg/L	300 mL	0.1 mg/L	300 µL

Test Concentration	Total Volume	Atrazine Substock Used	Volume Substock Added
0.1% DMSO	300 mL	DMSO	300 µL
100 µg/L	300 mL	100 mg/L	300 µL
50 µg/L	300 mL	50 mg/L	300 µL
5 µg/L	300 mL	10 mg/L	300 µL

Test Concentration	Total Volume	EE Stock Used	Volume Substock Added
0.1% DMSO	300 mL	DMSO	300 µL
0.1 µg/L	300 mL	0.1 mg/L	300 µL

Appendix B: Acute data

LEGEND: Groupings: L=loose, M=medium, T=Tight, VT=Very tight; **Dissolved O_2** in mg/L, C1(#) = Cell 1, T(#) = Test, A(#) = All – See below

Treatment starting date: Tuesday April 20 – Day 1

Test Chamber:	Atrazine, 100 µg/L												
Day	1	2	3	24	48	72	168	240	336	504	672	840	1008
# of Amphipods:	20	20	20	20	20	18	17	17	0	x	x	x	x
On water surface	0	0	0	0	0	1	0	0	0				
In overlying water	0	0	0	0	3	0	0	0	0				
On top of sediment	4	3	3	0	2	1	1	1	0				
Within sediment	16	17	17	20	3	5	9	0	0				
In groupings	0	11	17	13	3	5	0	0	0				
Dead	0	0	0	0	0	2	1	0	0				
Dissolved O_2									7.6				
Comments	14 d: pH=8.11, 534 µS/cm												
Test Chamber:	17α-ethinylestradiol, 0.1 µg/L												
Day	1	2	3	24	48	72	168	240	336	504	672	840	1008
# of Amphipods:	20	20	20	20	20	20	19	19	19	19	19	5	7
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	2	1	1	1	1	1	0	0	0	0	0	0	0
On top of sediment	0	0	0	0	4	1	0	0	0	0	0	1	0
Within sediment				15	15	5	9	10	8	7	3	4	7
In groupings				12	15	5	0	0	0	4	2	2	2
Dead	0	0	0	0	0	0	1	0	0	0	0	0	0
Dissolved O_2 (mg/L)						12	7.5				T55	T105	T158
Comments	Algal development, not as intense as CTRL; pH=8.46; >750 = 3												
Test Chamber:	Tributyltin, 50 µg/L												
Day	1	2	3	24	48	72	168	240	336	504	672	840	1008
# of Amphipods:	20	20	20	17	5	1	0	x	x	x	x	x	x
On water surface	5	4	2	0	0	0	0						
In overlying water				0	1	0	0						
On top of sediment				0	0	0	0						
Within sediment				17	2	1	0						
In groupings				5	0	0	0						
Dead	0	0	0	3	12	4	1						
Dissolved O_2													
Comments													
Test Chamber:	Atrazine, 5 µg/L												
Time (h)	1	2	3	24	48	72	168	240	336	504	672	840	1008
# of Amphipods:	20	20	20	20	19	19	19	19	19	18	18	6	12
On water surface				0	0	0	0	0	0	0	0	0	0
In overlying water				0	1	1	0	0	0	0	0	0	0
On top of sediment			4	0	3	0	1	0	0	0	0	1	0
Within sediment				15	15	18	3	6	4	6	5	5	12
In groupings				15	12	18	0	3	0	0	0	3	4
Dead	0	0	0	0	1	0	0	0	0	1	0	0	0
Dissolved O_2											T56	T107	T160
Comments	Highest water clarity; pH = 8.39; >750 = 6												
Test Chamber:	Control – No treatment												
Day	1	2	3	24	48	72	168	240	336	504	672	840	1008
# of Amphipods:	20	20	20	20	20	20	18	18	18	18	18	18	16
On water surface				0	0	0	0	0	0	0	0	0	0
In overlying water				0	6	0	1	0	0	0	0	0	1

On top of sediment			6	0	2	2	0	0	1	0	0	3	3
Within sediment				16	8	6	4	2	6	4	6	12	12
In groupings				10	8	4	0	0	0	0	0	8	4
Dead	0	0	0	0	0	0	2	0	0	0	0	0	2
Dissolved O_2									7.7		T54	T106	T159
Comments	Significant algal development; pH = 8.45; >750 = 7; 497 uS/cm; ORP = 18.3; T = 21.2 deg. C												

Treatment starting date: Wednesday April 21 – Day 2

Test Chamber:	Tributyltin, 10 µg/L												
Day	1	2	3	24	48	72	168	240	336	504	672	840	1008
# of Amphipods:	20	20	20	18	18	18	18	17	16	2	2	2	2
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	10	6	3	2	0	0	0	0	0	0	0	0	0
On top of sediment	0	0	1	1	0	0	0	0	0	0	0	0	0
Within sediment	10	14	17	12	16	10	10	7	6	2	2	2	2
In groupings	0	0	0	9	14	10	10	0	2	0	0	0	0
Dead	0	0	0	2	0	0	0	1	1	14	0	0	0
Dissolved O_2										T16	T63	T111	A2
Comments	D1 – least active @3hr; D42 - Significant algal development; pH = 9.06; >750 = 1												
Test Chamber:	Control – DMSO, 0.1%												
Day	1	2	3	24	48	72	168	240	336	504	672	840	1008
# of Amphipods:	20	20	20	20	19	19	18	18	18	18	18	15	10
On water surface	0	0	0	0	0	0	1	0	0	0	0	0	0
In overlying water	4	4	3	1	0	0	1	0	0	0	0	0	3
On top of sediment	0	0	1	2	0	0	0	2	1	0	0	0	2
Within sediment	16	16	16	19	14	7	10	5	7	5	6	9	10
In groupings	4	4	4	15	14	3	6	0	2	0	0	3	0
Dead	0	0	0	0	1	0	1	0	0	0	0	3	5
Dissolved O_2	MH4 = 0.25ppm						6.9			T15	T64	T109	A3
Comments	Mild algal development; pH = 8.82; >750 = 5												
Test Chamber:	Atrazine, 50 µg/L												
Day	1	2	3	24	48	72	168	240	336	504	672	840	1008
# of Amphipods:	20	20	20	20	20	19	19	18	18	18	18	8	8
On water surface	0	0	0	0	1	0	0	0	0	0	0	0	0
In overlying water	2	1	1	0	0	0	0	0	0	0	0	0	0
On top of sediment	3	3	4	0	0	0	0	0	3	0	0	0	1
Within sediment	15	16	15	20	18	8	10	7	4	5	6	6	8
In groupings	8	8	8	20	18t	7	8t	0	3	3	0	2	2
Dead	0	0	0	0	0	1	0	1	0	0	0	10	0
Dissolved O_2											T65	T110	A4
Comments	Highest clarity; brown gauze; pH = 8.16; >750 = 3												
Test Chamber:	Tributyltin, 10 µg/L												
Day	1	2	3	24	48	72	168	240	336	504	672	840	1008
# of Amphipods:	20	20	20	20	20	17	16	13	0	x	x	x	x
On water surface	0	0	0	1	1	0	0	0	0				
In overlying water	2	1	1	2	0	0	0	0	0				
On top of sediment	3	2	2	5	0	0	1	0	0				
Within sediment	15	17	17	10	16	5	3	1	0				
In groupings	3	3	3	10	8	0	0	0	0				
Dead	0	0	0	0	0	3	1	3	13				
Dissolved O_2							6.0		TestA				
Comments	Least active ~3Hrs												
Test Chamber:	17α-ethinylestradiol, 0.1 µg/L												
Day	1	2	3	24	48	72	168	240	336	504	672	840	1008
# of Amphipods:	20	20	20	20	20	20	20	20	20	20	20	7	9
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	3	3	4	3	1	0	2	1	1	0	0	0	0

On top of sediment	0	0	1	2	0	0	2	1	7	0	2	2	2
Within sediment	17	17	15	15	14	10	16	14	1	10	5	4	9
In groupings	5	5	5	15	14	0	0	8	0	7	5	0	0
Dead	0	0	0	0	0	0	0	0	0	0	0	0	2
Dissolved O_2							8.6			T14	T62	T108	A1
Comments	Most active ~3Hrs; Second highest clarity; pH = 8.7; >750 = 3												

Treatment starting date: Thursday April 22 – Day 3

Test Chamber:	Tributyltin, 50 µg/L												
Day	1	2	3	24	48	72	168	240	336	504	672	840	1008
# of Amphipods:	20			2	1	0	x	x	x	x	x	x	x
On water surface	0	0	0	2d	0	0							
In overlying water	8	4	1	0	0	0							
On top of sediment	0	0	0	0	0	0							
Within sediment	11	16	19	2	1	0							
In groupings	10	12	14	0	0	0							
Dead	0	0	0	18	1	1							
Dissolved O_2						7.0							
Comments	Most active ~3Hrs, highly active												
Test Chamber:	Control – DMSO, 0.1%												
Day	1	2	3	24	48	72	168	240	336	504	672	840	1008
# of Amphipods:	20			19	18	18	17	17	17	17	15	10	17
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	2	2	2	3	1	1	0	1	0	0	0	0	0
On top of sediment	0	0	0	0	1	0	2	0	0	0	0	0	0
Within sediment	18	18	18	16	12	19	12	13	9	5	4	10	17
In groupings	10	10	10	9	8L	19L	0	3	9	0	0	2	3
Dead	0	0	0	1	1	0	1	0	0	0	2	5	0
Dissolved O_2							7.6			T24	T66	T112	A18
Comments	>750=7; pH=8.5												
Test Chamber:	Tributlytin, 10 µg/L												
Day	1	2	3	24	48	72	168	240	336	504	672	840	1008
# of Amphipods:	20			19	19	18	15	9	0	x	x	x	x
On water surface	0	1	0	1	0	0	0	0	0				
In overlying water	3	0	0	1	0	0	0	0	0				
On top of sediment	2	2	0	0	1	0	1	0	0				
Within sediment	15	17	20	16	12	15	3	1	0				
In groupings	8	10	15	5	6T	15	0	0	0				
Dead	0	0	0	1	0	1	3	6	9				
Dissolved O_2									T2				
Comments	Med. Activity ~3Hr; pH = 7.82; DO = 7.4; tds = 238 ppm; T = 21.07 deg.; Cond = 476 uS												
Test Chamber:	Control – DMSO, 0.1%												
Day	1	2	3	24	48	72	168	240	336	504	672	840	1008
# of Amphipods:	20			20	20	20	20	17	17	15	15	14	19
On water surface	0	0	0	0	0	0	0	1d	0	0	0	0	0
In overlying water	2	3	0	3	0	0	1	0	0	0	0	0	0
On top of sediment	1	2	0	0	0	0	2	2	0	1	0	0	0
Within sediment	17	16	20	17	17	12	12	8	13	9	4	4	19
In groupings	15	15	15	7	17t	12t	0	4	10	8	0	6	6
Dead	0	0	0	0	0	0	0	3	0	2	0	1	0
Dissolved O_2										T23	T67	T113	A19
Comments	1 amplex @ 42d; >750=8; pH=8.74												
Test Chamber:	Atrazine, 50 µg/L												
Day	1	2	3	24	48	72	168	240	336	504	672	840	1008
# of Amphipods:	20			19	19	18	18	17	17	15	13	9	6
On water surface	0	0	0	1	0	0	0	0	0	1	0	0	0
In overlying water	0	0	0	0	0	0	1	1	0	0	0	0	0

On top of sediment	0	0	0	1	0	0	1	2	0	0	0	0	0
Within sediment	20	20	20	17	18	16	14	13	9	3	3	9	6
In groupings	20	20	02	12	18t	16	5	5t	0	0	0	9	0
Dead	0	0	0	1	0	1	0	1	0	2	2	4	3
Dissolved O_2										T22	T68	T114	A20
Comments	Sunk to bottom → Least activity; >750=3; pH=8.62												

Treatment starting date: Friday April 23 – Day 4

Test Chamber:	Control – No treatment												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	19	19	19	19	19	17	16	22
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	2	2	2	2	0	0	0	0	0	0	0	0	0
On top of sediment	0	0	0	0	2	1	2	2	1	0	0	2	0
Within sediment	18	18	18	14	16	12	8	7	8	7	3	14	21
In groupings	0	0	0	14	16	9	3	3	0	0	3	4	6
Dead	0	0	0	0	0	1	0	0	0	0	2	1	0
Dissolved O_2								C1#3		T26	T82	T115	A21
Comments	>750=6; pH=8.32												
Test Chamber:	Tributyltin, 100 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			11	0	x	x	x	x	x	x	x	x
On water surface	0	0	0	5d	0								
In overlying water	5	5	3	0	0								
On top of sediment	0	0	0	0	0								
Within sediment	15	15	17	4	0								
In groupings	15L	15L	17L	4	0								
Dead	0	0	0	9	11								
Dissolved O_2					8.0								
Comments	Most active, fastest swimming												
Test Chamber:	17α-ethinylestradiol, 0.1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	19	19	18	12	12
On water surface	0	0	0	0	0	0	0	0	1d	0	0	0	0
In overlying water	3	3	1	2	0	0	0	1	0	0	0	0	0
On top of sediment	0	0	0	0	0	0	4	0	0	0	0	0	0
Within sediment	17	17	19	8	20	12	9	15	13	7	5	9	12
In groupings	17L	17L	19L	0	16T	8L	0	11T	13T	4	2	3	3
Dead	0	0	0	0	0	0	0	0	1	0	1	0	0
Dissolved O_2									T3	T27	T83	T116	A22
Comments	24Hr – Active under gauze; >750=5; pH=8.45												
Test Chamber:	Atrazine, 100 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	20	20	18	7	7
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	1	0	0	1	1	0	0	0	0	0	0	0	0
On top of sediment	3	2	2	2	0	2	0	0	0	0	0	0	0
Within sediment	17	18	18	17	17	17	12	17	16	13	3	7	7
In groupings	15	15	15	17t	15t	12	9t	14	13t	10t	0	3	3
Dead	0	0	0	0	0	0	0	0	0	0	2	0	0
Dissolved O_2										T28	T85	T118	A24
Comments	Least active, sink to bottom of beaker; Highest clarity; Brown gauze; >750=2; pH=8.03												
Test Chamber:	17α-ethinylestradiol, 0.1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	19	19	18	18	18	18	7	11
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	4	4	1	0	1	0	0	0	0	0	0	0	0

On top of sediment	0	0	1	1	1	1	2	1	0	0	0	3	2
Within sediment	16	16	17	18	16	16	9	11	8	9	7	4	11
In groupings	12	12	12	18t	12L	13t	3	10	2	3	2	3	4
Dead	0	0	0	0	0	1	0	1	0	0	0	0	0
Dissolved O_2										T29	T84	T117	A23
Comments	>750=9; pH=8.62												

Treatment starting date: Saturday April 24 – Day 5

Test Chamber:	Atrazine, 5 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	20	20	20	8	6
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	3	2	1	0	0	0	3	0	1	0	0	0	0
On top of sediment	0	0	0	2	1	2	2	1	0	0	2	1	0
Within sediment	0	0	0	8	13	12	8	14	8	8	5	8	4
In groupings	17	18	19	0	10L	5	3	11L	0	2	0	0	0
Dead	0	0	0	0	0	0	0	0	0	0	0	0	1
Dissolved O_2										T40	T88	T135	A48
Comments	Highest clarity; >750=4; pH=8.62												
Test Chamber:	Tributyltin, 1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	20	19	18	6	4
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	3	3	1	1	0	0	0	0	1	2	0	0	0
On top of sediment	3	3	3	0	1	1	3	0	2	0	0	0	0
Within sediment	14	14	16	19	12	12	10	16	10	7	3	6	4
In groupings	0	0	0	10L	12L	0	6	16VT	6	3	0	2	2
Dead	0	0	0	0	0	0	0	0	0	1	1	0	1
Dissolved O_2										T43	T86	T138	A49
Comments	pH = 8.6; >750=3												
Test Chamber:	Atrazine, 50 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	19	18	18	3	4
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	3	3	1	1	0	0	0	2	0	0	0	0	0
On top of sediment	1	1	1	1	0	0	2	0	4	0	0	0	2
Within sediment	16	16	18	15	13	6	15	14	10	5	4	3	4
In groupings	0	0	0	12t	13t	6L	10	12	9	2	0	0	0
Dead	0	0	0	0	0	0	0	0	1	1	0	0	0
Dissolved O_2										T41	T89	T136	A47
Comments	Brownest gauze; pH = 8.46; >750=1												
Test Chamber:	Tributyltin, 1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	18	18	17	2	6
On water surface	0	0	0	0	0	0	0	0	2d	0	0	0	0
In overlying water	4	4	1	0	0	0	3	0	1	1	0	0	0
On top of sediment	2	2	2	0	1	0	0	1	2	0	0	0	0
Within sediment	14	14	17	16	15	16	11	11	10	6	4	2	6
In groupings	3	3	4	11L	15L	14T	11T	7VT	7VT	2	2	0	0
Dead	0	0	0	0	0	0	0	0	2	0	1	1	1
Dissolved O_2	pH = 7									T44	T87	T139	A50
Comments	Most fouling; pH=8.6; >750=4												
Test Chamber:	Atrazine, 100 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	19	19	19	19	18	8	13
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	1	1	1	0	1	1	1	2	0	0	0	0	0

On top of sediment	0	0	0	0	0	0	1	0	1	0	0	0	2
Within sediment	19	19	19	20	18	14	6	12	11	7	7	8	11
In groupings	0	0	0	16VT	16T	12T	0	4	2	3	2	3	3
Dead	0	0	0	0	0	0	1	0	0	0	1	0	2
Dissolved O_2							C1#4			T42	T90	T137	A46
Comments	Low clarity; pH=8.2; >750=11 (1d)												

Treatment starting date: Sunday April 25 – Day 6

Test Chamber:	Control – No treatment												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	18	18	17	16	25
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	1	1	1	0	0	0	0	1	0	0	0	0	0
On top of sediment	2	1	1	1	1	0	0	0	0	0	0	1	0
Within sediment	17	18	18	15	18	17	14	19	7	11	6	15	25
In groupings	0	0	0	10	15T	13L	7	15L	2	2	2	3	3
Dead	0	0	0	0	0	0	0	0	2	0	1	1	1
Dissolved O_2	7.34					6.6			T9	T48	T93	T140	A51
Comments	pH = 8.63, >750=19												
Test Chamber:	Tributyltin, 10 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	17	13	10	0	x	x	x	x
On water surface	0	0	0	0	0	0	0	0	0				
In overlying water	2	2	2	0	1	0	0	0	0				
On top of sediment	0	0	0	0	0	0	0	0	0				
Within sediment	18	18	18	20	9	9	4	3	0				
In groupings	0	0	0	20L	0	9T	0	3	0				
Dead	0	0	0	0	0	3	4	3	10				
Dissolved O_2									T8				
Comments	Highly active ~3Hrs; swimming under gauze; pH=8.1; mV = -67												
Test Chamber:	Atrazine, 100 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	19	19	18	17	9	8
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	0	0	0	0	0	0	1	0	0	0	0	0	0
On top of sediment	2	2	2	0	0	0	0	0	0	0	0	1	0
Within sediment	18	18	18	10	10	12	14	9	10	8	4	8	8
In groupings	14	14	14	8L	6T	12L	11	7	6	7	2	3	3
Dead	0	0	0	0	0	0	0	1	0	1	1	0	0
Dissolved O_2						6.0				T47	T94	T141	A52
Comments	Brown gauze, pH=8.01, >750=5												
Test Chamber:	Tributyltin, 0.1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	20	20	19	4	6
On water surface	0	0	0	0	0	0	0	0	0	1	0	0	0
In overlying water	1	1	1	1	0	0	0	0	0	3	0	0	0
On top of sediment	1	1	1	1	1	0	0	1	0	0	0	0	0
Within sediment	18	18	18	18	12	11	9	8	11	5	4	4	6
In groupings	10	10	10	18	8t	8t	0	7	10	3	0	0	0
Dead	0	0	0	0	0	0	0	0	0	0	1	0	0
Dissolved O_2										T45	T91	T143	A53
Comments	pH=8.52, >750=5												
Test Chamber:	Tributyltin, 0.1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	20	19	18	5	11
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	0	0	0	0	0	0	1	0	0	0	0	0	0

On top of sediment	2	2	2	0	0	0	0	0	0	0	0	0	0
Within sediment	18	18	18	12	13	13	12	16	14	7	2	5	11
In groupings	8	8	8	6	10t	10t	8	10	6	4	0	0	0
Dead	0	0	0	0	0	0	0	0	0	1	1	0	0
Dissolved O_2										T46	T92	T142	A54
Comments	pH=8.34, >750=4												

Treatment starting date: Monday April 26 – Day 7

Test Chamber:	Control – No treatment												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	19	19	19	18	16	18
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	1	1	0	0	0	0	0	0	0	0	0	1	0
On top of sediment	1	1	0	0	0	0	0	0	0	0	0	1	0
Within sediment	18	18	14	14	16	17	12	11	8	2	6	0	18
In groupings	8	8	14L	12 t	15 t	14 t	0	9	0	0	0	15	3
Dead	0	0	0	0	0	0	0	0	1	0	1	1	0
Dissolved O_2							C15		T10	T49	T98	T148	A55
Comments	1 amplex@35d, pH=8.56, >750=15												
Test Chamber:	Control – DMSO, 0.1%												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	19	19	19	19	19	17	13	13
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	1	0	0	0	0	0	0	0	0	0	0	0	0
On top of sediment	1	1	0	1	0	0	0	1	0	0	0	1	1
Within sediment	18	19	20	14	8	14	7	7	7	3	6	12	12
In groupings	6	6	8	7 T	5 T	10 L	0	0	4	0	0	3	3
Dead	0	0	0	0	0	1	0	0	0	0	2	0	0
Dissolved O_2					4.5	5				T52	T99	T146	A56
Comments	pH=8.62, >750=11												
Test Chamber:	Atrazine, 50 µg/L - 1												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	19	18	18	18	18	18	10	9
On water surface	0	1	0	0	1	0	0	0	0	0	0	0	0
In overlying water	2	2	1	0	0	0	0	0	0	0	0	0	0
On top of sediment	2	1	0	0	0	0	0	1	0	0	0	1	0
Within sediment	16	16	19	17	5	11	8	6	4	2	3	9	9
In groupings	5	6	11L	15T	2	6	5	3	0	0	0	0	0
Dead	0	0	0	0	0	1	1	0	0	0	0	0	1
Dissolved O_2							C16			T50	T95	T144	A57
Comments	High clarity, brown gauze, pH=8.35, >750=7												
Test Chamber:	Atrazine, 50 µg/L – 2												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	19	18	16	16	7	8
On water surface	0	0	0	0	1	0	0	0	0	0	0	0	0
In overlying water	2	3	0	0	0	0	0	0	0	0	0	0	0
On top of sediment	1	1	0	1	1	0	0	1	0	0	0	0	1
Within sediment	17	16	20	15	15	12	12	5	6	2	3	7	8
In groupings	17	16	20 T	13 T	12 T	7 T	0	0	0	0	0	4	3
Dead	0	0	0	0	0	0	0	1	1	2	0	0	0
Dissolved O_2									T11	T51	T96	T145	A58
Comments	High clarity, brown gauze, pH=8.15, >750=7												
Test Chamber:	Tributyltin, 0.1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	20	20	18	7	6
On water surface	0	1	0	0	0	0	0	0	0	0	0	0	0
In overlying water	3	6	0	1	1	0	0	0	0	0	0	0	0

On top of sediment	1	2	0	0	0	0	3	0	0	0	0	0	0
Within sediment	16	11	20	14	16	19	19	18	16	11	6	7	6
In groupings	6	8	20 T	8 T	12 T	15 VT	5	18 T	13 T	9 T	2	2	1
Dead	0	0	0	0	0	0	0	0	0	0	2	0	1
Dissolved O_2										T53	T97	T147	A59
Comments	pH=8.62, >750=5												

Treatment starting date: Tuesday April 27 – Day 8

Test Chamber:	Atrazine, 5 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	18	18	18	18	17	15	14	5	6
On water surface	0	0	0	0	0	0	0	1	0	0	0	0	0
In overlying water	0	0	1	0	0	1	1	2	0	0	0	0	0
On top of sediment	1	1	1	0	1	0	0	0	1	0	2	1	0
Within sediment	19	19	18	16	7	11	7	4	5	5	4	4	4
In groupings	10	11	12	12	0	3 L	5	0	0	0	3	0	2
Dead	0	0	0	0	2	0	0	0	1	2	1	0	0
Dissolved O_2									T12	T57	T104	T162	A71
Comments	Loose groupings, 2nd highest clarity, pH=8.33, >750=4												
Test Chamber:	Tributyltin, 1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	19	19	19	18	18	15	15	8	9
On water surface	0	0	0	1	1	0	0	0	0	0	0	0	0
In overlying water	0	0	1	1	0	0	1	1	0	0	0	1	0
On top of sediment	3	2	1	0	0	3	0	1d	1	0	0	0	0
Within sediment	17	18	18	16	18	9	15	11	11	4	3	7	9
In groupings	7	6	6	13 VT	15 VT	0	5	0	11	0	2	0	0
Dead	0	0	0	0	1	0	0	1	0	3	0	0	0
Dissolved O_2					5.8			T5		T58	T101	T164	A72
Comments	24Hr grouping – super tight <0.5cm^2, pH=8.65, >750=6												
Test Chamber:	Atrazine, 100 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	18	18	17	14	14	7	3
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	0	0	0	0	1	0	0	0	0	0	0	0	0
On top of sediment	2	2	3	1	0	0	0	0	0	0	0	0	0
Within sediment	18	18	17	20	16	14	14	7	4	3	3	7	3
In groupings	6	7	6	6 T	4 T	5	4	0	0	0	3t	0	0
Dead	0	0	0	0	0	0	2	0	1	3	0	0	1
Dissolved O_2									T13	T60	T100	T161	A70
Comments	24, 48 Hr grouping – tight, Highest clarity, brown gauze, pH=8.3, >750=3												
Test Chamber:	Tributyltin, 0.1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	20	19	19	6	16
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	0	1	1	1	0	0	1	0	0	0	0	0	0
On top of sediment	2	2	2	0	1	2	2	2	0	0	0	0	0
Within sediment	18	17	17	11	10	12	12	9	9	5	6	6	16
In groupings	0	0	0	10 L	10 T	7	7	7 T	9	2	3	0	0
Dead	0	0	0	0	0	0	0	0	0	1	0	0	0
Dissolved O_2										T59	T103	T165	A73
Comments	1 amplex @42d, pH=8.93, >750=11												
Test Chamber:	Control - DMSO, 0.1%												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	19	18	18	18	18	18	11	14
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	0	0	2	0	0	0	0	0	0	0	0	0	0

On top of sediment	2	2	1	0	0	0	1	1	0	0	2	1	0
Within sediment	18	18	17	20	20	15	6	9	9	6	9	10	14
In groupings	15	15	15	12 M	0	0	0	9	0	3	2	3	2
Dead	0	0	0	0	0	1	1	0	0	1	0	0	0
Dissolved O_2	6.9 mg/L, 76%			6		5.6	C17			T61	T102	T163	A69
Comments	1 amplex@42d, pH=8.54, >750=11												

Treatment starting date: Wednesday April 28 – Day 9

Test Chamber:	Tributyltin, 1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	19	18	16	15	15	2	6	4
On water surface	0	0	0	0	0	3	0	0	0	0	0	1	0
In overlying water	3	3	0	0	0	1	0	0	0	1	0	0	0
On top of sediment	3	3	0	0	0	2	1	1	0	0	1	0	1
Within sediment	14	14	20	17	14	8	11	6	6	5	1	6	4
In groupings	14	14	18VT	11t	6L	0	11	3	0	0	0	0	0
Dead	0	0	0	0	0	1	1	2	1	0	1	0	0
Dissolved O_2									T19	T71	T121	A7	A76
Comments	pH=8.72, >750=4												
Test Chamber:	Atrazine, 5 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	19	19	18	16	4	3	4
On water surface	0	0	0	0	0	0	1d	0	0	0	0	0	0
In overlying water	0	1	0	0	0	0	1	0	0	0	0	0	0
On top of sediment	0	0	0	0	0	2	0	0	0	0	1	0	0
Within sediment	20	20	20	12	13	11	12	10	6	5	3	3	4
In groupings	20L	20	20T	9	5	5	11	3	4	2	0	0	0
Dead	0	0	0	0	0	0	1	0	1	2	0	1	0
Dissolved O_2	5.5								T21	T70	T120	A6	A74
Comments	pH=8.60, >750=3												
Test Chamber:	Tributyltin, 10 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	16	11	0	x	x	x	x
On water surface	0	0	0	0	0	1	0	0	0				
In overlying water	0	0	0	0	0	1	0	0	0				
On top of sediment	2	2	0	0	0	0	1	0	0				
Within sediment	18	18	20	16	15	15	8	3	0				
In groupings	11	12	12	6	11T	12	2	3	0				
Dead	0	0	0	0	0	0	4	5	11				
Dissolved O_2									T20				
Comments	pH = 8.47; -81mV												
Test Chamber:	Tributyltin, 0.1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	19	18	18	18	18	6	7	7
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	4	5	0	0	0	0	0	0	0	0	0	0	0
On top of sediment	1	1	0	0	1	7	0	0	0	0	0	0	0
Within sediment	15	14	20	14	16	3	10	10	6	5	6	7	7
In groupings	0	5	7L	8T	12	3	9	9VT	4	2	0	2	0
Dead	0	0	0	0	0	1	1	0	0	0	1	0	0
Dissolved O_2	7.0								T18	T72	T122	A8	A77
Comments	pH=8.67, >450=5												
Test Chamber:	17α-ethinylestradiol, 0.1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	19	19	19	19	19	6	6	11
On water surface	0	0	0	0	0	1d	0	0	0	0	0	0	0
In overlying water	2	2	1	2	0	0	0	1	0	0	0	0	0

On top of sediment	2	1	0	0	0	1	0	0	0	0	0	0	0
Within sediment	16	17	19	17	18	9	10	10	14	9	6	6	8
In groupings	10	17L	19T	9	17	3	6	6	14	3	3	2	2
Dead	0	0	0	0	0	1	0	0	0	0	0	0	1
Dissolved O_2									T17	T69	T119	A5	A75
Comments	pH=8.58, >750=8												

Treatment starting date: Thursday April 29 – Day 10

Test Chamber:	Atrazine, 5 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	19	19	19	19	17	16	8	7	6
On water surface	0	0	0	0	0	0	1	0	0	0	0	0	0
In overlying water	3	3	2	1	0	1	0	0	0	0	0	0	0
On top of sediment	2	0	0	0	0	0	0	0	0	0	0	0	0
Within sediment	15	17	18	9	15	13	8	9	4	4	8	7	6
In groupings	0	10	10VT	0	13	7	4	3	0	0	0	0	0
Dead	0	0	0	0	1	0	0	0	2	1	1	1	0
Dissolved O_2	7.8								T25		T125	A27	A79
Comments	pH=8.02, >750=11												
Test Chamber:	Tributyltin, 1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	20	20	8	11	11
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	2	0	0	0	0	1	0	0	0	0	0	0	0
On top of sediment	1	2	1	1	0	0	0	0	0	0	0	0	0
Within sediment	17	18	19	15	13	4	15	8	8	7	8	11	11
In groupings	0	6	10	0	0	7	15T	3	5	0	3	4	3
Dead	0	0	0	0	0	0	0	0	0	0	1	0	0
Dissolved O_2	6.8								T26		T123	A26	A80
Comments	pH=8.02, >750=11												
Test Chamber:	Control – No treatment												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	18	18	16	18	18
On water surface	0	0	0	0	0	0	0	1	0	0	0	0	10
In overlying water	3	0	0	0	0	0	2	0	0	0	0	0	1
On top of sediment	2	0	0	0	0	2	1	0	0	0	3	0	1
Within sediment	15	20	20	9	15	15	12	11	6	7	12	18	18
In groupings	0	10	10T	0	10T	7	4	6	0	0	4	6	6
Dead	0	0	0	0	0	0	0	0	2	0	0	0	0
Dissolved O_2	6.0					C1#8			T27		T124	A25	A78
Comments	1 amplex@42d, pH=8.48, >750=12												

Treatment starting date: Friday May 7 – Day 11.1

Test Chamber:	Control – No treatment												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	20	17	17	17	31
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	0	2	2	0	0	0	2	0	0	6	0	0	0
On top of sediment	2	0	1	1	2	3	3	3	3	2	2	1	1
Within sediment	18	18	17	18	17	16	12	11	10	11	10	14	29
In groupings	0	2	2	4	3	3L	4	4	3	3	4	3	9
Dead	0	0	0	0	0	0	0	0	0	0	0	0	0
Dissolved O_2							T35		T76	T126	A28	A90	A117
Comments	Active below surface of gauze; 14d – 1 amplex; 35d – 1 amplex, Amplex d42=4; pH=8.41; >750=17												
Test Chamber:	Control - DMSO, 0.1%												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	20	15	14	14	19

On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	1	2	3	1	1	0	1	1	0	2	1	0	0
On top of sediment	3	2	3	0	3	5	6	4	2	0	1	0	0
Within sediment	17	16	14	17	17	16	10	12	17	13	12	12	19
In groupings	0	2	2	8	9	10L	5	5	5	5L	0	0	0
Dead	0	0	0	0	0	0	0	0	0	0	1	0	0
Dissolved O_2							T36		T79	T130	A29	A91	A118
Comments	Active below surface of gauze; Amplex d42=1; pH=7.95; >750=18												
Test Chamber:	17α-ethinylestradiol, 0.1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	20	14	14	9	7
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	5	4	4	2	1	0	0	0	0	0	1	0	0
On top of sediment	3	3	4	0	0	1	2	2	2	0	2	0	0
Within sediment	12	13	12	18	18	17	12	13	13	14	14	9	7
In groupings	0	0	0	3	3	3	6L	8	10	0	0	0	0
Dead	0	0	0	0	0	0	0	0	0	0	0	0	1
Dissolved O_2							T31		T75	T131	A33	A92	A119
Comments	Most active, not settled <3Hrs; inactive at 72Hrs; pH=5.34; >750=4												
Test Chamber:	Tributyltin, 10 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	19	10	10	9	13
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	0	2	1	0	0	0	1	0	0	0	1	0	0
On top of sediment	2	2	0	0	0	1	2	1	0	0	1	2	0
Within sediment	18	16	19	20	18	16	12	12	12	10	10	9	13
In groupings	0	2	3	14T	15	15L	4	4	5	4	0	0	0
Dead	0	0	0	0	0	0	0	0	1	0	0	0	0
Dissolved O_2							T37		T80	T128	A30	A93	A120
Comments	Active below surface of gauze; slow; d35-most algae; pH=7.09; >750=12												
Test Chamber:	Tributyltin, 1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	20	9	4	11	35
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	1	1	3	0	0	0	0	0	0	0	0	0	0
On top of sediment	1	1	2	0	0	0	0	0	0	0	0	0	0
Within sediment	18	18	15	18	17	15	18	17	16	9	9	11	35
In groupings	0	2	3	16T	15L	15L	9T	11	12	3L	3	5	6
Dead	0	0	0	0	0	0	0	0	0	0	0	0	0
Dissolved O_2							T34		T78	T127	A31	A94	A121
Comments	Active below surface of gauze; 35d-1amplex; pH=7.80; >750=15												

Treatment starting date: Friday May 7 – Day 11.2

Test Chamber:	Tributyltin, 0.1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	20	10	9	9	17
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	0	1	2	0	0	0	0	0	0	0	1	0	0
On top of sediment	2	2	0	0	0	0	0	0	0	0	0	0	0
Within sediment	18	17	18	14	15	14	9	9	10	10	9	9	17
In groupings	0	2	4	9	7	6	4	4	4	0	0	2	2
Dead	0	0	0	0	0	0	0	0	0	0	1	0	3
Dissolved O_2							T39		T81	T129	A32	A95	A122
Comments	pH=5.34; >750=14												
Test Chamber:	Atrazine, 100 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	20	20	4	6	6	5

On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	0	3	0	0	1	1	1	1	0	0	0	0	0
On top of sediment	2	3	2	1	0	2	2	2	1	0	0	0	0
Within sediment	18	14	18	19	15	13	12	10	7	4	6	6	5
In groupings	0	2	3	17	12	7	8	6	2	0	0	0	2
Dead	0	0	0	0	0	0	0	0	0	0	0	0	0
Dissolved O_2							T33		T77	T134	A34	A96	A123
Comments	Least active, slowest moving <3Hr; d35-highest clarity; d42-amplex=1; pH=5.28; >750=5												
Test Chamber:	Atrazine, 50 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	19	18	5	4	4	4
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	0	0	1	0	0	0	0	0	0	0	0	0	0
On top of sediment	1	1	0	0	0	0	0	0	2	0	0	0	0
Within sediment	19	19	19	18	15	11	11	7	5	4	4	4	4
In groupings	0	3	5	11T	11	8T	3T	3	2	0	0	0	0
Dead	0	0	0	0	0	0	0	1	1	0	0	0	0
Dissolved O_2							T32		T74	T133	A35	A97	A124
Comments	Most settled – Sank to bottom, immediate inactivity; pH=5.39; >750=4												
Test Chamber:	Atrazine, 5 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	20			20	20	20	20	16	14	4	4	2	0
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	2	2	1	2	0	0	0	0	0	0	0	0	0
On top of sediment	1	0	1	0	0	0	2	0	0	0	0	0	0
Within sediment	17	18	18	14	18	17	12	6	3	4	4	2	0
In groupings	0	2	3	7	12	11	6L	3	0	0	0	0	0
Dead	0	0	0	0	0	0	0	4	2	1	0	0	0
Dissolved O_2							T30		T73	T132	A36	A98	A125
Comments	Active below surface of gauze; pH=4.89; >750=2(d)												

Treatment starting date: Monday May 31 – Day 12.1

Test Chamber:	Control – No treatment												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	53			53	53	53	53	53	53	53	53	53	55
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	0	0	0	0	0	0	1	3	1	0	2	2	0
On top of sediment	0	0	0	0	1	0	1	2	7	3	1	3	0
Within sediment	53	53	53	53	52	53	51	48	45	50	50	48	55
In groupings	0	18	23L	53T	40L	26L	8	8	25L	45	50	12	18
Dead	0	0	0	0	0	0	0	0	0	0	0	0	0
Dissolved O_2	T149			T166	A9	A57	A60	A81	A99	A108	A126	A135	A144
Comments	28d-Algal development, most secure amplexes, amplex@d42=5, pH=8.2, >750=38												

Test Chamber:	Control - DMSO, 0.1%												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	52			52	52	52	52	52	52	50	52	48	34
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	5	2	3	4	0	1	1	2	0	0	1	1	0
On top of sediment	5	4	3	3	1	1	1	7	11	4	2	5	0
Within sediment	42	46	46	45	51	50	50	43	41	46	49	42	34
In groupings	6	18	46	45L	51L	15L	5	5	8	3	5	6	10
Dead	0	0	0	0	0	0	0	0	0	0	0	4	14
Dissolved O_2	T150			T17	A10	A38	A61	A82	A100	A109	A127	A136	A145
Comments	21d-1amplex, 28d-1amplex, 42d-5 amplex, pH=7.4, >750=18												
Test Chamber:	17α-ethinylestradiol, 0.1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d

# of Amphipods:	55			55	55	55	55	55	55	55	55	55	67
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	4	5	6	0	1	1	2	2	2	0	0	3	0
On top of sediment	3	2	1	0	0	0	1	3	11	0	0	0	0
Within sediment	48	48	48	55	54	54	52	50	42	12	12	16	67
In groupings	5	7	43	55	29	15	9	10	8	8	7	6	12
Dead	0	0	0	0	0	0	0	0	0	0	0	0	0
Dissolved O_2	T151			T171	A14	A42	A68	A89	A107	A116	A134	A143	A153
Comments	28d-2amplex, 35d-2amplex, 42d-2amplex, most active during counting, pH=7.8, >750=28												
Test Chamber:	Tributyltin, 10 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	54			54	54	54	54	52	51	49	49	49	5
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	5	3	2	0	0	0	0	0	0	0	0	0	0
On top of sediment	3	4	3	0	0	0	1	0	2	1	1	1	0
Within sediment	46	47	49	54	54	54	51	6	9	2	1	3	5
In groupings	17	19	31	30	11	5	2	2	0	0	0	0	2
Dead	0	0	0	0	0	0	0	2	1	2	0	0	0
Dissolved O_2	T155			T172	A15	A39	A65	A86	A104	A113	A131	A140	A149
Comments	Lowest visibility@10d, pH=7.12, >750=5												
Test Chamber:	Tributyltin, 1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	51			51	51	51	51	50	48	46	45	44	7
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	0	0	0	0	0	0	0	0	0	0	0	0	0
On top of sediment	5	2	1	0	1	1	1	0	0	0	0	0	0
Within sediment	46	49	50	51	50	50	50	50	6	1	2	3	7
In groupings	0	30	46	51	50	10	0	4	0	0	0	0	2
Dead	0	0	0	0	0	0	0	1	2	2	1	1	37
Dissolved O_2	T156			T173	A16	A40	A66	A87	A105	A114	A132	A141	A150
Comments	pH=6.95, >750=7												

Treatment starting date: Monday May 31 – Day 12.2

Test Chamber:	Tributyltin, 0.1 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	53			53	53	53	53	53	53	53	53	50	13
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	0	0	3	0	0	0	4	0	0	0	0	0	0
On top of sediment	5	4	1	0	1	0	2	7	6	0	0	0	0
Within sediment	48	49	49	53	52	53	51	46	47	53	53	50	13
In groupings	0	12	37	53	52	9	3	4	3	3	2	2	3
Dead	0	0	0	0	0	0	0	0	0	0	0	3	37
Dissolved O_2	T157			T174	A17	A41	A67	A88	A106	A115	A133	A142	A151
Comments	pH=7.14, >750=11												
Test Chamber:	Atrazine, 100 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	50			50	50	50	50	50	50	50	50	50	29
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	6	3	5	0	0	0	1	1	0	0	0	0	0
On top of sediment	2	1	1	0	0	0	1	2	9	7	2	1	3
Within sediment	42	46	44	50	50	50	48	47	41	43	48	49	26
In groupings	3	31	44	50	50	27	3	0	0	5	6	12L	0
Dead	0	0	0	0	0	0	0	0	0	0	0	0	31
Dissolved O_2	T152			T168	A11	A43	A62	A83	A101	A110	A128	A137	A146
Comments	14d-Brown gauze, 28d-1amplex, Amplex@d42=5; pH=6.12, >750=27												
Test Chamber:	Atrazine, 50 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d

# of Amphipods:	51			51	51	51	51	51	51	49	47	8	11
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	3	1	1	0	2	0	1	1	0	0	0	2	0
On top of sediment	4	2	2	1	5	3	0	6	0	0	0	0	0
Within sediment	44	48	48	50	44	48	50	48	51	49	47	6	11
In groupings	0	16	30	25	0	0	0	12	0	0	0	6	3
Dead	0	0	0	0	0	0	0	0	0	2	2	39	0
Dissolved O_2	T153			T169	A12	A44	A63	A84	A102	A111	A129	A138	A147
Comments	14-35d, seems empty despite lack of carcasses – cannibalism?; pH=6.8, >750=8												
Test Chamber:	Atrazine, 5 µg/L												
Day	0-3 Hr			24 Hr	48 Hr	72 Hr	7 d	10 d	14 d	21 d	28 d	35 d	42 d
# of Amphipods:	50			50	50	50	50	50	50	47	46	5	4
On water surface	0	0	0	0	0	0	0	0	0	0	0	0	0
In overlying water	0	0	0	0	0	0	0	0	0	0	0	0	0
On top of sediment	0	1	2	0	2	1	1	1	1	0	1	1	0
Within sediment	50	49	48	50	48	49	49	49	7	4	4	4	4
In groupings	0	26	48L	50T	28	6	6	6	0	4	0	0	2
Dead	0	0	0	0	0	0	0	0	0	3	1	41	1
Dissolved O_2	T154			T170	A13	A45	A64	A85	A103	A112	A130	A139	A148
Comments	14d-seems empty, lack of carcasses, pH=7.1, >750=4												

LEGEND:

Groupings: L=loose, M=medium, T=Tight, VT=Very tight;

Dissolved O_2 in mg/L

C1(#) = Cell 1 – See CELL 1 Readouts under Appendix E

T(#) = Test-- See TEST Readouts under Appendix E

A(#) = All -- See ALL Readouts under Appendix E

Appendix C: 42-day results

Male measurements = Body length (mm) vs. Secondary gnathopod area (mm^2)

Female measurements = body length (mm) vs. Brood pouch area (mm^2)

Male measurements				Female measurements			
Length	Area	Day	Treatment	Length	Area	Day	Treatment
4.388	0.166	Apr-20	CTRL	3.379	0.114	Apr-20	CTRL
4.470	0.155	Apr-20	CTRL	3.686	0.295	Apr-20	CTRL
3.419	0.061	Apr-20	CTRL	4.367	0.334	Apr-20	CTRL
3.636	0.052	Apr-23	CTRL	2.812	0.044	Apr-20	CTRL
4.411	0.131	Apr-23	CTRL	3.257	0.111	Apr-20	CTRL
3.312	0.089	Apr-23	CTRL	3.770	0.078	Apr-20	CTRL
2.750	0.026	Apr-23	CTRL	4.402	0.163	Apr-20	CTRL
2.929	0.078	Apr-23	CTRL	4.298	0.057	Apr-20	CTRL
3.681	0.121	Apr-25	CTRL	4.223	0.174	Apr-20	CTRL
3.873	0.131	Apr-25	CTRL	3.672	0.222	Apr-20	CTRL
4.753	0.283	Apr-25	CTRL	3.971	0.151	Apr-20	CTRL
4.008	0.084	Apr-25	CTRL	4.691	0.330	Apr-20	CTRL
4.740	0.098	Apr-25	CTRL	4.133	0.196	Apr-20	CTRL
4.194	0.084	Apr-25	CTRL	3.218	0.100	Apr-20	CTRL
2.698	0.031	Apr-25	CTRL	4.666	0.214	Apr-20	CTRL
4.089	0.140	Apr-25	CTRL	4.039	0.129	Apr-23	CTRL
6.112	0.412	Apr-26	CTRL	3.880	0.163	Apr-23	CTRL
3.107	0.083	Apr-26	CTRL	3.919	0.131	Apr-23	CTRL
4.779	0.168	Apr-26	CTRL	3.031	0.035	Apr-23	CTRL
4.894	0.344	Apr-26	CTRL	2.389	0.032	Apr-23	CTRL
5.811	0.264	Apr-29	CTRL	3.528	0.112	Apr-23	CTRL
4.509	0.233	Apr-29	CTRL	3.410	0.129	Apr-23	CTRL
5.374	0.343	Apr-29	CTRL	3.075	0.131	Apr-23	CTRL
5.596	0.307	Apr-29	CTRL	3.384	0.035	Apr-23	CTRL
4.373	0.140	Apr-29	CTRL	2.908	0.090	Apr-23	CTRL
4.821	0.216	Apr-29	CTRL	2.020	0.052	Apr-23	CTRL
5.598	0.326	Apr-29	CTRL	2.485	0.021	Apr-23	CTRL
3.724	0.112	Apr-29	CTRL	3.631	0.112	Apr-25	CTRL
5.579	0.352	Apr-29	CTRL	4.343	0.223	Apr-25	CTRL
4.829	0.247	Apr-29	CTRL	4.569	0.274	Apr-25	CTRL
5.924	0.455	May-07	CTRL	3.330	0.021	Apr-25	CTRL
5.873	0.418	May-07	CTRL	4.535	0.220	Apr-25	CTRL
5.891	0.409	May-07	CTRL	4.682	0.450	Apr-25	CTRL
5.967	0.533	May-07	CTRL	3.814	0.059	Apr-25	CTRL
5.614	0.364	May-07	CTRL	4.143	0.267	Apr-25	CTRL
6.201	0.367	May-07	CTRL	3.077	0.051	Apr-25	CTRL

5.568	0.370	May-07	CTRL	4.327	0.080	Apr-25	CTRL
6.317	0.396	May-07	CTRL	4.410	0.258	Apr-25	CTRL
4.102	0.169	May-07	CTRL	4.426	0.024	Apr-25	CTRL
6.103	0.476	May-07	CTRL	4.762	0.308	Apr-25	CTRL
4.065	0.147	May-31	CTRL	3.991	0.255	Apr-25	CTRL
4.222	0.094	May-31	CTRL	3.202	0.167	Apr-25	CTRL
3.829	0.066	May-31	CTRL	3.824	0.129	Apr-25	CTRL
4.599	0.172	May-31	CTRL	3.369	0.431	Apr-26	CTRL
3.969	0.146	May-31	CTRL	4.168	0.444	Apr-26	CTRL
4.824	0.111	May-31	CTRL	3.553	0.153	Apr-26	CTRL
5.032	0.296	May-31	CTRL	4.594	0.362	Apr-26	CTRL
4.153	0.218	May-31	CTRL	4.727	0.719	Apr-26	CTRL
4.708	0.258	May-31	CTRL	4.627	0.739	Apr-26	CTRL
4.439	0.111	May-31	CTRL	4.511	0.640	Apr-26	CTRL
4.495	0.169	May-31	CTRL	4.894	0.486	Apr-26	CTRL
3.280	0.042	May-31	CTRL	4.846	0.687	Apr-26	CTRL
4.394	0.221	May-31	CTRL	4.407	0.446	Apr-26	CTRL
3.979	0.109	May-31	CTRL	3.303	0.103	Apr-26	CTRL
4.035	0.109	May-31	CTRL	3.061	0.122	Apr-26	CTRL
5.225	0.242	May-31	CTRL	2.852	0.182	Apr-26	CTRL
3.642	0.046	May-31	CTRL	6.245	0.815	Apr-26	CTRL
6.241	0.281	May-31	CTRL	4.786	0.266	Apr-29	CTRL
5.885	0.379	May-31	CTRL	3.688	0.344	Apr-29	CTRL
4.253	0.100	Apr-21	DMSO	4.070	0.196	Apr-29	CTRL
4.202	0.136	Apr-21	DMSO	4.622	0.523	Apr-29	CTRL
3.604	0.061	Apr-21	DMSO	5.933	0.549	May-07	CTRL
5.024	0.248	Apr-22	DMSO 1	5.440	0.690	May-07	CTRL
2.922	0.034	Apr-22	DMSO 1	4.818	0.450	May-07	CTRL
4.452	0.216	Apr-22	DMSO 2	5.664	0.635	May-07	CTRL
4.495	0.169	Apr-22	DMSO 2	5.500	0.568	May-07	CTRL
4.458	0.177	Apr-22	DMSO 2	5.805	0.590	May-07	CTRL
4.883	0.123	Apr-26	DMSO	5.304	0.668	May-07	CTRL
4.285	0.119	Apr-26	DMSO	5.142	0.640	May-07	CTRL
4.749	0.221	Apr-26	DMSO	4.216	0.274	May-31	CTRL
4.571	0.322	Apr-26	DMSO	3.565	0.060	May-31	CTRL
4.271	0.159	Apr-26	DMSO	3.918	0.052	May-31	CTRL
5.055	0.215	Apr-26	DMSO	4.166	0.115	May-31	CTRL
4.562	0.153	Apr-27	DMSO	4.597	0.387	May-31	CTRL
4.732	0.154	Apr-27	DMSO	5.090	0.145	May-31	CTRL
4.708	0.275	Apr-27	DMSO	3.991	0.085	May-31	CTRL
4.489	0.100	May-07	DMSO	3.790	0.047	May-31	CTRL
5.316	0.368	May-31	DMSO	3.852	0.103	May-31	CTRL
5.868	0.385	May-31	DMSO	4.247	0.208	May-31	CTRL
5.843	0.294	May-31	DMSO	4.154	0.051	May-31	CTRL
6.095	0.510	May-31	DMSO	4.655	0.331	May-31	CTRL

5.871	0.358	May-31	DMSO	4.933	0.295	May-31	CTRL
3.886	0.076	Apr-20	EE	4.170	0.178	May-31	CTRL
2.514	0.058	Apr-20	EE	4.756	0.317	May-31	CTRL
3.757	0.163	Apr-21	EE	3.439	0.054	May-31	CTRL
4.212	0.188	Apr-23	EE 1	4.184	0.136	May-31	CTRL
4.457	0.138	Apr-23	EE 1	3.749	0.143	May-31	CTRL
3.680	0.062	Apr-23	EE 2	4.064	0.081	May-31	CTRL
6.048	0.339	Apr-28	EE	3.134	0.038	May-31	CTRL
5.387	0.332	Apr-28	EE	3.479	0.037	May-31	CTRL
4.693	0.170	Apr-28	EE	3.664	0.039	May-31	CTRL
5.423	0.323	Apr-28	EE	3.734	0.128	May-31	CTRL
4.646	0.208	May-07	EE	3.434	0.068	May-31	CTRL
5.615	0.230	May-31	EE	4.507	0.173	May-31	CTRL
6.325	0.370	May-31	EE	3.307	0.026	Apr-21	DMSO
6.834	0.529	May-31	EE	2.316	0.036	Apr-21	DMSO
5.762	0.341	May-31	EE	3.270	0.051	Apr-21	DMSO
6.194	0.418	May-31	EE	2.445	0.013	Apr-21	DMSO
5.484	0.311	May-31	EE	2.918	0.038	Apr-21	DMSO
6.101	0.322	May-31	EE	4.101	0.237	Apr-21	DMSO
5.822	0.295	May-31	EE	4.403	0.309	Apr-22	DMSO 1
6.560	0.318	May-31	EE	4.413	0.211	Apr-22	DMSO 1
6.024	0.283	May-31	EE	4.243	0.134	Apr-22	DMSO 1
6.418	0.456	May-31	EE	4.668	0.384	Apr-22	DMSO 1
5.673	0.335	May-31	EE	3.768	0.267	Apr-22	DMSO 1
5.948	0.475	May-31	EE	3.267	0.113	Apr-22	DMSO 1
6.138	0.418	May-31	EE	3.332	0.064	Apr-22	DMSO 1
6.199	0.439	May-31	EE	3.038	0.057	Apr-22	DMSO 1
6.185	0.329	May-31	EE	3.420	0.092	Apr-22	DMSO 1
6.123	0.334	May-31	EE	3.052	0.043	Apr-22	DMSO 1
5.890	0.303	May-31	EE	4.325	0.097	Apr-22	DMSO 1
3.829	0.028	Apr-23	A100	3.386	0.108	Apr-22	DMSO 1
4.761	0.177	Apr-24	A100	4.153	0.168	Apr-22	DMSO 2
4.691	0.200	Apr-25	A100	3.314	0.030	Apr-22	DMSO 2
5.933	0.296	May-07	A100	4.007	0.153	Apr-22	DMSO 2
5.326	0.326	May-07	A100	3.823	0.212	Apr-22	DMSO 2
5.592	0.386	May-31	A100	3.949	0.061	Apr-22	DMSO 2
5.492	0.249	May-31	A100	4.366	0.256	Apr-22	DMSO 2
4.903	0.184	May-31	A100	3.875	0.058	Apr-22	DMSO 2
5.525	0.382	May-31	A100	3.203	0.134	Apr-22	DMSO 2
5.216	0.121	May-31	A100	2.339	0.136	Apr-22	DMSO 2
5.513	0.352	May-31	A100	2.685	0.025	Apr-22	DMSO 2
4.238	0.211	Apr-22	A50	2.693	0.026	Apr-22	DMSO 2
4.075	0.107	Apr-22	A50	3.304	0.103	Apr-22	DMSO 2
5.127	0.092	Apr-26	A50 2	3.960	0.031	Apr-22	DMSO 2
5.163	0.115	Apr-26	A50 2	3.011	0.053	Apr-22	DMSO 2

4.722	0.156	May-07	A50	3.815	0.192	Apr-22	DMSO 2
4.677	0.191	May-31	A50	4.256	0.015	Apr-26	DMSO
3.741	0.116	Apr-24	A5	3.921	0.120	Apr-26	DMSO
3.961	0.056	Apr-24	A5	5.128	0.530	Apr-26	DMSO
5.020	0.271	Apr-24	A5	4.104	0.085	Apr-26	DMSO
3.213	0.034	Apr-27	A5	4.836	0.509	Apr-26	DMSO
4.691	0.139	Apr-27	A5	4.161	0.147	Apr-26	DMSO
3.872	0.084	Apr-28	A5	3.635	0.154	Apr-26	DMSO
5.699	0.363	Apr-28	A5	4.488	0.161	Apr-27	DMSO
4.476	0.133	May-31	A5	4.659	0.609	Apr-27	DMSO
5.954	0.362	May-07	T10	3.697	0.197	Apr-27	DMSO
5.673	0.390	May-07	T10	3.554	0.130	Apr-27	DMSO
4.222	0.175	May-07	T10	4.262	0.313	Apr-27	DMSO
5.513	0.422	May-07	T10	5.208	0.369	Apr-27	DMSO
4.103	0.103	May-07	T10	3.966	0.320	Apr-27	DMSO
4.934	0.256	May-07	T10	3.987	0.258	Apr-27	DMSO
4.949	0.246	May-07	T10	3.814	0.054	Apr-27	DMSO
5.578	0.286	May-31	T10	5.605	0.617	May-07	DMSO
4.395	0.167	Apr-24	T1 2	4.480	0.490	May-07	DMSO
4.455	0.164	Apr-27	T1	5.239	0.512	May-07	DMSO
3.888	0.074	Apr-28	T1	5.379	0.403	May-07	DMSO
4.403	0.160	Apr-28	T1	6.142	1.109	May-07	DMSO
5.230	0.233	Apr-29	T1	3.991	0.247	May-07	DMSO
4.726	0.186	Apr-29	T1	4.600	0.542	May-07	DMSO
5.079	0.138	Apr-29	T1	5.249	0.435	May-07	DMSO
5.983	0.314	May-07	T1	5.675	0.244	May-07	DMSO
5.586	0.381	May-07	T1	5.052	0.081	May-07	DMSO
5.974	0.364	May-07	T1	3.822	0.243	May-07	DMSO
5.865	0.298	May-07	T1	5.756	0.488	May-07	DMSO
5.387	0.349	May-07	T1	5.251	0.727	May-07	DMSO
5.380	0.343	May-31	T1	4.444	0.444	May-07	DMSO
3.955	0.107	May-31	T1	3.559	0.059	May-07	DMSO
5.546	0.280	May-31	T1	6.704	0.788	May-31	DMSO
3.927	0.100	May-31	T1	5.499	0.444	May-31	DMSO
4.560	0.199	May-31	T1	5.492	0.436	May-31	DMSO
4.138	0.129	Apr-25	T0.1 1	5.838	0.384	May-31	DMSO
3.782	0.033	Apr-25	T0.1 2	6.232	0.863	May-31	DMSO
4.124	0.157	Apr-25	T0.1 2	5.460	0.434	May-31	DMSO
3.655	0.114	Apr-26	T0.1	5.835	0.758	May-31	DMSO
4.535	0.148	Apr-27	T0.1	5.368	0.534	May-31	DMSO
5.172	0.293	Apr-27	T0.1	5.270	0.662	May-31	DMSO
4.579	0.218	Apr-27	T0.1	5.679	0.718	May-31	DMSO
4.555	0.122	Apr-27	T0.1	5.571	0.691	May-31	DMSO
5.219	0.318	Apr-27	T0.1	3.710	0.104	Apr-20	EE

5.010	0.269	Apr-27	T0.1
5.285	0.278	May-07	T0.1
5.394	0.170	May-07	T0.1
5.075	0.256	May-07	T0.1
4.933	0.267	May-07	T0.1
5.337	0.310	May-07	T0.1
5.490	0.229	May-07	T0.1
4.879	0.200	May-07	T0.1
4.332	0.209	May-07	T0.1
4.691	0.173	May-07	T0.1
6.057	0.256	May-31	T0.1
5.942	0.288	May-31	T0.1
6.295	0.469	May-31	T0.1
6.253	0.385	May-31	T0.1
5.615	0.376	May-31	T0.1
5.362	0.311	May-31	T0.1
6.415	0.305	May-31	T0.1

Juvenile measurements
Body length (mm)

Length	Day	Treatment
3.276	Apr-20	CTRL
2.262	Apr-23	CTRL
2.518	Apr-23	CTRL
2.502	Apr-23	CTRL
3.066	Apr-23	CTRL
2.391	Apr-23	CTRL
3.357	Apr-23	CTRL
2.738	Apr-23	CTRL
2.884	Apr-23	CTRL
2.935	Apr-25	CTRL
3.822	Apr-25	CTRL
2.623	Apr-25	CTRL
3.716	Apr-25	CTRL
2.918	Apr-26	CTRL
2.139	Apr-26	CTRL
2.685	Apr-29	CTRL
3.769	Apr-29	CTRL
3.181	Apr-29	CTRL
2.665	Apr-29	CTRL
1.148	Apr-29	CTRL
3.04	Apr-29	CTRL
3.095	Apr-29	CTRL
3.867	May-07	CTRL

3.227	0.091	Apr-20	EE
3.472	0.105	Apr-20	EE
2.974	0.047	Apr-20	EE
3.380	0.091	Apr-21	EE
3.014	0.085	Apr-21	EE
3.177	0.243	Apr-21	EE
2.711	0.034	Apr-21	EE
3.669	0.096	Apr-21	EE
2.587	0.063	Apr-21	EE
2.557	0.020	Apr-21	EE
4.301	0.165	Apr-23	EE 1
3.487	0.192	Apr-23	EE 1
4.809	0.323	Apr-23	EE 1
3.263	0.044	Apr-23	EE 1
2.899	0.017	Apr-23	EE 1
3.902	0.188	Apr-23	EE 2
4.309	0.210	Apr-23	EE 2
3.241	0.139	Apr-23	EE 2
3.774	0.150	Apr-23	EE 2
4.103	0.215	Apr-23	EE 2
4.037	0.110	Apr-23	EE 2
4.140	0.336	Apr-23	EE 2
4.203	0.089	Apr-23	EE 2
3.230	0.017	Apr-23	EE 2
5.034	0.700	Apr-28	EE
5.150	0.938	Apr-28	EE
5.255	0.554	Apr-28	EE
4.829	0.406	Apr-28	EE
5.054	0.537	Apr-28	EE
3.607	0.043	May-07	EE
3.645	0.132	May-07	EE
3.699	0.180	May-07	EE
3.702	0.181	May-07	EE
4.575	0.247	May-07	EE
3.351	0.053	May-07	EE
3.307	0.054	May-07	EE
5.994	0.452	May-31	EE
5.679	0.480	May-31	EE
5.396	0.746	May-31	EE
6.196	0.695	May-31	EE
5.159	0.670	May-31	EE
6.818	1.380	May-31	EE
5.616	0.713	May-31	EE
5.991	0.637	May-31	EE
5.436	0.709	May-31	EE

3.741	May-07	CTRL
2.849	May-07	CTRL
2.919	May-07	CTRL
1.699	May-07	CTRL
1.28	May-07	CTRL
1.275	May-07	CTRL
1.596	May-07	CTRL
1.444	May-07	CTRL
1.343	May-07	CTRL
1.364	May-07	CTRL
1.033	May-07	CTRL
1.289	May-07	CTRL
0.929	May-07	CTRL
1.105	May-07	CTRL
1.269	May-07	CTRL
1.166	May-07	CTRL
1.306	May-07	CTRL
3.091	May-31	CTRL
2.586	May-31	CTRL
3.526	May-31	CTRL
3.144	May-31	CTRL
2.565	May-31	CTRL
2.236	May-31	CTRL
3.03	May-31	CTRL
2.575	Apr-22	DMSO 1
3.170	Apr-22	DMSO 1
3.080	Apr-22	DMSO 2
2.355	Apr-22	DMSO 2
2.964	Apr-22	DMSO 2
2.233	Apr-22	DMSO 2
2.174	Apr-22	DMSO 2
3.119	Apr-22	DMSO 2
2.275	Apr-22	DMSO 2
3.496	Apr-27	DMSO
3.205	Apr-27	DMSO
3.207	May-07	DMSO
1.663	May-31	DMSO
1.524	May-31	DMSO
1.651	May-31	DMSO
1.643	May-31	DMSO
1.509	May-31	DMSO
1.643	May-31	DMSO
1.898	May-31	DMSO
1.228	May-31	DMSO
1.514	May-31	DMSO

6.284	0.694	May-31	EE
4.992	0.562	May-31	EE
6.221	0.549	May-31	EE
6.375	0.868	May-31	EE
6.461	1.117	May-31	EE
5.883	0.411	May-31	EE
5.352	0.439	May-31	EE
5.648	0.463	May-31	EE
4.559	0.230	Apr-23	A100
3.463	0.149	Apr-23	A100
2.485	0.032	Apr-23	A100
2.694	0.053	Apr-23	A100
3.792	0.071	Apr-24	A100
3.818	0.251	Apr-24	A100
3.732	0.206	Apr-24	A100
4.212	0.263	Apr-24	A100
5.256	0.465	Apr-24	A100
5.251	0.385	Apr-24	A100
4.203	0.235	Apr-24	A100
4.493	0.246	Apr-24	A100
4.390	0.117	Apr-24	A100
4.506	0.256	Apr-24	A100
3.730	0.162	Apr-25	A100
5.230	0.309	Apr-25	A100
3.575	0.021	Apr-25	A100
3.543	0.059	Apr-25	A100
3.126	0.023	Apr-25	A100
4.323	0.214	Apr-27	A100
4.357	0.191	Apr-27	A100
4.750	0.362	Apr-27	A100
5.516	0.798	May-07	A100
5.377	0.747	May-07	A100
5.277	0.669	May-07	A100
5.116	0.294	May-31	A100
5.346	0.438	May-31	A100
5.303	1.005	May-31	A100
4.377	0.179	May-31	A100
5.449	0.252	May-31	A100
5.134	0.634	May-31	A100
4.399	0.307	May-31	A100
4.915	0.460	May-31	A100
4.863	0.290	May-31	A100
5.932	0.373	May-31	A100
5.058	0.371	May-31	A100
4.613	0.349	May-31	A100

1.49	May-31	DMSO	5.277	0.644	May-31	A100
1.469	May-31	DMSO	5.493	0.797	May-31	A100
1.609	May-31	DMSO	4.524	0.140	May-31	A100
1.528	May-31	DMSO	4.809	0.245	May-31	A100
1.657	May-31	DMSO	4.871	0.310	May-31	A100
1.213	May-31	DMSO	5.595	0.379	May-31	A100
1.618	May-31	DMSO	4.329	0.493	May-31	A100
1.572	May-31	DMSO	5.441	0.894	May-31	A100
1.686	May-31	DMSO	5.212	0.283	May-31	A100
2.255	Apr-20	EE	3.937	0.023	May-31	A100
2.668	Apr-21	EE	3.454	0.131	Apr-21	A50
3.320	Apr-23	EE 1	4.021	0.046	Apr-21	A50
2.880	Apr-23	EE 1	2.645	0.024	Apr-21	A50
2.282	Apr-23	EE 1	2.747	0.018	Apr-21	A50
2.048	Apr-23	EE 1	4.377	0.350	Apr-21	A50
2.18	Apr-23	EE 1	2.891	0.054	Apr-21	A50
2.113	Apr-23	EE 1	3.755	0.102	Apr-22	A50
4.685	Apr-28	EE	3.410	0.109	Apr-22	A50
1.322	Apr-28	EE	3.152	0.098	Apr-22	A50
1.2	Apr-28	EE	4.584	0.346	Apr-24	A50
1.367	Apr-28	EE	3.118	0.055	Apr-24	A50
1.304	May-07	EE	3.101	0.026	Apr-24	A50
2.186	May-31	EE	4.470	0.295	Apr-26	A50 1
1.895	May-31	EE	4.929	0.163	Apr-26	A50 1
2.03	May-31	EE	3.521	0.214	Apr-26	A50 1
1.865	May-31	EE	4.375	0.362	Apr-26	A50 1
1.929	May-31	EE	3.696	0.179	Apr-26	A50 1
2.358	May-31	EE	4.126	0.359	Apr-26	A50 1
1.46	May-31	EE	5.350	0.500	Apr-26	A50 2
1.861	May-31	EE	4.075	0.259	Apr-26	A50 2
1.695	May-31	EE	5.234	0.519	Apr-26	A50 2
1.548	May-31	EE	4.522	0.369	Apr-26	A50 2
1.37	May-31	EE	4.017	0.367	Apr-26	A50 2
1.6	May-31	EE	4.584	0.153	Apr-26	A50 2
1.483	May-31	EE	4.862	0.367	May-07	A50
1.975	May-31	EE	6.096	0.440	May-07	A50
2.451	May-31	EE	5.852	0.784	May-07	A50
1.503	May-31	EE	5.286	0.462	May-07	A50
1.329	May-31	EE	4.023	0.064	May-31	A50
1.293	May-31	EE	3.809	0.190	May-31	A50
1.877	May-31	EE	5.283	0.773	May-31	A50
1.397	May-31	EE	4.946	0.692	May-31	A50
1.23	May-31	EE	4.650	0.522	May-31	A50
1.392	May-31	EE	4.732	0.425	May-31	A50
1.816	May-31	EE	5.328	0.721	May-31	A50

1.292	May-31	EE
1.222	May-31	EE
1.541	May-31	EE
1.503	May-31	EE
1.375	May-31	EE
1.522	May-31	EE
1.312	May-31	EE
1.355	May-31	EE
1.436	May-31	EE
1.536	May-31	EE
1.403	May-31	EE
1.547	May-31	EE
1.339	May-31	EE
1.193	May-31	EE
1.211	May-31	EE
1.167	May-31	EE
1.297	May-31	EE
1.223	May-31	EE
1.306	May-31	EE
1.315	May-31	EE
1.281	May-31	EE
1.229	May-31	EE
2.709	Apr-23	A100
2.473	Apr-23	A100
2.827	Apr-24	A100
2.117	Apr-24	A100
2.931	Apr-25	A100
1.400	May-31	A100
1.312	May-31	A100
1.267	May-31	A100
2.379	Apr-21	A50
2.707	Apr-21	A50
3.166	Apr-22	A50
3.933	Apr-22	A50
3.425	Apr-22	A50
2.530	Apr-24	A50
2.492	Apr-24	A50
2.892	Apr-26	A50 1
2.76	Apr-26	A50 1
3.953	Apr-26	A50 1
3.998	Apr-26	A50 1
3.105	Apr-26	A50 2
1.238	May-31	A50
1.239	May-31	A50

4.625	0.173	May-31	A50
3.487	0.085	May-31	A50
3.939	0.148	Apr-20	A5
3.409	0.018	Apr-20	A5
2.708	0.018	Apr-20	A5
2.596	0.010	Apr-20	A5
2.809	0.021	Apr-20	A5
2.287	0.014	Apr-20	A5
2.687	0.009	Apr-20	A5
3.916	0.363	Apr-20	A5
3.567	0.238	Apr-20	A5
4.379	0.254	Apr-20	A5
3.981	0.312	Apr-20	A5
3.114	0.092	Apr-20	A5
4.484	0.342	Apr-24	A5
3.680	0.203	Apr-24	A5
3.688	0.171	Apr-24	A5
3.785	0.340	Apr-27	A5
4.196	0.331	Apr-27	A5
3.490	0.444	Apr-27	A5
4.277	0.513	Apr-28	A5
2.893	0.075	Apr-29	A5
3.556	0.040	Apr-29	A5
3.553	0.119	Apr-29	A5
3.867	0.231	Apr-29	A5
3.870	0.247	Apr-29	A5
4.030	0.337	Apr-29	A5
4.033	0.424	May-07	A5
4.218	0.358	May-31	A5
5.512	0.409	May-31	A5
4.709	0.338	May-31	A5
4.122	0.033	May-31	A5
3.562	0.225	Apr-21	T10
2.595	0.083	Apr-21	T10
4.621	0.397	May-07	T10
5.602	0.466	May-07	T10
3.546	0.015	May-07	T10
5.464	0.538	May-07	T10
5.029	0.631	May-07	T10
4.986	0.065	May-07	T10
5.779	0.491	May-07	T10
7.628	0.825	May-31	T10
5.347	0.512	May-31	T10
4.526	0.183	May-31	T10

2.922	Apr-24	A5
2.432	Apr-24	A5
3.037	Apr-27	A5
3.253	Apr-27	A5
3.382	Apr-27	A5
2.882	Apr-28	A5
3.530	Apr-29	A5
1.622	May-07	A5
2.681	May-07	A5
4	May-31	A5
1.15	May-07	T10
1.675	Apr-24	T1 2
2.375	Apr-24	T1 2
2.233	Apr-27	T1
2.462	Apr-27	T1
2.443	Apr-27	T1
2.252	May-07	T1
1.349	May-07	T1
1.474	May-07	T1
1.492	May-07	T1
1.297	May-07	T1
1.379	May-07	T1
1.266	May-07	T1
2.314	May-07	T1
2.302	May-07	T1
1.68	May-07	T1
1.325	May-07	T1
1.311	May-07	T1
1.439	May-07	T1
1.191	May-07	T1
1.187	May-07	T1
1.786	May-07	T1
1.725	May-07	T1
1.508	May-07	T1
1.624	May-07	T1
2.045	May-07	T1
1.599	May-07	T1
1.209	May-07	T1
1.21	May-07	T1
1.955	May-07	T1
2.431	Apr-25	T0.1 1
2.384	Apr-25	T0.1 2
3.144	Apr-25	T0.1 2
2.722	Apr-25	T0.1 2
2.799	Apr-25	T0.1 2

5.317	0.300	May-31	T10
4.070	0.038	Apr-24	T1 1
4.788	0.339	Apr-24	T1 1
3.305	0.281	Apr-24	T1 1
2.789	0.077	Apr-24	T1 2
3.204	0.097	Apr-24	T1 2
3.163	0.068	Apr-24	T1 2
3.480	0.025	Apr-24	T1 2
4.931	0.569	Apr-27	T1
4.317	0.228	Apr-27	T1
4.190	0.413	Apr-27	T1
3.710	0.060	Apr-27	T1
4.338	0.062	Apr-27	T1
3.151	0.021	Apr-27	T1
3.791	0.196	Apr-28	T1
3.554	0.031	Apr-28	T1
6.002	0.370	Apr-29	T1
4.083	0.073	Apr-29	T1
4.160	0.056	Apr-29	T1
3.487	0.166	Apr-29	T1
3.570	0.076	Apr-29	T1
3.548	0.123	Apr-29	T1
4.198	0.097	Apr-29	T1
4.039	0.091	Apr-29	T1
4.026	0.131	Apr-29	T1
4.805	0.416	May-07	T1
5.828	0.700	May-07	T1
5.418	0.628	May-07	T1
5.213	0.832	May-07	T1
5.936	0.896	May-07	T1
5.553	0.916	May-07	T1
6.508	1.276	May-07	T1
6.382	0.854	May-07	T1
4.268	0.317	May-31	T1
5.124	0.314	May-31	T1
3.955	0.368	May-31	T1
4.354	0.052	May-31	T1
4.449	0.085	May-31	T1
4.324	0.361	May-31	T1
4.469	0.330	Apr-25	T0.1 1
4.880	0.400	Apr-25	T0.1 1
5.354	0.411	Apr-25	T0.1 1
4.169	0.336	Apr-25	T0.1 1
4.355	0.240	Apr-25	T0.1 2
4.933	1.041	Apr-25	T0.1 2

3.431	Apr-25	T0.1 2
4.373	Apr-26	T0.1
2.709	Apr-27	T0.1
2.561	Apr-27	T0.1
2.666	Apr-27	T0.1
2.461	Apr-27	T0.1
2.542	Apr-27	T0.1
2.601	Apr-27	T0.1
3.474	Apr-28	T0.1
2.855	Apr-28	T0.1
1.064	May-07	T0.1
1.13	May-07	T0.1
1.417	May-31	T0.1
3.317	May-31	T0.1

3.541	0.245	Apr-25	T0.1 2
3.241	0.069	Apr-25	T0.1 2
4.633	0.458	Apr-26	T0.1
4.523	0.268	Apr-26	T0.1
5.446	0.600	Apr-26	T0.1
5.400	0.297	Apr-27	T0.1
3.415	0.114	Apr-27	T0.1
5.066	0.375	Apr-27	T0.1
5.293	0.894	Apr-27	T0.1
4.862	0.796	Apr-27	T0.1
4.644	0.600	Apr-27	T0.1
3.506	0.043	Apr-27	T0.1
4.112	0.134	Apr-28	T0.1
4.491	0.153	Apr-28	T0.1
3.785	0.068	Apr-28	T0.1
3.218	0.159	Apr-28	T0.1
3.261	0.187	Apr-28	T0.1
3.519	0.034	Apr-28	T0.1
5.186	0.415	May-07	T0.1
5.306	0.599	May-07	T0.1
4.974	0.481	May-07	T0.1
5.011	0.446	May-07	T0.1
4.560	0.446	May-07	T0.1
3.775	0.041	May-07	T0.1
5.251	0.734	May-31	T0.1
5.328	0.563	May-31	T0.1
5.770	0.444	May-31	T0.1
5.379	0.379	May-31	T0.1
6.313	0.750	May-31	T0.1
6.345	0.757	May-31	T0.1
6.161	1.118	May-31	T0.1

Appendix D: Gender Ratio data

	Control			DMSO			EE		
	M	F	J	M	F	J	M	F	J
	3	15	1	3	6	0	2	4	1
	5	12	8	2	12	3	1	7	1
	8	16	4	3	15	6	2	5	6
	4	14	2	6	7	0	1	9	0
	10	4	7	3	9	2	4	5	4
	10	8	18	1	15	1	1	7	1
	19	25	7	5	11	18	18	17	45
Total	59	94	47	23	75	30	29	54	58
Total/n	0.295	0.470	0.235	0.180	0.586	0.234	0.206	0.383	0.411
%Total/n	29.50	47.00	23.50	17.97	58.59	23.44	20.57	38.30	41.13
%M/F	38.56	61.44		23.47	76.53		34.94	65.06	

	A100			A50			A5		
	M	F	J	M	F	J	M	F	J
	1	4	2	0	6	2	0	11	1
	1	10	2	2	3	3	3	3	2
	1	5	1	0	3	2	2	3	3
	0	3	0	0	6	4	2	1	1
	2	3	0	2	6	1	0	6	1
	6	22	3	1	4	0	0	1	2
				1	9	2	1	4	1
Total	11	47	8	6	37	14	8	29	11
Total/n	0.167	0.712	0.121	0.105	0.649	0.246	0.167	0.604	0.229
%Total/n	16.67	71.21	12.12	10.53	64.91	24.56	16.67	60.42	22.92
%M/F	18.97	81.03		13.95	86.05		21.62	78.38	

	T10			T1			T0.1		
	M	F	J	M	F	J	M	F	J
	0	2	0	0	3	1	1	4	1
	0	0	0	1	4	2	2	4	5
	0	0	0	1	6	3	1	3	1
	7	7	1	2	2	0	6	7	6
	1	4	0	3	9	0	0	6	2
				5	8	24	9	6	2
				5	6	0	7	7	2
Total	8	13	1	17	38	30	26	37	19
Total/n	0.364	0.591	0.045	0.200	0.447	0.353	0.317	0.451	0.232
%Total/n	36.36	59.09	4.55	20.00	44.71	35.29	31.71	45.12	23.17
%M/F	38.10	61.90		30.91	69.09		41.27	58.73	

Appendix E: Hanna Instruments 9828 Multi-Parameter Probe Readouts

These readings were taken in chronological order, starting with CELL 1, then proceeding to TEST, then ending with ALL. These were filenames created within the multi-parameter probe. There was a malfunction with the pH probe after CELL 1 readings were collected, so a new filename, TEST, had to be generated, which lacks pH readings. The pH probe was re-inserted, and thus the machine prompted the creation of a new filename; thus, ALL was created. However, in ALL, the pH readings are inaccurate, as the probe was still faulty. All other readings should fare accurate.

CELL 1

HI 92000 - 4.5

Model HI 9828 v1.3
Id:
Lot name Cell 1
N. samples 11
Starting date 20/07/2007
Starting time 8:56 AM

Date	Time	°F	pH	pH mV	ORP	DO %	DO ppm	µS/cm	µS/cm A	TDS ppm	Salinity
2007/07/20	08:56:25	69.74	7.90	-59.3	158.4	62.9	5.49	686	634	343	0.33
2007/07/22	13:05:44	75.55	7.68	-46.7	149.6	61.5	5.16	617	608	309	0.30
2007/07/31	12:26:48	86.33	8.47	-91.9	113.6	111.2	8.34	565	620	282	0.27
2010/05/04	15:07:26	70.14	8.09	-76.1	19.2	79.7	6.97	494	459	247	0.24
2010/05/04	15:21:26	69.93	7.87	-63.8	116.8	97.0	8.49	531	492	266	0.26
2010/05/04	15:51:50	65.61	7.73	-55.5	144.6	97.3	8.93	461	405	230	0.22
2010/05/04	16:05:08	68.11	8.25	-84.9	168.0	95.2	8.49	477	432	239	0.23
2010/05/04	16:20:53	68.84	7.92	-66.5	176.2	93.0	8.22	417	381	209	0.20
2010/05/04	16:31:50	67.99	8.33	-89.9	203.6	92.5	8.25	447	404	223	0.22
2010/05/04	16:42:32	70.51	8.10	-77.0	162.5	91.5	7.95	418	389	209	0.20
2010/05/04	18:51:03	68.78	8.87	-120.8	215.0	96.9	8.57	394	359	197	0.19

TEST

HI 92000 - 4.5

Model HI 9828 v1.3
Id:
Lot name Test
N. samples 174
Starting date 05/05/2010
Starting time 9:54 PM

Date	Time	°F	DO %	DO ppm	μS/cm	μS/cm A	TDS ppm	Salinity
2010/05/05	21:54:15	71.88	96.9	8.25	514	486	257	0.25
2010/05/06	19:51:31	69.95	79.7	7.01	475	440	238	0.23
2010/05/07	18:28:45	68.06	99.5	8.90	473	428	236	0.23
2010/05/07	18:29:06	68.21	101.7	9.09	0	0	0	0.00
2010/05/07	19:41:19	66.05	101.5	9.27	483	427	242	0.23
2010/05/07	20:46:44	70.68	96.5	8.37	322	300	161	0.15
2010/05/07	20:46:57	70.68	94.9	8.23	283	264	141	0.13
2010/05/10	15:45:21	69.47	91.4	8.18	460	424	230	0.22
2010/05/10	15:57:56	66.06	95.8	8.91	478	423	239	0.23
2010/05/10	16:54:20	67.03	92.7	8.53	474	424	237	0.23
2010/05/10	16:54:48	67.31	92.4	8.48	496	446	248	0.24
2010/05/11	20:01:47	70.38	95.8	8.44	499	464	250	0.24
2010/05/11	20:08:08	65.61	99.1	9.21	494	435	247	0.24
2010/05/12	19:39:13	65.33	103.9	9.69	573	503	287	0.28
2010/05/12	19:51:30	66.57	101.5	9.34	587	523	294	0.29
2010/05/12	19:59:23	67.71	93.7	8.51	591	533	296	0.29
2010/05/12	20:09:32	66.31	96.4	8.91	452	401	226	0.22
2010/05/12	20:19:46	68.68	89.9	8.09	445	406	222	0.21
2010/05/12	20:20:31	67.87	94.5	8.59	468	423	234	0.23
2010/05/12	20:20:54	67.83	96.3	8.75	461	416	230	0.22
2010/05/12	20:37:38	68.17	91.7	8.30	447	406	224	0.22
2010/05/13	21:34:05	68.71	78.7	7.03	498	454	249	0.24
2010/05/13	21:40:06	68.18	89.5	8.03	611	554	306	0.30
2010/05/13	21:40:45	68.22	92.7	8.32	528	479	264	0.26
2010/05/13	21:45:05	68.62	88.2	7.88	495	452	248	0.24
2010/05/14	14:54:25	70.44	86.2	7.55	486	452	243	0.23
2010/05/14	17:14:49	68.75	88.4	7.88	557	508	278	0.27
2010/05/14	17:22:26	68.08	87.5	7.86	534	483	267	0.26
2010/05/14	17:23:22	70.11	88.5	7.78	552	512	276	0.27
2010/05/14	17:36:15	66.21	90.4	8.30	454	402	227	0.22
2010/05/14	18:34:18	70.38	85.5	7.49	402	374	201	0.19
2010/05/14	18:37:45	68.07	82.4	7.40	459	415	229	0.22
2010/05/14	18:40:37	67.55	89.6	8.09	495	446	248	0.24
2010/05/14	18:46:22	68.60	87.8	7.84	410	374	205	0.20
2010/05/14	18:48:59	67.86	85.9	7.73	419	378	209	0.20
2010/05/14	18:53:18	67.43	89.1	8.06	440	395	220	0.21
2010/05/14	18:57:58	68.12	88.4	7.93	387	351	194	0.19
2010/05/14	19:01:39	67.65	87.8	7.91	590	532	295	0.29
2010/05/14	19:06:37	68.01	87.4	7.85	393	356	197	0.19
2010/05/15	22:39:05	71.34	86.8	7.59	480	451	240	0.23
2010/05/15	22:41:14	70.09	85.9	7.61	490	454	245	0.24
2010/05/15	22:50:38	69.70	88.2	7.85	491	453	246	0.24
2010/05/15	22:55:05	68.84	90.8	8.16	501	457	250	0.24
2010/05/15	22:59:05	70.28	87.5	7.74	505	469	252	0.24
2010/05/15	23:04:41	68.93	92.1	8.26	471	431	236	0.23
2010/05/15	23:05:03	68.46	92.4	8.33	590	537	295	0.29
2010/05/15	23:11:49	69.03	82.2	7.37	507	464	254	0.25

2010/05/15	23:14:38	69.83	87.5	7.78	465	430	232	0.22
2010/05/17	19:47:55	69.61	90.2	8.01	505	465	252	0.24
2010/05/17	19:56:30	70.02	79.6	7.04	524	485	262	0.25
2010/05/17	19:56:55	69.35	84.7	7.55	576	530	288	0.28
2010/05/17	20:06:25	69.64	87.9	7.81	526	485	263	0.25
2010/05/17	20:08:35	69.12	87.1	7.77	579	531	289	0.28
2010/05/18	16:25:03	73.17	85.4	7.28	625	600	313	0.30
2010/05/18	16:31:03	69.68	86.9	7.70	431	397	215	0.21
2010/05/18	16:38:19	70.30	81.6	7.18	619	575	309	0.30
2010/05/18	16:59:22	70.98	84.7	7.39	541	507	271	0.26
2010/05/18	17:06:29	70.41	86.5	7.60	592	551	296	0.29
2010/05/18	17:19:01	71.48	86.8	7.54	385	362	192	0.18
2010/05/18	17:47:11	69.73	85.6	7.57	473	437	237	0.23
2010/05/18	17:48:21	69.52	88.3	7.83	539	497	270	0.26
2010/05/20	20:18:54	72.88	82.8	7.07	576	551	288	0.28
2010/05/20	20:27:15	72.61	80.9	6.94	528	503	264	0.25
2010/05/20	20:31:22	72.40	79.2	6.81	492	468	246	0.24
2010/05/20	20:34:22	72.26	84.1	7.24	604	574	302	0.29
2010/05/20	21:05:57	72.28	87.0	7.47	525	499	263	0.25
2010/05/20	21:10:18	72.12	86.6	7.46	561	532	281	0.27
2010/05/20	21:14:06	72.30	84.4	7.26	482	458	241	0.23
2010/05/20	21:17:45	72.13	82.8	7.14	411	389	205	0.20
2010/05/20	21:21:58	71.60	79.0	6.85	450	424	225	0.22
2010/05/20	21:24:55	71.04	81.8	7.13	457	428	228	0.22
2010/05/20	21:27:13	71.42	88.1	7.65	504	474	252	0.24
2010/05/21	15:18:30	73.07	81.6	6.99	430	413	215	0.21
2010/05/21	15:18:46	71.97	84.0	7.28	491	465	245	0.24
2010/05/21	15:19:22	72.52	83.7	7.21	412	393	206	0.20
2010/05/21	15:20:25	71.38	84.0	7.33	464	437	232	0.22
2010/05/21	15:20:58	71.87	84.4	7.32	453	429	227	0.22
2010/05/21	15:21:30	72.64	84.4	7.26	442	422	221	0.21
2010/05/21	15:21:56	72.43	85.6	7.38	446	424	223	0.21
2010/05/21	15:22:45	71.44	84.0	7.32	420	396	210	0.20
2010/05/21	15:23:28	71.61	80.4	7.00	433	408	216	0.21
2010/05/24	17:29:38	81.32	78.1	6.19	503	526	252	0.24
2010/05/24	17:30:37	80.77	84.0	6.70	568	591	284	0.27
2010/05/24	17:31:03	78.12	88.1	7.21	611	618	305	0.29
2010/05/24	17:32:12	80.34	71.7	5.74	592	613	296	0.29
2010/05/24	17:33:36	79.35	79.5	6.43	570	584	285	0.27
2010/05/24	17:34:07	78.28	80.3	6.56	582	590	291	0.28
2010/05/24	17:35:12	79.81	83.9	6.75	544	560	272	0.26
2010/05/24	17:35:21	79.43	85.2	6.88	591	607	296	0.28
2010/05/24	17:35:34	78.85	83.1	6.75	617	629	308	0.30
2010/05/24	17:36:45	78.74	85.5	6.96	460	468	230	0.22
2010/05/24	17:36:56	78.83	87.2	7.09	621	633	310	0.30
2010/05/24	17:37:36	80.74	84.3	6.72	497	517	249	0.24
2010/05/24	17:37:49	79.69	77.2	6.22	581	597	290	0.28
2010/05/24	17:38:57	79.88	77.1	6.20	530	546	265	0.25
2010/05/24	17:39:08	79.34	82.4	6.66	610	625	305	0.29
2010/05/24	17:39:51	80.18	85.7	6.87	569	588	285	0.27

2010/05/24	17:40:21	79.40	86.5	6.99	542	555	271	0.26
2010/05/24	17:40:33	78.99	86.9	7.05	615	628	308	0.30
2010/05/25	21:01:59	80.77	81.6	6.46	470	488	235	0.22
2010/05/25	21:02:36	78.65	85.1	6.89	494	503	247	0.24
2010/05/25	21:03:04	77.37	86.3	7.08	485	486	242	0.23
2010/05/25	21:04:10	76.69	78.2	6.46	459	458	230	0.22
2010/05/25	21:04:43	77.03	79.8	6.57	488	488	244	0.23
2010/05/28	14:45:40	68.06	68.8	6.19	500	452	250	0.24
2010/05/28	14:46:21	70.97	79.1	6.90	442	414	221	0.21
2010/05/28	14:47:06	71.15	78.7	6.84	574	539	287	0.28
2010/05/28	15:50:32	68.66	76.0	6.78	532	486	266	0.26
2010/05/28	15:51:03	69.74	78.9	6.96	525	485	263	0.25
2010/05/28	15:51:34	69.76	82.3	7.26	601	555	301	0.29
2010/05/28	15:52:18	69.09	82.5	7.33	556	510	278	0.27
2010/05/28	17:05:14	69.08	82.6	7.33	443	406	222	0.21
2010/05/28	17:05:31	69.21	84.0	7.45	610	559	305	0.30
2010/05/28	17:06:21	68.40	77.2	6.91	560	509	280	0.27
2010/05/28	17:23:37	68.86	85.5	7.61	374	342	187	0.18
2010/05/28	17:23:51	68.70	87.9	7.84	472	431	236	0.23
2010/05/28	17:24:06	68.29	88.6	7.94	540	490	270	0.26
2010/05/28	17:24:33	68.90	82.1	7.30	481	440	241	0.23
2010/05/28	17:42:04	69.15	76.5	6.80	409	375	205	0.20
2010/05/28	17:42:32	68.85	84.4	7.52	414	378	207	0.20
2010/05/28	17:42:53	68.50	87.8	7.85	418	380	209	0.20
2010/05/28	17:43:07	68.35	90.5	8.10	492	447	246	0.24
2010/05/28	19:08:15	68.51	87.6	7.82	438	398	219	0.21
2010/05/28	19:08:48	68.49	81.1	7.24	485	441	242	0.23
2010/05/28	19:09:14	69.05	82.8	7.35	463	424	232	0.22
2010/05/28	19:22:59	68.61	86.0	7.67	487	443	243	0.24
2010/05/28	19:23:18	68.50	88.2	7.87	538	489	269	0.26
2010/05/28	19:23:49	68.53	87.9	7.84	548	499	274	0.27
2010/05/28	19:24:16	68.58	79.8	7.12	514	468	257	0.25
2010/05/28	19:24:45	68.36	85.2	7.63	449	408	224	0.22
2010/05/28	19:25:02	68.26	87.2	7.81	481	436	240	0.23
2010/05/28	19:25:52	68.73	85.7	7.65	492	449	246	0.24
2010/05/28	19:26:08	68.44	88.6	7.93	581	528	290	0.28
2010/05/28	19:26:23	68.34	87.7	7.85	609	554	305	0.30
2010/05/31	19:37:48	72.79	81.7	6.95	517	494	258	0.25
2010/05/31	19:38:02	72.39	80.9	6.91	610	580	305	0.30
2010/05/31	19:38:17	71.98	82.4	7.07	590	559	295	0.29
2010/05/31	19:38:57	72.70	75.5	6.43	494	471	247	0.24
2010/05/31	19:39:16	72.34	80.7	6.90	575	547	287	0.28
2010/05/31	21:39:41	71.50	79.5	6.85	407	384	204	0.20
2010/05/31	21:39:52	71.45	81.3	7.00	575	541	287	0.28
2010/05/31	21:40:16	71.45	83.7	7.22	474	446	237	0.23
2010/05/31	21:40:27	71.37	82.2	7.10	481	452	240	0.23
2010/05/31	22:03:44	70.93	82.7	7.17	459	429	229	0.22
2010/05/31	22:03:55	71.05	81.2	7.03	553	518	276	0.27
2010/05/31	22:04:26	70.89	86.6	7.52	489	458	245	0.24
2010/05/31	22:04:53	71.07	83.7	7.25	501	470	251	0.24

2010/05/31	22:05:17	71.08	85.1	7.37	471	442	236	0.23
2010/05/31	23:31:37	71.78	80.4	6.92	308	291	154	0.15
2010/05/31	23:31:57	71.41	84.4	7.29	328	308	164	0.16
2010/05/31	23:32:15	71.51	85.7	7.40	327	308	164	0.16
2010/05/31	23:33:21	71.46	84.2	7.28	299	281	149	0.14
2010/05/31	23:33:36	71.51	86.0	7.42	326	307	163	0.16
2010/05/31	23:33:45	71.74	85.4	7.36	325	307	163	0.16
2010/05/31	23:34:09	71.72	85.7	7.38	297	280	148	0.14
2010/05/31	23:34:21	71.46	86.1	7.44	327	308	164	0.16
2010/05/31	23:34:30	71.48	85.0	7.35	327	308	163	0.16
2010/06/01	13:12:09	71.92	78.3	6.72	464	439	232	0.22
2010/06/01	13:13:03	71.86	80.9	6.95	380	360	190	0.18
2010/06/01	13:13:23	71.87	82.7	7.10	484	458	242	0.23
2010/06/01	13:29:14	74.27	74.3	6.21	437	425	219	0.21
2010/06/01	13:29:30	73.14	77.5	6.56	539	517	270	0.26
2010/06/01	13:31:03	72.91	68.0	5.78	484	463	242	0.23
2010/06/01	13:31:23	72.32	78.9	6.75	546	519	273	0.26
2010/06/01	13:31:44	72.00	79.0	6.77	483	457	241	0.23
2010/06/01	14:28:55	72.19	76.0	6.50	294	279	147	0.14
2010/06/01	14:29:29	71.12	73.2	6.34	331	310	165	0.16
2010/06/01	14:30:05	71.35	76.8	6.63	331	311	165	0.16
2010/06/01	14:30:32	71.41	75.7	6.54	331	311	165	0.16
2010/06/01	14:30:51	71.81	79.8	6.86	327	309	163	0.16
2010/06/01	14:31:50	71.93	76.8	6.60	284	269	142	0.13
2010/06/01	14:32:16	72.19	80.6	6.90	296	281	148	0.14
2010/06/01	14:32:33	71.30	79.5	6.87	329	309	164	0.16
2010/06/01	14:32:44	71.19	81.5	7.06	330	310	165	0.16

ALL

HI 92000 - 4.5

Model HI 9828 v1.3
Id:
Lot name All2
N. samples 152
Starting date 02/06/2010
Starting time 3:13 PM

Date	Time	°F	pH	pH mV	ORP	DO %	DO ppm	µS/cm	µS/cm A	TDS ppm	Salinity
2010/06/02	15:13:09	72.52	8.57	-89.6	69.3	76.0	6.46	486	463	243	0.23
2010/06/02	15:16:47	71.99	8.78	-101.5	41.3	79.4	6.80	462	438	231	0.22
2010/06/02	15:17:44	70.81	9.34	-132.8	9.5	78.5	6.80	442	413	221	0.21
2010/06/02	15:18:48	71.97	8.82	-103.5	36.3	72.8	6.23	505	478	253	0.24
2010/06/02	15:45:11	72.20	9.35	-134.2	6.5	78.2	6.68	404	384	202	0.19
2010/06/02	15:46:24	71.67	9.00	-114.0	26.6	81.5	7.01	387	365	194	0.19
2010/06/02	15:46:52	71.92	9.65	-151.2	-9.7	82.2	7.05	389	369	195	0.19
2010/06/02	15:47:05	71.71	9.12	-120.9	18.2	83.4	7.17	450	425	225	0.22

2010/06/02	17:44:11	72.62	10.62	-206.5	-39.3	73.0	6.18	299	285	150	0.14
2010/06/02	17:44:31	71.66	11.15	-236.1	-95.1	81.2	6.96	333	314	166	0.16
2010/06/02	17:45:02	72.07	10.64	-207.2	-40.9	77.9	6.64	332	315	166	0.16
2010/06/02	17:45:17	71.89	10.74	-213.0	-45.7	83.0	7.10	332	314	166	0.16
2010/06/02	17:45:29	72.37	10.70	-210.7	-42.3	83.2	7.08	329	313	165	0.16
2010/06/02	17:47:19	71.42	10.08	-175.3	-25.6	83.5	7.18	316	297	158	0.15
2010/06/02	17:47:44	72.63	10.11	-177.1	-27.5	82.3	6.98	300	287	150	0.14
2010/06/02	17:47:57	71.53	10.28	-186.7	-35.1	85.3	7.33	331	312	166	0.16
2010/06/02	17:48:07	71.04	9.98	-169.4	-18.9	85.9	7.41	335	314	168	0.16
2010/06/03	14:25:37	71.34	8.89	-107.3	-41.8	73.2	6.31	405	381	203	0.19
2010/06/03	14:25:57	71.27	7.77	-43.8	7.0	80.4	6.94	541	509	271	0.26
2010/06/03	14:26:20	71.12	8.48	-84.4	-24.7	79.2	6.84	521	489	261	0.25
2010/06/04	18:11:42	73.84	7.93	-53.1	-22.6	74.6	6.27	345	334	173	0.16
2010/06/04	18:12:00	72.32	8.03	-58.6	-28.4	79.8	6.82	423	402	211	0.20
2010/06/04	18:12:15	72.23	7.60	-34.4	-6.3	81.5	6.97	494	469	247	0.24
2010/06/04	18:12:57	73.54	7.83	-47.6	-17.1	69.9	5.90	453	437	227	0.22
2010/06/04	21:17:58	72.06	7.35	-19.7	11.5	77.7	6.65	444	421	222	0.21
2010/06/04	21:18:14	71.53	8.80	-102.7	-62.3	79.3	6.82	445	419	222	0.21
2010/06/04	21:19:05	71.93	7.86	-48.7	-14.7	74.0	6.34	470	445	235	0.23
2010/06/04	21:24:33	71.60	7.32	-18.0	12.7	77.4	6.65	449	423	224	0.22
2010/06/04	21:25:01	71.26	7.30	-16.9	14.1	82.3	7.10	481	451	240	0.23
2010/06/04	21:25:23	71.28	7.53	-30.0	2.6	83.7	7.22	486	456	243	0.23
2010/06/04	21:25:38	71.40	7.69	-39.0	-5.3	84.8	7.31	463	436	232	0.22
2010/06/04	21:25:57	71.39	7.51	-28.8	3.6	84.9	7.31	465	437	232	0.22
2010/06/04	21:26:32	71.94	7.40	-22.9	8.0	82.1	7.04	460	435	230	0.22
2010/06/04	21:27:21	71.37	7.59	-33.4	-1.5	80.8	6.96	517	486	258	0.25
2010/06/04	21:28:14	71.66	6.94	3.6	31.6	75.8	6.52	412	389	206	0.20
2010/06/04	21:28:30	71.47	8.58	-89.8	-50.0	81.8	7.04	456	429	228	0.22
2010/06/04	21:47:42	72.38	9.19	-125.1	-81.9	74.5	6.35	282	268	141	0.13
2010/06/04	21:47:57	70.78	9.52	-143.4	-102.0	81.4	7.06	345	323	173	0.17
2010/06/04	21:48:09	72.35	9.59	-147.7	-106.1	78.5	6.69	339	322	169	0.16
2010/06/04	21:48:24	71.14	9.46	-139.8	-99.0	83.9	7.25	340	319	170	0.16
2010/06/04	21:48:36	70.45	9.44	-138.5	-96.9	82.3	7.17	349	324	174	0.17
2010/06/04	21:49:02	71.15	9.61	-148.3	-107.4	83.0	7.17	339	318	170	0.16
2010/06/04	21:49:21	71.87	9.34	-133.0	-92.7	80.4	6.90	300	284	150	0.14
2010/06/04	21:49:30	71.75	9.20	-125.2	-83.4	83.1	7.14	338	319	169	0.16
2010/06/04	21:49:38	72.11	9.22	-126.4	-85.2	83.3	7.13	337	319	168	0.16
2010/06/05	16:27:42	70.21	9.75	-156.3	-82.6	76.4	6.65	492	457	246	0.24
2010/06/05	16:27:55	69.93	9.56	-145.1	-75.5	79.7	6.96	468	433	234	0.23
2010/06/05	16:28:07	69.79	8.61	-91.4	-31.9	81.6	7.13	513	474	256	0.25
2010/06/05	16:28:52	71.67	8.71	-97.5	-35.8	78.8	6.75	468	442	234	0.23
2010/06/05	16:29:03	71.21	8.74	-99.0	-37.6	81.7	7.03	498	468	249	0.24
2010/06/06	15:58:19	72.70	8.92	-109.7	-39.4	79.1	6.71	413	394	207	0.20
2010/06/06	15:58:45	73.15	9.95	-168.4	-85.1	76.2	6.43	520	499	260	0.25
2010/06/06	16:00:58	74.22	10.05	-174.2	-96.4	70.3	5.87	431	418	215	0.21
2010/06/06	16:01:14	73.93	9.46	-140.7	-70.0	76.1	6.37	516	499	258	0.25
2010/06/07	16:09:43	73.76	7.60	-34.4	26.4	70.8	6.00	448	433	224	0.22
2010/06/07	16:09:56	73.41	8.14	-65.2	11.9	82.5	7.01	489	471	245	0.24
2010/06/07	16:10:08	73.05	7.94	-53.7	18.2	80.0	6.83	513	492	257	0.25
2010/06/07	16:10:22	72.83	7.70	-39.9	25.2	76.7	6.56	541	517	270	0.26

2010/06/07	16:11:48	73.25	7.49	-28.0	29.6	72.9	6.21	455	437	228	0.22
2010/06/07	17:40:48	71.68	10.02	-172.0	-50.1	78.0	6.75	312	295	156	0.15
2010/06/07	17:41:02	70.52	9.94	-167.0	-52.3	81.8	7.17	353	329	177	0.17
2010/06/07	17:41:15	71.21	9.84	-161.8	-51.1	81.5	7.09	342	321	171	0.16
2010/06/07	17:41:26	71.15	10.02	-172.0	-58.1	82.4	7.18	342	321	171	0.16
2010/06/07	17:41:39	71.38	9.71	-154.1	-46.2	82.7	7.19	341	320	170	0.16
2010/06/07	17:42:03	71.98	9.78	-158.3	-47.3	81.1	7.00	321	304	161	0.15
2010/06/07	17:42:18	70.45	9.79	-158.4	-53.0	82.1	7.21	349	325	174	0.17
2010/06/07	17:42:33	69.99	9.78	-157.7	-54.3	83.3	7.35	366	339	183	0.18
2010/06/07	17:43:13	70.84	9.88	-163.5	-51.3	81.2	7.10	355	332	178	0.17
2010/06/08	15:54:39	72.96	8.40	-80.0	9.9	76.9	6.61	458	439	229	0.22
2010/06/08	15:55:13	72.53	8.41	-80.3	10.8	73.0	6.30	476	453	238	0.23
2010/06/08	15:55:31	72.38	8.36	-77.7	11.9	77.0	6.66	492	468	246	0.24
2010/06/08	15:56:00	73.42	8.15	-65.8	16.2	83.1	7.11	480	462	240	0.23
2010/06/08	15:56:15	72.85	8.65	-94.2	-1.5	84.8	7.30	440	421	220	0.21
2010/06/09	16:01:28	70.33	8.21	-68.5	43.8	80.5	7.02	419	389	209	0.20
2010/06/09	16:02:18	71.76	8.08	-61.4	53.4	76.7	6.59	434	410	217	0.21
2010/06/09	16:02:47	72.21	8.44	-82.3	49.3	79.8	6.83	359	340	179	0.17
2010/06/09	16:03:02	71.93	8.21	-68.9	50.4	83.6	7.18	418	396	209	0.20
2010/06/10	14:18:18	70.60	7.79	-45.1	34.4	79.8	6.97	426	397	213	0.20
2010/06/10	14:20:42	70.16	8.52	-86.3	22.2	80.0	7.02	452	419	226	0.22
2010/06/10	14:35:08	71.33	9.14	-121.5	-10.1	73.9	6.40	707	664	353	0.34
2010/06/10	20:53:50	71.60	8.73	-98.4	9.6	74.5	6.46	335	315	167	0.16
2010/06/10	20:54:08	71.95	8.92	-109.3	-1.7	75.2	6.49	345	326	172	0.16
2010/06/10	20:54:24	71.50	8.86	-106.1	-3.3	77.9	6.76	346	326	173	0.17
2010/06/10	20:54:38	71.44	9.01	-114.6	-9.8	78.8	6.84	348	328	174	0.17
2010/06/10	20:54:52	71.07	8.96	-111.3	-6.7	81.8	7.13	341	320	171	0.16
2010/06/10	20:56:24	70.99	8.59	-90.2	13.0	79.9	6.97	339	318	170	0.16
2010/06/10	20:56:42	71.16	8.64	-93.1	9.7	81.2	7.08	356	334	178	0.17
2010/06/10	20:56:57	71.12	9.04	-115.9	-7.8	82.2	7.17	371	348	186	0.18
2010/06/10	20:57:39	72.50	8.95	-110.9	2.6	75.4	6.48	316	301	158	0.15
2010/06/11	13:39:22	70.87	7.93	-53.0	16.3	82.4	7.24	456	426	228	0.22
2010/06/11	13:39:38	71.12	7.34	-19.3	27.0	83.0	7.28	487	457	244	0.23
2010/06/11	13:39:57	71.35	8.27	-72.5	8.6	81.1	7.09	495	466	248	0.24
2010/06/11	13:41:07	72.29	8.95	-110.9	5.2	86.1	7.46	448	426	224	0.22
2010/06/11	13:41:20	72.43	8.23	-70.2	18.0	84.1	7.27	457	435	228	0.22
2010/06/11	13:41:47	72.54	8.20	-68.2	17.5	79.4	6.86	443	422	221	0.21
2010/06/11	13:42:49	72.66	7.68	-39.0	32.1	76.4	6.59	457	436	229	0.22
2010/06/11	13:43:11	72.23	7.41	-23.6	36.6	79.5	6.89	462	439	231	0.22
2010/06/11	13:43:25	72.07	7.65	-37.3	31.6	81.9	7.11	414	393	207	0.20
2010/06/15	17:30:07	72.17	7.92	-52.5	-7.5	86.4	7.48	355	337	178	0.17
2010/06/15	17:30:22	71.79	7.90	-51.3	-4.6	87.8	7.64	363	343	182	0.17
2010/06/15	17:30:39	71.67	7.96	-54.7	-5.0	87.2	7.59	363	343	182	0.17
2010/06/15	17:30:55	71.77	7.93	-52.9	-0.9	90.2	7.85	369	349	185	0.18
2010/06/15	17:31:15	71.25	7.92	-52.5	-0.8	90.5	7.92	365	343	182	0.17
2010/06/15	17:32:00	71.17	7.86	-49.1	3.1	89.4	7.82	370	347	185	0.18
2010/06/15	17:32:16	71.63	7.46	-26.2	12.4	89.0	7.75	380	359	190	0.18
2010/06/15	17:32:33	71.16	8.00	-56.6	0.1	89.0	7.80	409	384	205	0.20
2010/06/15	17:32:58	72.73	8.13	-64.3	-14.0	88.4	7.61	326	311	163	0.16
2010/06/21	16:30:50	73.73	8.07	-61.3	8.4	80.6	6.86	344	332	172	0.16

2010/06/21	16:31:07	73.76	8.20	-68.6	1.6	79.7	6.79	352	339	176	0.17
2010/06/21	16:31:22	73.67	8.18	-67.4	1.6	77.8	6.63	366	353	183	0.17
2010/06/21	16:31:35	73.81	8.17	-67.1	0.2	80.3	6.83	364	352	182	0.17
2010/06/21	16:31:51	73.69	8.22	-69.6	-2.9	83.1	7.08	358	346	179	0.17
2010/06/21	16:33:09	73.69	8.18	-67.5	-2.7	82.3	7.01	364	351	182	0.17
2010/06/21	16:33:22	73.92	8.40	-80.1	-16.9	83.8	7.12	374	362	187	0.18
2010/06/21	16:33:38	73.87	8.24	-71.0	-7.0	81.6	6.94	386	374	193	0.18
2010/06/21	16:34:18	74.07	8.13	-64.7	17.0	83.4	7.08	326	316	163	0.16
2010/06/22	15:09:30	73.45	6.67	18.9	23.3	79.0	6.70	461	444	231	0.22
2010/06/22	15:10:01	73.05	6.67	18.7	21.9	78.5	6.69	514	493	257	0.25
2010/06/22	15:11:22	72.91	6.57	24.6	25.4	78.1	6.66	588	562	294	0.28
2010/06/22	15:12:54	74.11	6.75	14.5	24.2	81.2	6.84	469	455	235	0.23
2010/06/22	15:13:11	74.00	6.53	26.9	28.8	80.8	6.82	312	302	156	0.15
2010/06/22	15:13:29	73.91	6.59	23.2	31.0	80.9	6.83	564	545	282	0.27
2010/06/22	15:14:13	75.01	6.71	16.8	38.0	76.0	6.34	488	478	244	0.23
2010/06/22	15:14:28	74.50	6.79	12.1	33.7	81.7	6.85	477	464	238	0.23
2010/06/22	15:14:45	74.19	6.68	18.3	32.9	80.2	6.76	482	468	241	0.23
2010/06/28	22:52:07	73.59	8.51	-86.4	-70.8	80.9	6.80	313	302	157	0.15
2010/06/28	22:52:23	73.71	8.18	-67.7	-24.9	80.5	6.76	361	348	180	0.17
2010/06/28	22:52:38	74.54	8.41	-80.9	-41.9	79.3	6.59	387	377	194	0.18
2010/06/28	22:52:53	74.88	8.54	-88.4	-53.2	80.5	6.67	395	386	197	0.19
2010/06/28	22:53:09	73.51	8.43	-81.5	-46.5	81.3	6.84	394	380	197	0.19
2010/06/28	22:53:55	73.47	8.41	-80.3	-74.8	82.3	6.93	354	341	177	0.17
2010/06/28	22:54:12	73.67	8.54	-88.1	-71.9	81.9	6.87	393	379	196	0.19
2010/06/28	22:54:26	73.76	8.56	-88.9	-80.4	83.0	6.96	372	359	186	0.18
2010/06/28	22:54:52	73.79	8.38	-79.1	-72.3	82.9	6.95	306	295	153	0.15
2010/07/05	20:09:21	75.29	10.43	-196.7	-117.4	79.7	6.64	375	368	187	0.18
2010/07/05	20:09:37	75.77	10.35	-192.1	-108.9	81.3	6.74	396	390	198	0.19
2010/07/05	20:09:51	76.50	10.89	-223.5	-124.9	83.0	6.83	394	392	197	0.19
2010/07/05	20:10:05	75.60	10.39	-194.6	-96.9	83.4	6.93	402	396	201	0.19
2010/07/05	20:10:17	75.10	10.24	-185.8	-76.3	84.6	7.06	411	403	205	0.20
2010/07/05	20:18:34	74.73	10.19	-182.4	-87.9	81.2	6.81	403	393	202	0.19
2010/07/05	20:18:50	74.81	10.06	-175.2	-56.9	83.3	6.98	414	404	207	0.20
2010/07/05	20:19:03	74.72	9.99	-171.0	-46.8	83.5	7.00	410	401	205	0.20
2010/07/05	20:19:33	74.91	10.20	-183.0	-79.6	85.1	7.12	336	329	168	0.16
2010/07/12	16:59:53	69.57	11.52	-256.1	-231.1	81.4	7.15	380	350	190	0.18
2010/07/12	17:00:08	70.49	10.85	-218.5	-189.1	80.5	7.00	389	363	195	0.19
2010/07/12	17:00:23	70.52	11.19	-238.2	-210.1	82.4	7.17	383	356	191	0.18
2010/07/12	17:00:34	69.58	10.62	-205.5	-158.4	84.2	7.40	392	361	196	0.19
2010/07/12	17:00:46	69.14	11.17	-236.0	-164.1	83.8	7.40	417	382	208	0.20
2010/07/12	17:01:38	69.35	10.28	-185.9	-130.9	79.0	6.96	369	339	185	0.18
2010/07/12	17:01:53	69.16	10.14	-177.9	-105.8	82.4	7.27	406	372	203	0.20
2010/07/12	17:02:06	69.17	10.03	-171.9	-89.8	83.1	7.33	410	376	205	0.20
2010/07/12	17:02:45	70.05	10.89	-220.5	-159.4	81.1	7.09	348	323	174	0.17

2010/07/12	17:02:45	70.05	10.89	-220.5	-159.4	81.1	7.09	348	323	174	0.17

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