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The utilization of the freshwater invertebrates hyalella azteca and daphnia magna for use in assessing potential endocrine distruption in aquatic systems

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

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The chronic physiological effects of the compounds Atrazine, Tributyltin and 17α-ethinylestradiol were tested on the freshwater aquatic organisms; *Hyalella azteca* and *Daphnia magna*. *Daphnia magna* were exposed to a series of conditions designed to minimize the parthenogenetic cycle of and maximize the sexual cycle resulting in the formation of males and ephippia to be used as a screening assay for potentially endocrine disrupting chemicals. *Hyalella azteca* were exposed to the three contaminants during a 42-day chronic toxicity assay and analyzed for morphological changes to male secondary gnathopods, female brood pouches and gender ratios. It was found that atrazine, tributyltin and 17α-ethinylestradiol had no significant effects on the gender ratios or secondary sexual physiology of *Hyalella azteca*.

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LIST OF ABBREVIATIONS

AG = androgenic gland
AGH = androgenic gland hormone
AR = androgen receptor
BPA = bisphenol-A
DBT = dibutyltin
DEHP = di-2-ethyl hexyl phthalate
DES = diethylstilbestrol
DMSO = dimethylsulfoxide
DRL = dose-response lag
E2 = 17β-estradiol
EcR = ecdysone receptor
EDC = endocrine disrupting compond
EE = 17α-ethinylestradiol
FSH = follicle stimulating hormone
g = gram
JH = juvenile hormone
JHE= juvenile hormone esterase
JHEH= juvenile hormone epoxidehydrolase
L = Litre
LH = luteinizing hormone
MBT = monobutyltin
MF = methyl farnesoate
mg = milligram
mL = millilitre
mm = millimeter
MPP = Hanna Instruments 9828 multi-parameter probe
µm = micrometer
µS = microsiemens
NOEC = No observable effect concentration
P450 = cytochrome P450
PME = pulp-mill effluent
PVC = polyvinyl chloride
RXR = retinoid X receptor
SCN = suprachiasmatic nuclei
StAR = steroidogenic acute regulatory protein
TBT = Tributyltin
USP = ultraspiracle
VTG = vitellogenin

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INTRODUCTION

A wide variety of man-made chemicals with the capacity to disrupt the endocrine system are present in the aquatic environment (Alzieu and Heral, 1984; Colborn *et al*., 1993; Baldwin *et al*., 1995; Ankley *et al*., 1998; Dodson *et al.,* 1999; Graymore *et al.,* 2001; Ford *et al.*, 2004; Bowman *et al*., 2005; Bandelj *et al.*, 2006; Bonefeld-Jørgensen *et al.*, 2007; Coray and Bard, 2007; Dietrich *et al.,* 2010; Chang *et al.,* 2011) . 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 in subsequently increasing awareness of the long-term effects of many anthropogenic chemicals. A major group of contaminants includes endocrine-disrupting compounds or EDCs, a phrase first coined by Colborn in 1993 (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).

Chemicals may directly leach off of 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 EDCs being released in pulp mill effluents from the breakdown of plant sterols (Howell *et al.*, 1989; Hewitt *et al.*, 2000; Ellis *et al.*, 2003). 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). 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.

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 obtain 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, including parthenogenesis, 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 and have differing reproductive strategies. These include *Daphnia magna,* a free-swimming crustacean which typically inhabits the water column and *Hyalella azteca*, an amphipod 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.

One of the goals of this experiment is to manipulate the photoperiod and temperature and observe the sexual strategy utilized by *Daphnia* under stressful conditions, such as exposure to contaminants. Another objective of this experiment is to induce the conditions necessary to induce production of both ephippial females and males.

1.0 LITERATURE REVIEW

1.1 Important Potential Endocrine-Disrupting Compounds (EDCs)

While many potential endocrine-disrupting compounds (EDCs) have been documented in the scientific literature, only a few will be detailed here.

Diethylstilbestrol

An early example of a dangerous endocrine disrupting compound is diethylstilbestrol (DES); a synthetic, nonsteroidal estrogen synthesized in 1938 (Dodds *et al.*, 1938). In the early 1940's DES was aggressively prescribed 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 women undergoing seemingly normal pregnancies. Dieckmann *et al.*, (1953) found that there was no benefit to taking DES during pregnancy, however until 1970 it was given to pregnant women with the belief it would reduce the risk of pregnancy complications and spontaneous abortions. However, by 1971 several studies had shown that DES caused 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).

Bisphenol-A

Another example of such a compound that has made headlines in recent years is the chemical bisphenol-A or BPA. Once used as an antioxidant to plasticize bottles, including baby and water bottles; it is now linked to several incidences of disease. For instance, excessive BPA exposure is associated with reduced foetal body weight and survival in rodents and sheep (Ranjit *et al.*, 2010). It is associated with an increase in metabolic disorders in mice and humans (Lang *et al.*,

2008; Vom Saal *et al.*, 2008; Ranjit *et al.*, 2010). It has been shown to bind to thyroid hormone receptor *in vitro* and shown to increase levels of growth hormone during *in vivo* rat studies (Rubin and Soto, 2009). It is also believed to have adverse effects on ovarian function and mammary gland development in mice and humans (Roy *et al.*, 2009; Ranjit *et al.*, 2010). Bonefeld-Jørgensen *et al.* (2007) performed in vitro bioassays in human cell lines and found that BPA inhibits aromatase activity. They also found that the compound is an agonist of estrogen receptor and an antagonist of androgen receptor *in vitro* (Bonefeld-Jørgensen *et al.*, 2007). Perinatal exposure of mice 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 reduced fertility and cancers (Maffini *et al.*, 2006). In addition, 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 in polyvinyl chloride (PVC) (Rakkestad *et al.*, 2007), which is used in clothing, furnishings, packaging material, tubing, building materials, electronics, inflatable structures and many other products (Greenpeace, 2003). 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 such as mice, including impacts on the development of the male reproductive system and sperm production in young animals (Mylchreest *et al.*, 2000; Henley and Korach, 2006; European Union, 2007). These results have also been witnessed in humans through epidemiological studies (Mylchreest *et al.*, 2000). Phthalates may also play a role in disrupting masculine neurological development when male rodents are exposed prenatally (Swan *et al.*, 2009). Phthalates can also be easily released into the environment as they are not covalently bound 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 (2-ethyl-5-hydroxyl-hexylphthalate and 2-ethyl-5-oxo-hexylphthalate) 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). 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 (Mylchreest *et al.*, 2000; Henley and Korach, 2006). One 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 one of the most commonly used agrichemicals, sold in hardware and garden stores by its more common name, Roundup (OMAFRA, 2008). 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, 2003). It functions by inhibiting synthesis of tyrosine, tryptophan, and phenylalanine in target weeds (Schonbrunn *et al.*, 2001). However, it has also been shown to modulate plant cytochrome P450 (Lamb *et al.*, 1998).

It has been found that agricultural workers working with glyphosate are at risk of developing pregnancy issues such as miscarriage and preterm delivery (Savitz *et al.*, 1997). Roundup has been shown to inhibit steroidogenesis *in vitro* by disrupting the steroidogenic acute regulatory protein (StAR); important in mediating a rate-limiting of steroidogenesis in mammals (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 indicating endocrine disruptive properties. 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. Richard *et al*. (2005) also found that glyphosate disrupts aromatase activity and its mRNA levels. Glyphosate interacts with the active site of the purified aromatase enzyme in a competitive manner and inhibits its gene expression. Adjuvants, such as the surfactant polyethoxylated tallowamine, allow the compound to enter plant cells and enhance glyphosate bioavailability and bioaccumulation (Bonn, 2005; Richard *et al*., 2005). Roundup, due to the added adjuvants, is always more toxic than its active ingredient, glyphosate (Richard *et al*., 2005).

Triclosan

Triclosan, a chlorophenol, is widely used as a broad-spectrum antibacterial and antifungal agent in different types of commercial preparations, from hand sanitizer to toothpaste (Zorilla *et al*., 2009). Due to its ubiquity it is frequently detected in urban effluent waters with concentrations measured up to 37.8 µg/L and surface waters up to 431 ng/L (Zorilla *et al*., 2009).

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 decreases thyroid hormone concentrations in the male juvenile rat in a dose-dependent manner at 30 mg/kg and higher. They also found that rat serum testosterone levels were decreased at exposures of 200 mg/kg. Kumar *et al.*, (2009) found a reduced level of

StAR protein in rat testicular Leydig cells after exposure to triclosan. Moreover, there was a significant decrease in the level of serum luteinizing hormone (LH), follicle stimulating hormone (FSH), cholesterol, pregnenolone, and testosterone. The decreased the synthesis of androgens and reduced sperm production in the treated male rats which could be mediated by decreased synthesis of LH and FSH thus involving the 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 xeno-steroids in pulp mill effluent is suggested 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 analyzing 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 (Munkittrick *et al.*, 1997; Ellis *et al.*, 2003), 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.

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). Since many potential EDCs are analogues of vertebrate hormones, it is important to examine both the vertebrate endocrine system and the invertebrate endocrine system to determine the impact of these contaminants.

1.2.1 Vertebrates

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). 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 is specialized for vertebrates needs, it is homologous to the X-organ-sinus gland complex found in crustaceans such as *Hyalella*, responsible for regulating moulting, 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 titre, 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 vertebrate P450s have the potential to interfere with invertebrate P450s as well.

Endocrine disruption

Several types of endocrine disruption in vertebrate systems can occur. Chemicals, such as DES, can be estrogen mimicking and is therefore considered estrogenic (Roy *et al.,* 2009; Kolle *et al.*, 2011). Chemicals such as testosterone or PMEs can be androgen mimicking, and are considered androgenic (Bandelj *et al.,* 2006; Kolle *et al.*, 2011). Chemicals can also inhibit hormones by blocking the receptor, interfering with the carrier compound used to transport it through the circulatory system or within the nucleus to activate the DNA machinery to produce an end-product, 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 titres decrease, it does not necessarily mean that estrogen titres will increase, as testosterone is a precursor to estrogen.

1.2.2 Invertebrates

While much is known about vertebrate endocrinology, much less is known about the invertebrate endocrine system. Although it is known that invertebrates share several analogous glands, hormones and enzymes to vertebrates, such as the sinus gland, androgens and P450 (Lafont, 2000), 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). While *Daphnia* and *Hyalella* will be mentioned briefly in this ensuing section, more detailed information in future sections will be forthcoming.

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 (Schafellner *et al.*, 2008; Sim and Denlinger, 2008; Ikeno *et al.*, 2010). They also regulate polyphenisms, or multiple discrete phenotypes arising from a single genotype, controlling caste differentiation in social insects (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 relates slightly 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. It is unknown whether they work in exactly the same way, 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 moult, 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 moult (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 hive bees becoming 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 moulting 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 moulting 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 moulting 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).

Ecdysones can be used as insecticides by prematurely inducing moulting, thereby protecting crops from insects (Fujita and Nakagawa, 2007). Insecticide RH-5992 is an insecticide which mimics ecdysone (Kreutzweiser *et al.*, 1994). Acute toxicity tests were conducted on the amphipod *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 moulting (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 was not available in the literature.

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. VTG is an egg yolk precursor protein expressed in insects, and many oviparous vertebrates and is 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 insects 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 titre 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).

Crustaceans

Crustaceans, including *Daphnia* and *Hyalella*, have several endocrine glands specific to the subphylum, such as the eyestalk (sinus gland and X-organ) and pericardial organ (Fingerman, 1997). However, the production and activity 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). 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 of the subphylum Crustacea 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 such as *Hyalella*, the glands are present in the head close to the optic centers (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.

1.3 PARTHENOGENESIS

Parthenogenesis is the growth and development of an embryo or seed without fertilization (HHMI, 2000). It has been known to occur in several species of plants, invertebrates and very few vertebrates (Lampert, 2009). It can occur in a variety of forms, such as i) thelytoky, where only female offspring are produced and no mating is observed ii) pseudogamy, where mating occurs and the eggs require activation by entry of sperm, but only the maternal chromosomes are expressed iii) automixis, in which the eggs undergo meiosis, and iv) apomixis, in which the eggs do not undergo meiosis (Kirkendall and Normark, 2003). Plant parthenogenesis is outside of the range of this discussion.

In vertebrates, parthenogenesis, while relatively rare compared to sexual reproduction is known to take place in several species of reptiles, fish, birds and sharks. There are no known cases of mammals in the wild as many mammalian genes are maternally or paternally imprinted, or silenced (HHMI, 2000). However, parthenogenesis can be induced in some laboratory animals when imprinting is circumvented (HHMI, 2000; Kono *et al*., 2004; Lampert, 2009; Sritanaudomchai *et al.*, 2010). For example, Kono *et al*., (2004) created a parthenogenetic mouse, solely from female cells. Parthenogenesis does however; occur naturally in some species of invertebrates (Lampert, 2009).

1.3.1 Cyclical Parthenogenesis

Abiotic factors

Cyclical parthenogenesis is a reproductive strategy that occurs when organisms typically reproduce asexually producing clones. However, when a combination of several key abiotic factors occurs, asexual reproduction halts and reproduction becomes sexual (Stross and Hill, 1965).

Synchronization to relative time of day and seasonal cycles is key to survival (Kumar *et al.*, 1997). Night-time and day-time environments differ in illumination, temperature, food supplies and predators. Organisms have, therefore, developed highly specialized temporal programmes to be better adapted to activity during night or day conditions (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), which 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 (Stross and Hill, 1965; Kumar, 1997). 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 (pineal gland) 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*.

Rotifers

Monogononts are a mostly freshwater class of rotifers which tend to have a reduced corona (ciliated region around the head used for locomotion and feeding) 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 (shortened daylength, reduced temperatures); asexual females produce sexual females via mixis, or sexual reproduction (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. Asexual reproduction tends to predominate in this species (Carmona *et al.*, 2009). However, under stressful conditions, sexual reproduction is induced. *Brachionus calyciflorus*, are thought to be sensitive to most stressors such as 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 (Preston *et al.*, 2000).

In Summary

Parthenogenesis is an important reproductive strategy for many organisms. Cyclical parthenogenesis and induction to a sexual reproductive strategy, is especially important, and provides an interesting tool to study endocrine disruption. However, as seen by the dearth of literature on this subject, the endocrinology of parthenogenesis and cyclical parthenogenesis is poorly understood.

1.4 BIOASSAY ORGANISMS

1.4.1 *Daphnia magna*

Daphnia magna are small freshwater crustaceans commonly used in ecotoxicology 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; McCarthy et al. 2005; 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 filter setae (Figure 1) 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 phytoplankton and detritus 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, short lifecycle, and parthenogenic reproductive strategy make them ideal candidates as model organisms in this study. Additionally, their importance and relevance to the ecosystem make their potential decline due to exposure to EDCs catastrophic.

Daphnia Behaviour

Daphnia magna are pelagic or open-water organisms and 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 (Figure 1) (Dodson and Hanazato, 1995; Marshall, 2009). Their normal motile 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 laboratory, which typically consists of short water columns, their 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).

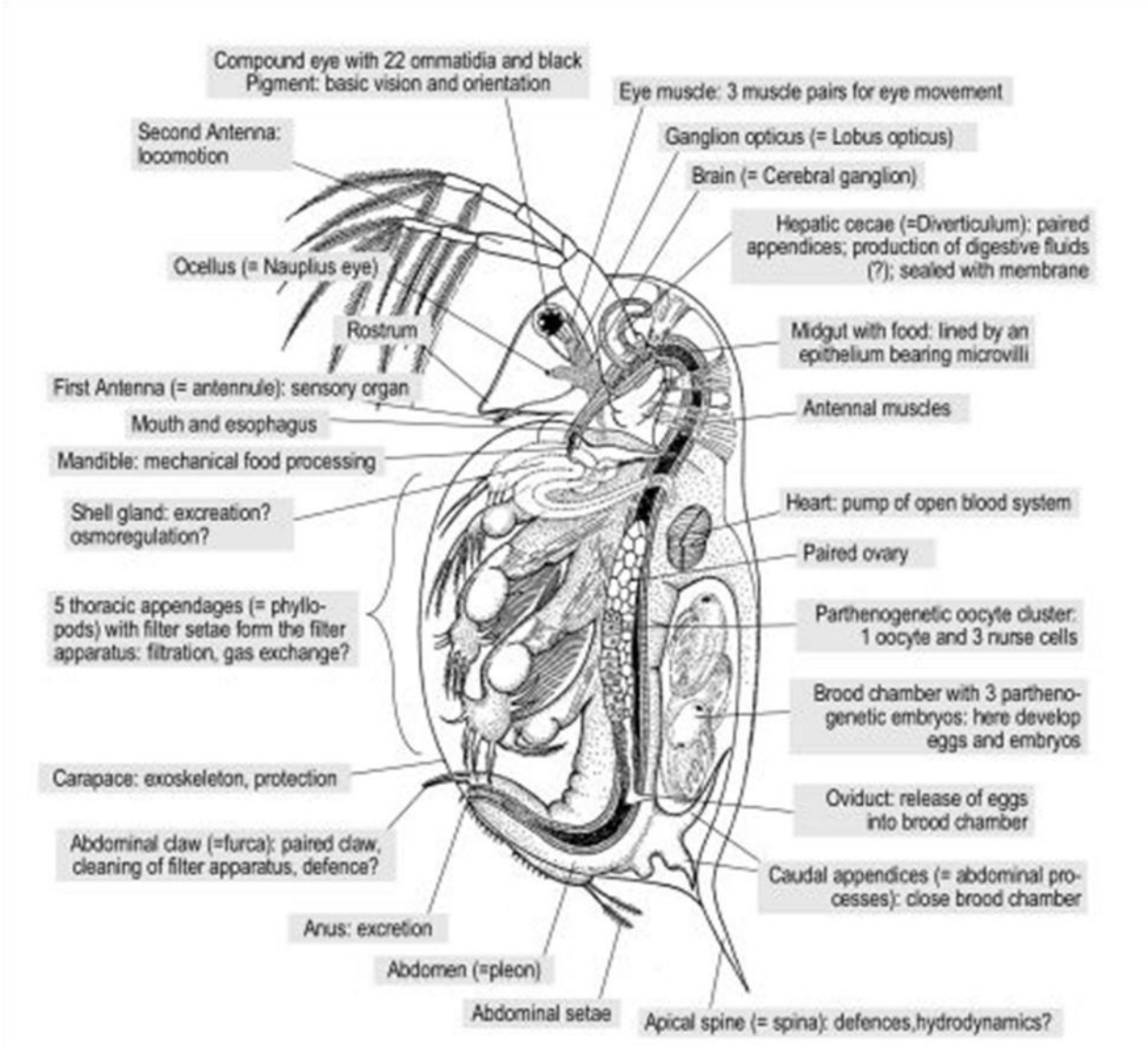

Figure 1: Anatomy of *Daphnia magna*. (Ebert, 2005a). Note: Second Antenna for locomotion and Brood chamber for reproduction.

Daphnia have been shown to be highly sensitive to a variety of common contaminants, such as atrazine and tributyltin, 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. Offspring are produced by ameiotic (nonreduction of chromosomes) 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 (Figure 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. These include a photopertiod less than 9 hours, food limitation and crowding (Kleiven *et al.*, 1992). Once the sexual trigger is set, instead of producing solely females, *Daphnia* begin to produce haploid males (Figures 2 and 3) by parthenogenesis, and the females lay meiotically-produced haploid eggs known as ephippia that require fertilization by males (Hebert 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 (Figures 1 and 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 ecotoxicological 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. It is one of the goals of this experiment to induce the conditions necessary to induce production of both ephippial females and males.

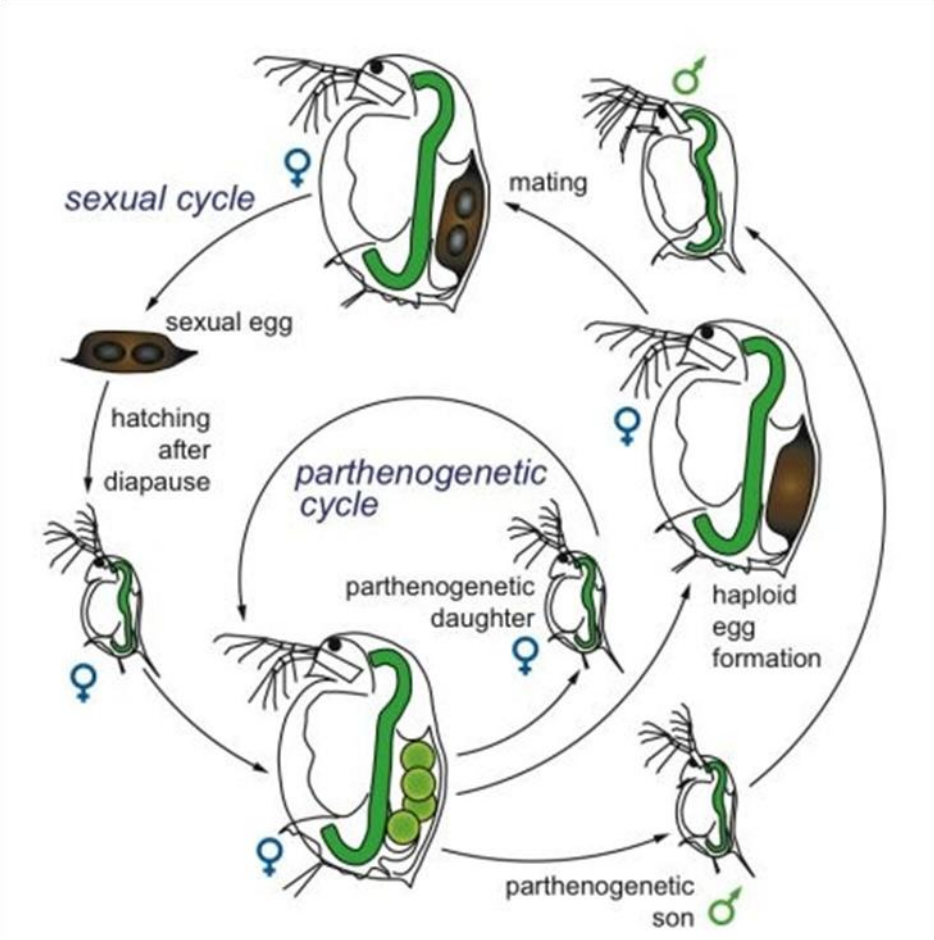

Figure 2: Life cycle of a cyclic parthenogenetic Daphnia. Note the ephippia in the brood chamber (Ebert, 2005b).

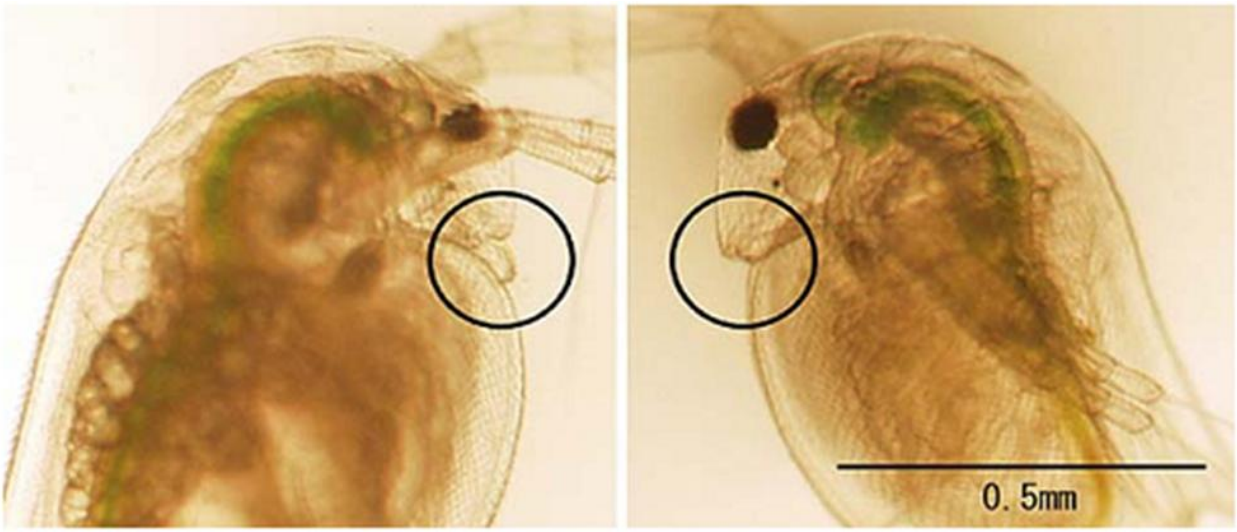

Figure 3: Differences between male (left) and female (right) Daphnia (Tatarazako and Oda, 2007). The circled areas feature the primary antenna, elongated in the male.

1.4.1.1 *Daphnia magna* Endocrinology

Studies have revealed that juvenile hormones (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 (Olmstead and LeBlanc, 2002, 2003; Tatarazako *et al.*, 2003; Oda *et al.*, 2005).

Daphnia are also known to express the vitelligenin (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).

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 (Kashian and Dodson 2004). 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.

Dodson *et al.* (1999a) showed an exposure-response relationship between *Daphnia* sex ratio and atrazine. They found that exposure to atrazine at 0.5 µg/L caused a detectable shift in sex determination towards males. These shifts also occur at ecologically relevant doses, in levels commonly found in aquatic environments (Dodson *et al*., 1999a).

Brennan *et al.* (2006) tested the effects of 4-NP, DES, 17β-estradiol (E2) and BPA 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 a disparity as to what exactly the vertebrate hormones do in the 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 Endocrine *Daphnia magna* Bioassays

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. They were 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.

1.4.2 *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; McCarthy *et al*. 2004; 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 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 6^{th} instar by examination of the secondary gnathopod. Males have an enlarged secondary gnathopod (Figures 4, 14, and 15), while females possess a brood pouch or marsupium, which can be identified as a large mass beneath their thorax (Figure 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 (Figure 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, or brood pouch, 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).

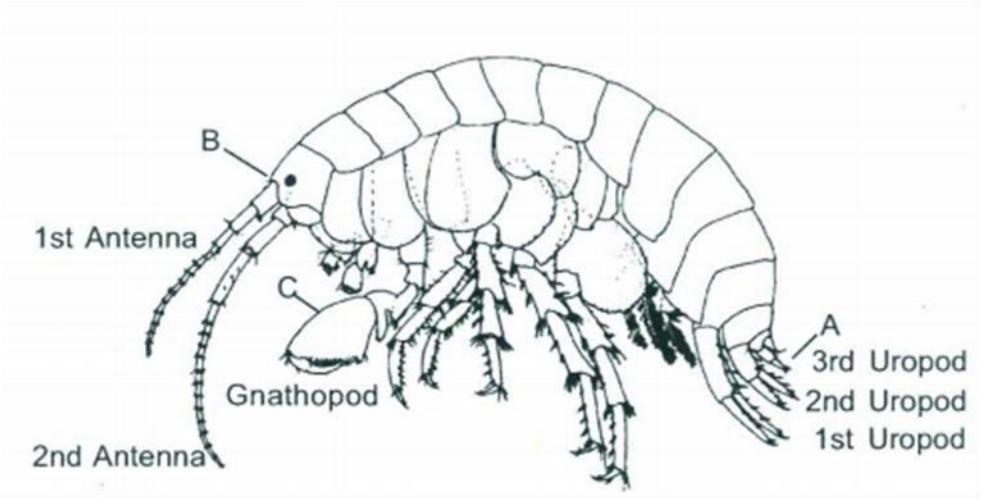

Figure 4: Hyalella azteca. (A) denotes the uropods; (B) denotes the base of the first antennae; (C) denotes the male secondary gnathopod used for grasping females. Measurement of length is made from the base of the 3rd uropod (A) to (B). Females are recognized by the presence of egg cases and the absence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

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 and 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 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 some concentrations of atrazine exposure (Marshall, 2009). Despite the observations, there is little known as to the reason why *Hyalella* group as a stress response.

1.4.2.1 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.

A study by Flick *et al*., (2001) studied differential analysis of RNA between *Hyalella* exposed to 17α-ethinylestradiol (EE) and control organisms. *Hyalella* showed a genetic response to vertebrate hormones. 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, a protein that 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 in a pulse-dose exposure scenario (weekly BPA application). On day 103 they measured the proportions of juveniles and breeding females and found that those in the highest BPA treatment were 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 including, 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 chronic 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*.

Hyalella Endocrinology

The endocrine system of *Hyalella* is poorly understood, as there is a dearth of information in the literature. It is usually assumed that their endocrine systems follow similar patterns to those of other sexual crustaceans, such as shrimp, mysids, crayfish, etc (Vandenbergh *et al*., 2003). 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 gender using secondary rather than primary sexual characteristics allows the reduction of handling organisms (Vandenbergh *et al*., 2003). 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

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.

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 (Barbosa *et al.*, 2008; CFBD, 2011).

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.

1.5.1 Atrazine

Atrazine (Figure 5) 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.

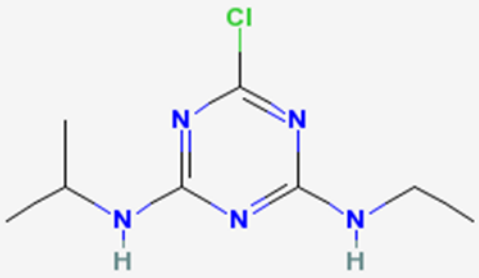

Figure 5: Chemical structure of atrazine (NCBI, 2011a).

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 $C_8H_{14}ClN_5$ (Figure 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 potentially toxic contaminant in aquatic environments, as it is able to associate and partition to sediments and become bioavailable to organisms such as *Hyalella*.

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-isopropylamelide 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 dicot plant chloroplasts 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 around the world (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 canola 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 of 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).

Potential Toxicity of Atrazine

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 has been seen in concentrations less than 10 µg/L (Graymore *et al.,* 2001). Atrazine can inhibit nitrification in sediments exposed to concentrations of 50 and 100 µg/L, stimulate denitrification under light at 10 µg/L, and inhibit denitrification in the dark at 100 µg/L; therefore, can significantly affect nitrogen and carbon cycling in streams, affect stream recovery from nitrate pollution and reduce total organic

matter retention (Laursen and Carlton, 1999). 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). Studies in mice have shown that 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 also. 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 in rats (Health Canada, 1993).

There have been no conclusive findings regarding atrazine as a human 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 animals 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 a high level of exposure that is unlikely, but possible 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 had an LC_{50} of 410 µg/L (Detenbeck *et al.*,1996).

Several bioassays have been conducted (Macek *et al*., 1976; Wan *et al*., 2006; Marshall, 2009) in the past examining the effects of atrazine on the survival of *Daphnia magna* and *Hyalella azteca,*. LC_{50} values for various periods of time have been reported for both organisms. The USEPA (2003) reports that *Daphnia magna* have a 48-hour LC_{50} value of 49 mg/L atrazine and

that *Hyalella azteca* have 48-hour LC_{50} value of 14.7 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).

Marshall (2009) studied behavioural endpoint of *Daphnia* and *Hyalella* during 24 hour subacute bioassays. Marshall (2009) found that atrazine affected swimming height, spinning behaviour and caused immobilization of *Daphnia magna* in the water column during a 24 hour subacute bioassay, validating the use of those endpoints during subacute studies. Marshall (2009) also found that atrazine decreased swimming behaviour and substrate crawling, while increasing immobilization, burrowing and grouping behaviour of *Hyalella azteca* during the 24 hour subacute bioassay. In addition, Marshall (2009) studied the effects of atrazine on respiration rates in *Hyalella azteca* and *Daphnia magna* and found that at 50 µg/L respiration rates increased in *Hyalella* and at 100 µg/L, respirations increased in both *Hyalella* and *Daphnia*. These results validate behavioural endpoints and pose ecological concerns of this compound, as altered behaviour in the wild could compromise survival of these organisms.

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). 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*.

1.5.2 Tributyltin

Tributyltin is potentially one of the most toxic chemicals ever deliberately introduced into the aquatic environment (Chau *et al.*, 1997). Tributyltin (TBT) (Figure 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 octanol-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).

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 have 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

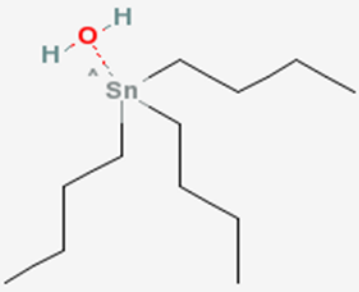

Figure 6: Chemical structure of TBT hydroxide (NCBI, 2011b)

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). 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 as an Endocrine Disruptor

Several experiments have been conducted looking at TBT as an endocrine disrupting compound. In the dog whelk (*Nucella 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 *Nucella lapillus* but not to the 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 androgen 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 *Xenopus 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 to 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).

TBT Bioassays

Several bioassays have been performed in the past using *Daphnia* and *Hyalella* (Marshall, 2009). 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 assay organisms (Fent and Looser, 1995).

Bioassays examining the changes in swimming behaviour of *Daphnia magna* in response to TBT have also been performed (Marshall, 2009). 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). No observable effect concentrations (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.

Marshall (2009) performed a 72 hour mortality assay with TBT studying *Daphnia magna* and *Lumbriculus variegatus* and found that after 72 hours, concentrations of 10, 50 and 100 µg/L had 100% mortality on *Daphnia*, while 50 µg/L had approximately 20% lethality, while 100 µg/L had approximately 100% lethality. These results indicate that TBT is quite toxic to aquatic organisms. The research also covered behavioural aspects of *Daphnia* and *Hyalella* during 24 hour subacute bioassays. Marshall (2009) found that TBT affected swimming height, spinning behaviour and caused immobilization of *Daphnia magna* in the water column during a 24 hour subacute bioassay, validating the use of those endpoints during subacute studies. Marshall (2009) also found that TBT decreased swimming behaviour and substrate crawling, while causing immobilization, increasing burrowing and grouping behaviour of *Hyalella azteca* during the 24 hour subacute bioassay. In addition, Marshall (2009) studied the effects of TBT on respiration rates in *Hyalella azteca* and *Daphnia magna* and found that at 100 µg/L, TBT caused increased respiration increased in both *Hyalella* and *Daphnia*. These results validate behavioural endpoints and pose ecological concerns of this compound, as altered behaviour in the wild could compromise survival of these organisms.

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). 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.

1.5.3 17α-Ethinylestradiol

17α-Ethinylestradiol (EE) (Figure 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). It has been approved for use by the FDA in 1943 and has been used ever since (FDA, 2004). With respect to the current study, it is used both as a positive 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 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

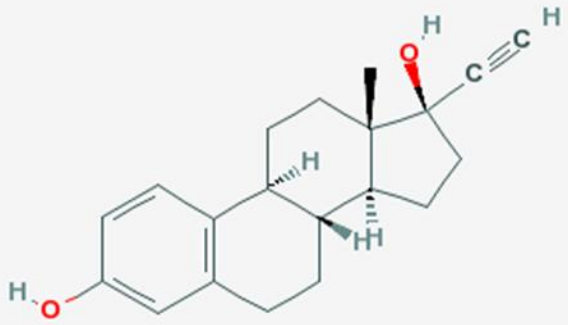

Figure 7: Chemical structure of Ethinyl Estradiol (NCBI, 2011d)

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 behaviour 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, 25 EE-exposed phenotypic female zebrafish were held 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 had a significant effect on zebrafish.

Despite the research examining the synthetic human estradiols on fish, the effects 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 (Jeng 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 EE. 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*.

1.5.4 Dimethyl Sulfoxide

Toxicity bioassays involving hydrophobic chemicals hinge heavily on the use of carrier solvents as delivery systems (Hallare *et al*., 2006; Marshall, 2009). Dimethysulfoxide (DMSO) (Figure 8) is a commonly used organic solvent in toxicity testing (Marshall, 2009). DMSO was used in the current 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

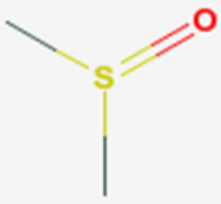

Figure 8: Chemical structure of Dimethyl sulfoxide (DMSO) (NCBI, 2011c)

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).

Marshall (2009) used DMSO to study the effects of atrazine and tributyltin on *Daphnia magna*, *Hyalella azteca* and *Lumbriculus variegatus* and found no significant difference between DMSO and no treatment control.

1.6 OBJECTIVES

Although chemical analysis of industrial air and water effluents and various soil, plant and food toxicant tests may provide some predictability of the quality of drinking water to humans, the ultimate monitors are those organisms having metabolic activities that are comparable to man (Buck, 1979). The goal of this thesis is to observe two aquatic invertebrates to see if they can alert us to unseen and perhaps devastating environmental hazards with respect to potentially endocrine disrupting compounds. The compounds of interest in this thesis are present in significant concentrations 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). 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*.

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 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.

For the *Daphnia* assays, 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.

For the *Hyalella* assays, 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.

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 laboratory 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 ethinylestradiol (EE) is based on results from Vandenbergh et al. (2003) and test solutions all had a final concentration of 0.1% v/v dimethylsulfoxide (DMSO), a value for this carrier compound 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; Marshall 2009; Ren et al*.,* 2009).

Despite the wealth of knowledge of acute responses of *Daphnia* and *Hyalella* to tributyltin (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% DMSO. 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*.

Additionally, a concentration of 0.1 µg/L of EE was used, as it was the lowest concentration that Vandenbergh *et al*. (2003) had success with. For the experiment with *Hyalella azteca*, EE served as a control. However, with the *Daphnia magna* experiment, EE was to serve as a test compound.

All dilutions were made from stock solutions of 100 mg/L TBT in DMSO, 100 mg/L atrazine in DMSO and 100 mg/L EE in DMSO. In all bioassays, reference and 0.1% DMSO control treatments were either performed at the same time as the TBT and atrazine treatments or randomly distributed throughout the assay 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 for *Hyalella azteca*. See appendix A for dilution charts.

2.3 *DAPHNIA MAGNA* BIOASSAY

2.3.1 *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 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 (Pers. Comm. – Karen Puddephat). 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 submerded several centimeters below the water surface to allow airflow, while keeping dust, debris, and potential contamination out of the aquarium. *Daphnia* were fed 3 times 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 toxic nitrites, or nitrates which can cause stress. A 200 L carboy at the same room temperature was aerated continuously for such a task.

2.3.2 *Daphnia magna* Sexual Induction Bioassay

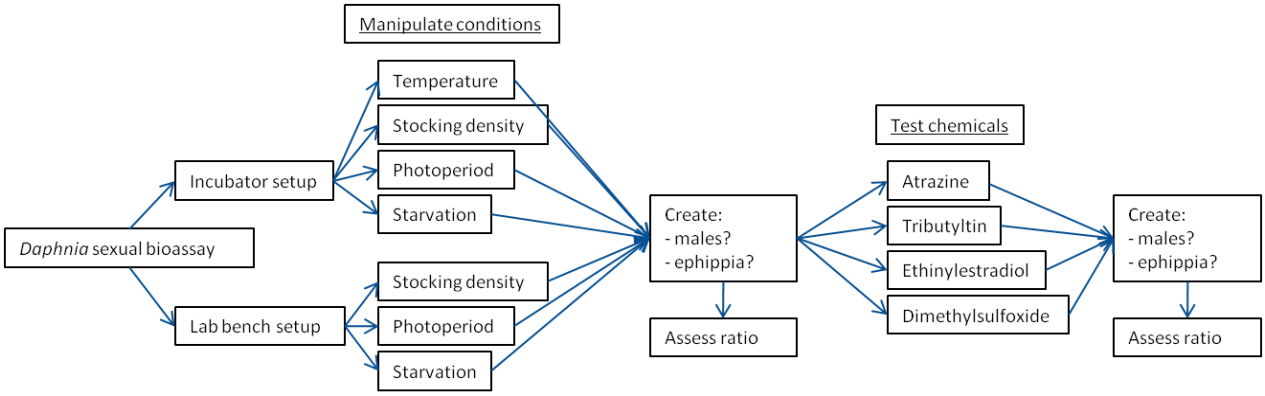

Figure 9: Outline of Daphnia sexual bioassay to determine ratios of male/ephippial female Daphnia between controls and chemical treatments to determine if the chemicals of interest disrupt endocrine pathways.

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 Chapter 1. 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 (Figure 9). 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 Celsius. 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 Celsius. 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 was placed with an airline tube inserted within it, tightly sealed with parafilm™. Each tube was then connected

through a network of gang valves to an air pump. The airflow rate in each vessel was set 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 in both setups were fed in weekly intervals of approximately $7.5x10^5$ cells of

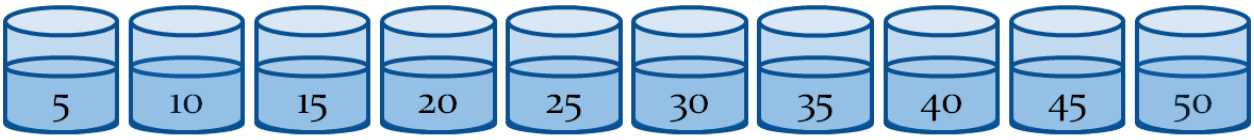

Figure 10: Experimental setup. Numbers represent numbers of *Daphnia* in each vessel

Selenastrum per vessel per feeding session.

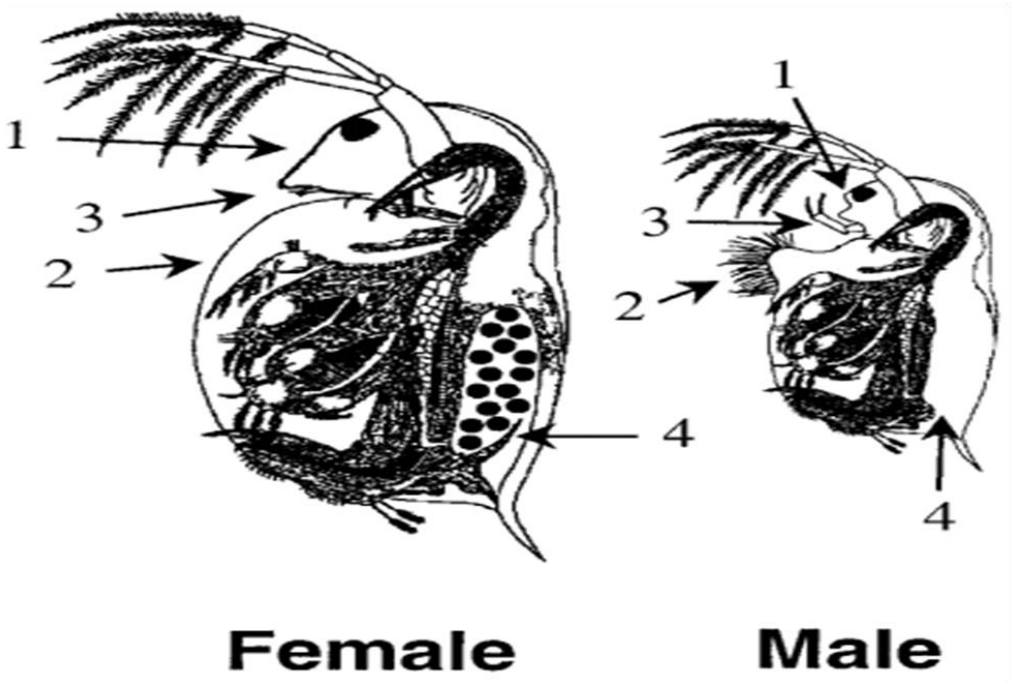

Figure 11: Secondary sex characteristics in adult daphnids. In the female, an elongated rostrum is present on the head capsule (1); carapace edge below the head capsule is smooth with no setae present (2); first antennae are diminutive (3); and a long abdominal process forms the posterior boundary of the brood chamber (4). In the male, frontal portion of the head capsule is flattened (1); carapace edge below the head capsule is modified and lined with setae (2); first antennae are elongated (3); and abdominal process is absent (4). (Olmstead and LeBlanc, 2000)

During the assay

During the assay, *Daphnia* were monitored for signs of males and developing ephippia. Organisms were monitored according to Figure 11.

2.4 *HYALELLA AZTECA* BIOASSAY

2.4.1 *Hyalella azteca* culturing

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*.

Additionally, 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.

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 Hanna Instruments 9828 Multi-Parameter Probe (MPP). Two litres of silica sand with four 10 cm x 10 cm gauze sheets gauze sheets overlaying 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 by Environment Canada (1997) and a photoperiod of 16hr light: 8hr dark. Glass sheets were placed above the aquaria in such a way as to keep dust and debris out of the aquarium, but allowing enough air to enter. *Hyalella* were fed 3 times a week on non-consecutive days using 8-12 milligrams of Tetramin™ flakes ground and sieved through a 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 toxic nitrites, or nitrates which can cause stress. 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

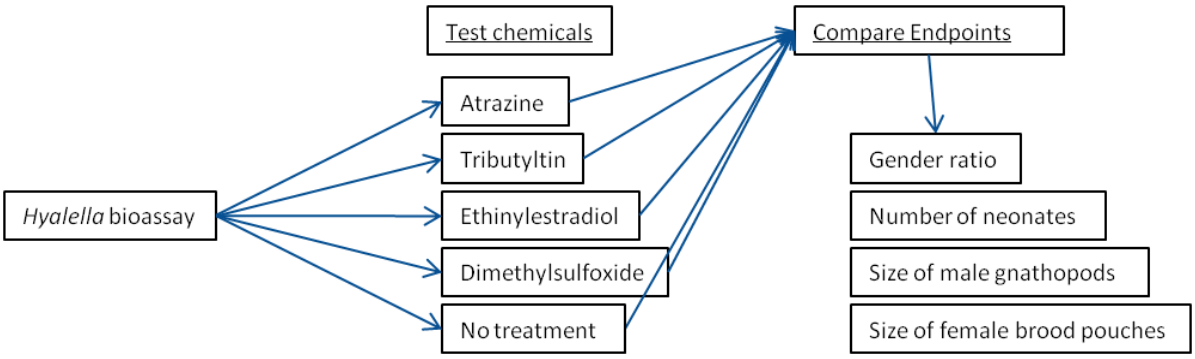

Figure 12: Outline of *Hyalella* bioassay highlighting the major endocrine endpoints

This bioassay was conducted to determine if there were potentially endocrine disruptive effects of atrazine, TBT and EE on *Hyalella azteca*. Protocols for this experiment were based on USEPA (2000) protocols for *Hyalella azteca* 42-d Test for Measuring the Effects of Sediment Associated 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 (It was decided later to have 18 more experiments for a total of 63). 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 initiation of bioassays 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. A block randomization procedure was developed for initiating bioassays using 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. Treatments that were started on a given day were selected at random. See Appendix B for full list of treatments.

Culture vessels

Each beaker was fitted with two 4-ply 5 cm x 5 cm 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 that were smaller than the grains of silica would be impossible 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 (Figure 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, which included presence in the water column, or presence within the sediment and grouping behaviour. 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.

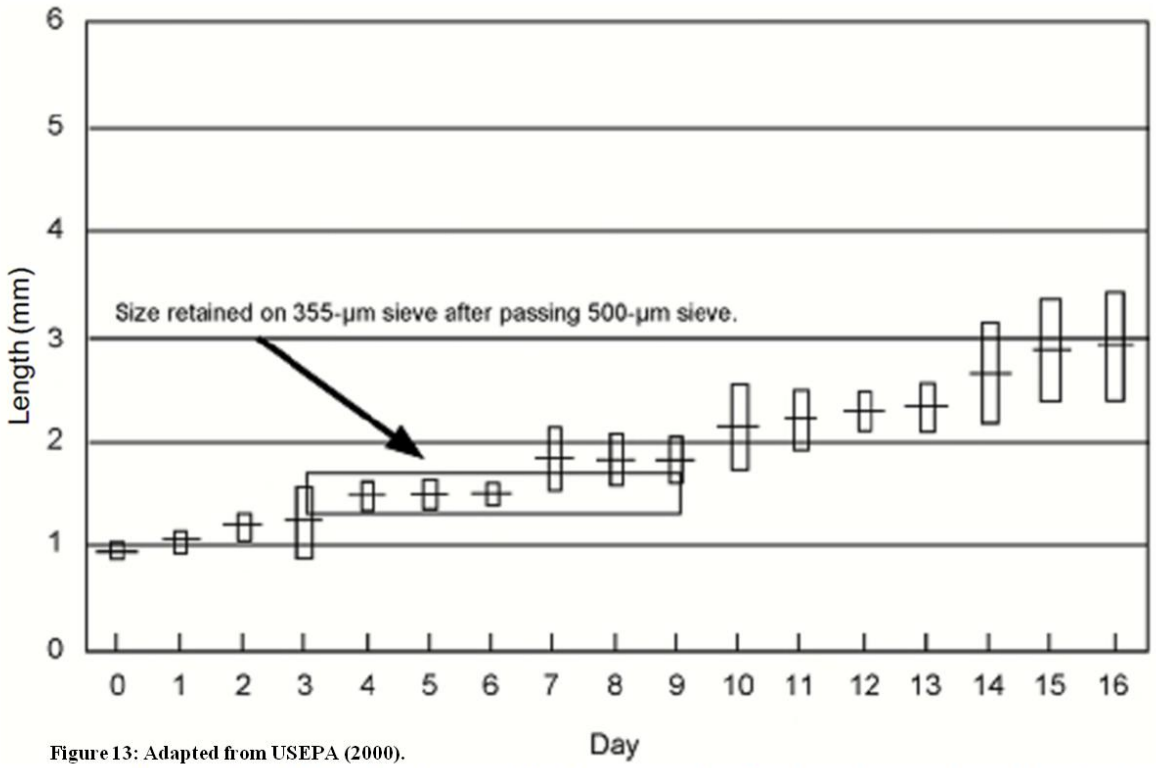

Figure 13: Adapted from USEPA (2000).

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.

During the Assay

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 and can be found in Appendix B. These included *Hyalella* presence in the water column, or presence within the sediment as well as grouping behaviour. Chemical parameters shown in Appendix E, were logged with the 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. Observations were made regarding the number of amplexus events, the number of *Hyalella* in groups of two or more, and the location of the *Hyalella* within the test chamber.

End of Assay

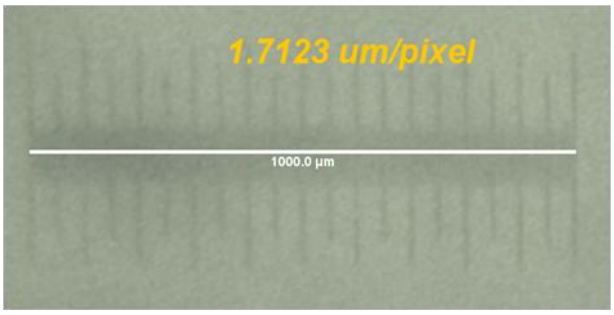

Figure 14: Calibration for image analysis

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. To analyze the images, ImageJ image analysis software was used. 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 (Figure 14). All images were taken with those exact settings to maintain consistency.

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. The data was recorded and can be found in Appendix B. After sieving, 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 (Figure 15) 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 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. After image capture, all surviving organisms used in the experiment were then placed in a retirement tank.

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.

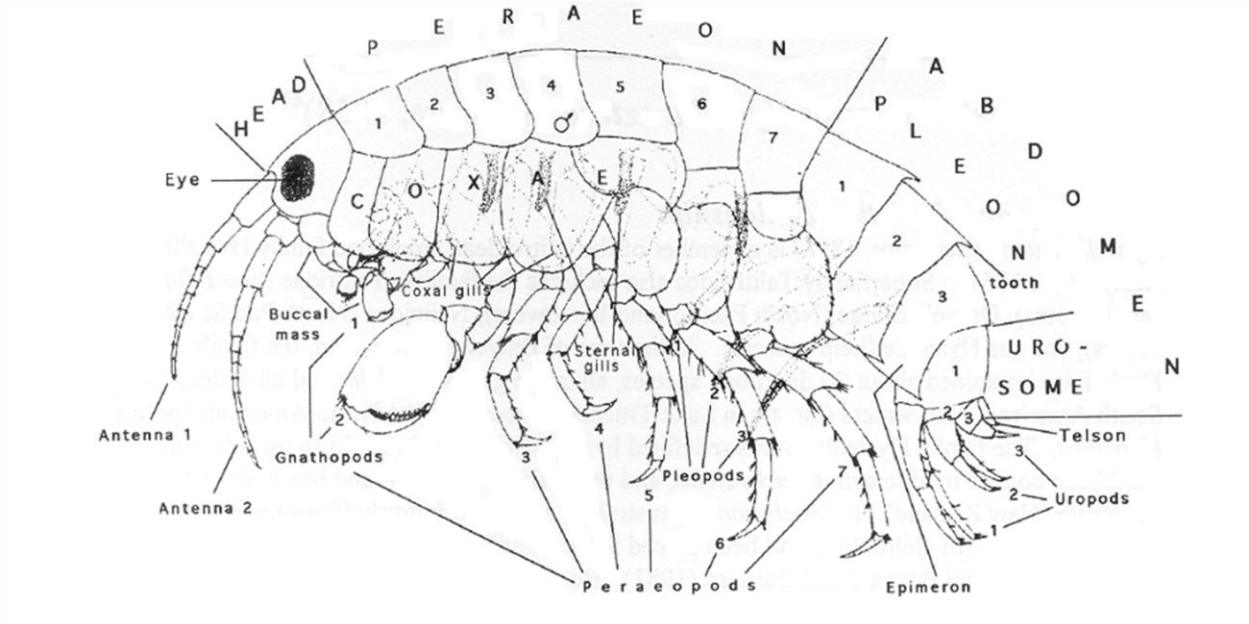

Figure 15: Anatomy of *Hyalella azteca* (Environment Canada, 1997; Updated from Bousfield, 1973)

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 presence of the characteristic male secondary gnathopod (Figure 15). Females were identified by the presence of the dark cloudy brood pouch (Figure 15) and by absence of the male secondary gnathopod. Juveniles were categorized as such if their gender could not be determined visually. If an individual *Hyalella* did not appear to have secondary gnathopods or a brood pouch, 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 16: Image of a) male and b) female *Hyalela 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 Figure 4 for anatomy. "A" is the area of the secondary gnathopod in the male and the brood pouch in the female. Both are 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 95% confidence intervals, were used for generating bar graphs. In order to run parametric tests, the data had to meet the requirements for parametric testing, which includes continuous data that is normally distributed with homogeneous variances. 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 and female area had to be transformed using a log transformation. Variances were tested for equality using Levene's test.

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

While early experiments in this study started with the water-column organism *Daphnia magna*, the major focus through the course of the thesis switched to impacts observed with the benthic amphipod *Hyalella azteca* and thus, these critical experiments will be discussed first.

3.1 *Hyalella azteca*

3.1.1 *Hyalella azteca* Preliminary Assays

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 (MPP) 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

i. This is why organisms were sorted by size prior to the commencement of the bioassay, as it is an accurate means of age-synchronizing *Hyalella azteca* (USEPA 2000; Othman and Pascoe, 2004)

2. Food must be made from monocultures, sterilized, or at least come from a reliable source.
    a. Tetramin™ was found to be a suitable food 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 may affect results. Ammonia, nitrite and nitrate levels could also build up over time affecting results.
5. Test water should be aerated so that substrates could promote the growth of aerobic nitrifying bacteria to reduce ammonia build-up.

3.1.2 *Hyalella azteca* Death

Figure 17 shows the overall figures for death throughout the 42 day major bioassay. A nonparametric Kruskal-Wallis test for death vs. treatments generated a p-value of 0.012. A One-way ANOVA was run in order to utilize Games-Howell post-hoc comparison for unequal variances. A5, A50, T1 and T10 have significantly higher death rates than the no treatment control (p-values = 0.002, 0.007. 0.012 and 0.004, respectively). According to the confidence intervals, all compounds have significantly higher death rates, especially T10 and T50, which are significantly higher than DMSO. TBT is the most toxic substance in the bioassay in a seemingly dose-dependent manner. Although the value for TBT at 50 µg/L appears much higher than TBT at 100 µg/L, this is due to the fact that an additional trial of TBT at 50 µg/L was run compared to TBT at 100 µg/L, which raised the average of T50.

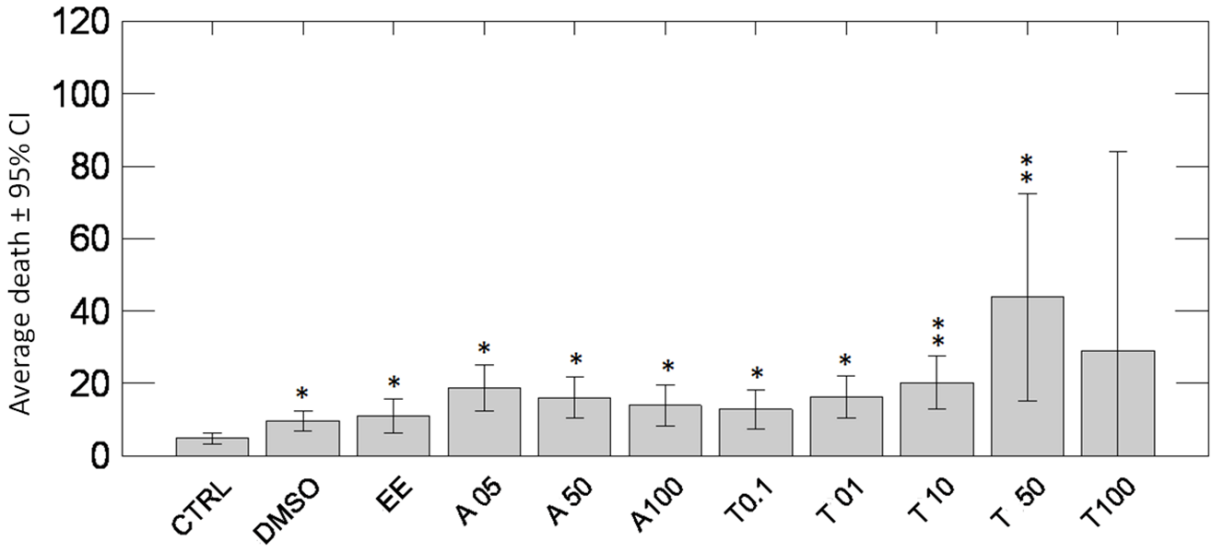

Figure 17: Average number of dead Hyalella per treatment. Error bard represent 95% confidence interval. CTRL = No treatment control. A05, A50, A100 = Atrazine at 5, 50 and 100 µg/L, respectively. T0.1, T01, T10, T50 and T100 = TBT at 0.1, 1, 10, 50 and 100 µg/L, respectively. Single asterisk above the error bar indicates a significant difference from CTRL. A double asterisk above the error bar indicates a significant difference from DMSO.

Figure 18 (a and b) show the cumulative occurrence of death over time by treatment as a proportion of the total. Error bars represent a 95% confidence interval. Naturally, the number of deaths increases over time. 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. All chemicals, including DMSO and EE appear to increase the occurrence of death throughout the experiment. The no treatment control (CTRL) averages 16.4% death per treatment on day 42. DMSO on the other hand, averages 35.7% death per treatment at the end of the assay. The reason for this may be due to the ability of DMSO to solubilise waste products that have accumulated over time, which become exposed to the test organisms.

Ethinylestradiol has a toxicity of 54.3% at the end of the 42 day assay, which is significantly higher than the toxicity of DMSO. All concentrations of atrazine have significantly higher toxicities than the carrier control DMSO, with average deaths per treatment starting at 80.3%

for 5 µg/L, 71.3% for 50 µg/L and 69.5% for 100 µg/L. The occurrence of death under exposure to atrazine does not appear to be dose-dependent for unknown reasons.

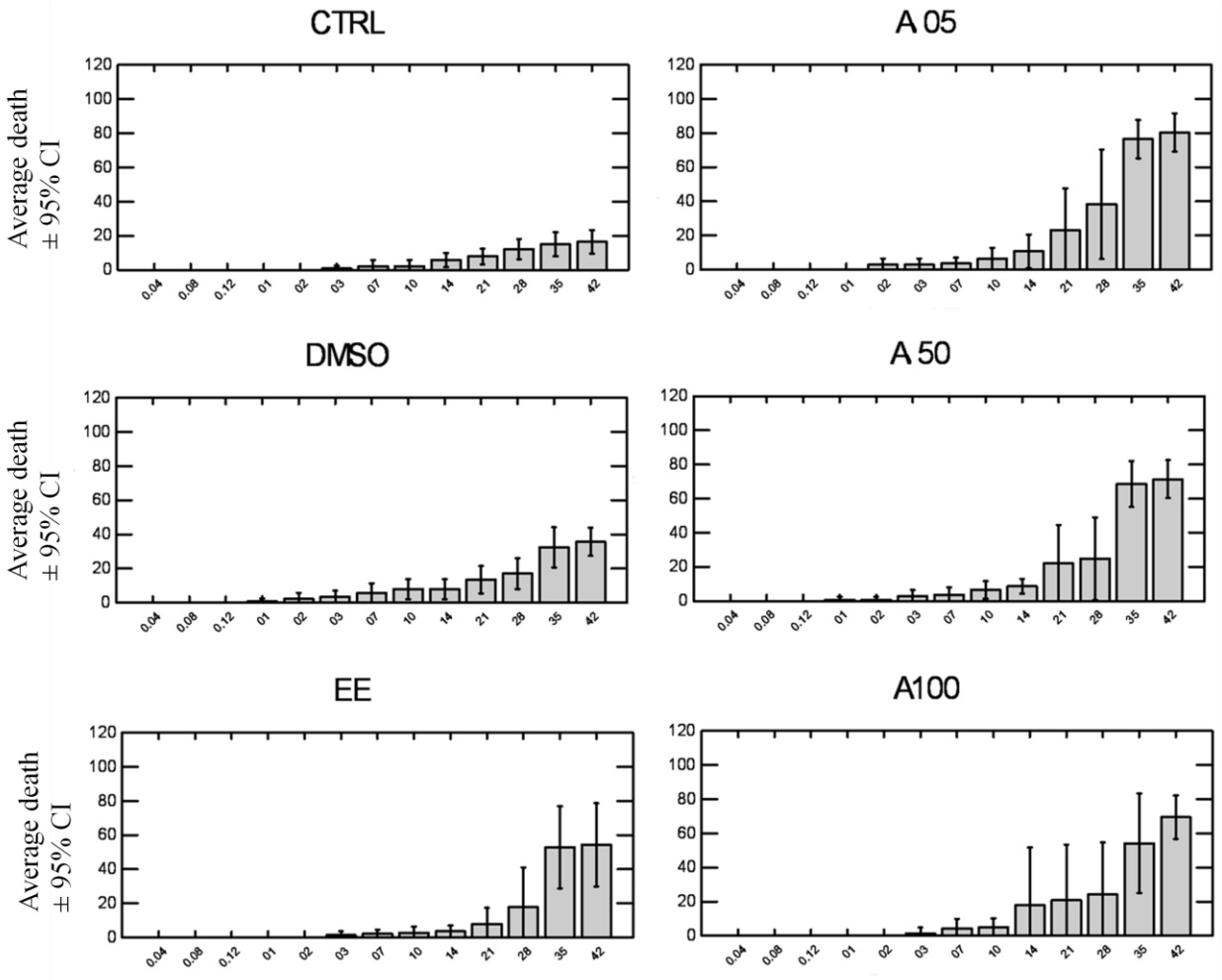

Figure 18a: Cumulative occurrence of death over time by treatment. Y-axis represents average death. X-axis represents day of treatment. 0.04, 0.08, 0.12 = 1, 2 and 3 hours, respectively. Error bars represent 95% confidence interval. CTRL = Control / No treatment; A05, A50 and A100 = Atrazine at 5, 50 and 100 µg/L respectively.

All concentrations of TBT have significantly higher toxicities than the carrier control DMSO, with average deaths per treatment starting at 70.8% for 0.1 µg/L and increasing in a dose-dependent manner (74.5 for 1 µg/L, 78.6 for 10 µg/L, 100% on day 7 for 50 µg/L and 100% on day 2 for 100 µg/L). The important result here is that T50 is 100% lethal to test organism within 7 test days and T100 is 100% lethal to test organisms within 2 test days. Some vessels of T10 reached 100%

lethality after 42 days while some did not, resulting in a large variance in the error bars. This data supports literature that TBT is both acutely and chronically toxic to aquatic organisms.

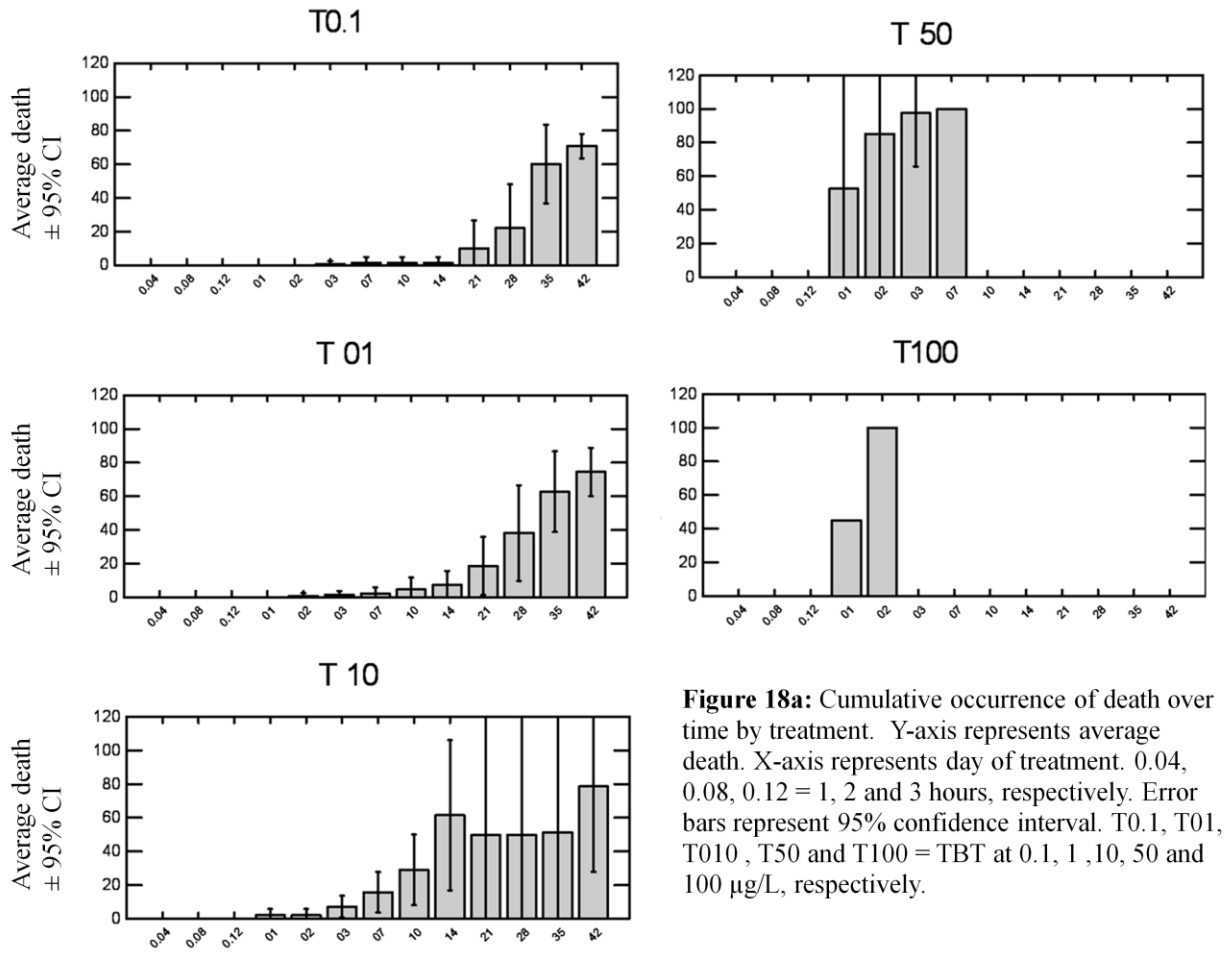

Figure 18a: Cumulative occurrence of death over time by treatment. Y-axis represents average death. X-axis represents day of treatment. 0.04, 0.08, 0.12 = 1, 2 and 3 hours, respectively. Error bars represent 95% confidence interval. T0.1, T01, T010 , T50 and T100 = TBT at 0.1, 1 ,10, 50 and 100 µg/L, respectively.

3.1.3 *Hyalella azteca* Mating

A Kruskal-Wallis test to determined that there was a significant difference among the treatments in amplex behaviour (p-value =0.002). However, post-hoc analysis was not able to determine a significant relationship due to the zero values.

Figure 19 shows the number of amplexus or precopulation events that were counted over the course of the 42 day bioassay. Figure 19 A shows the average amplex events during the course of the 42 day bioassay. CTRL, DMSO, EE and A100 had the highest overall occurrences of

amplex behaviour. A100 has the highest level of variance of this behaviour in the assay. The reason for the high level of variance is unknown, but could be due to the compound increasing the tendency of *Hyalella* to behave erratically.

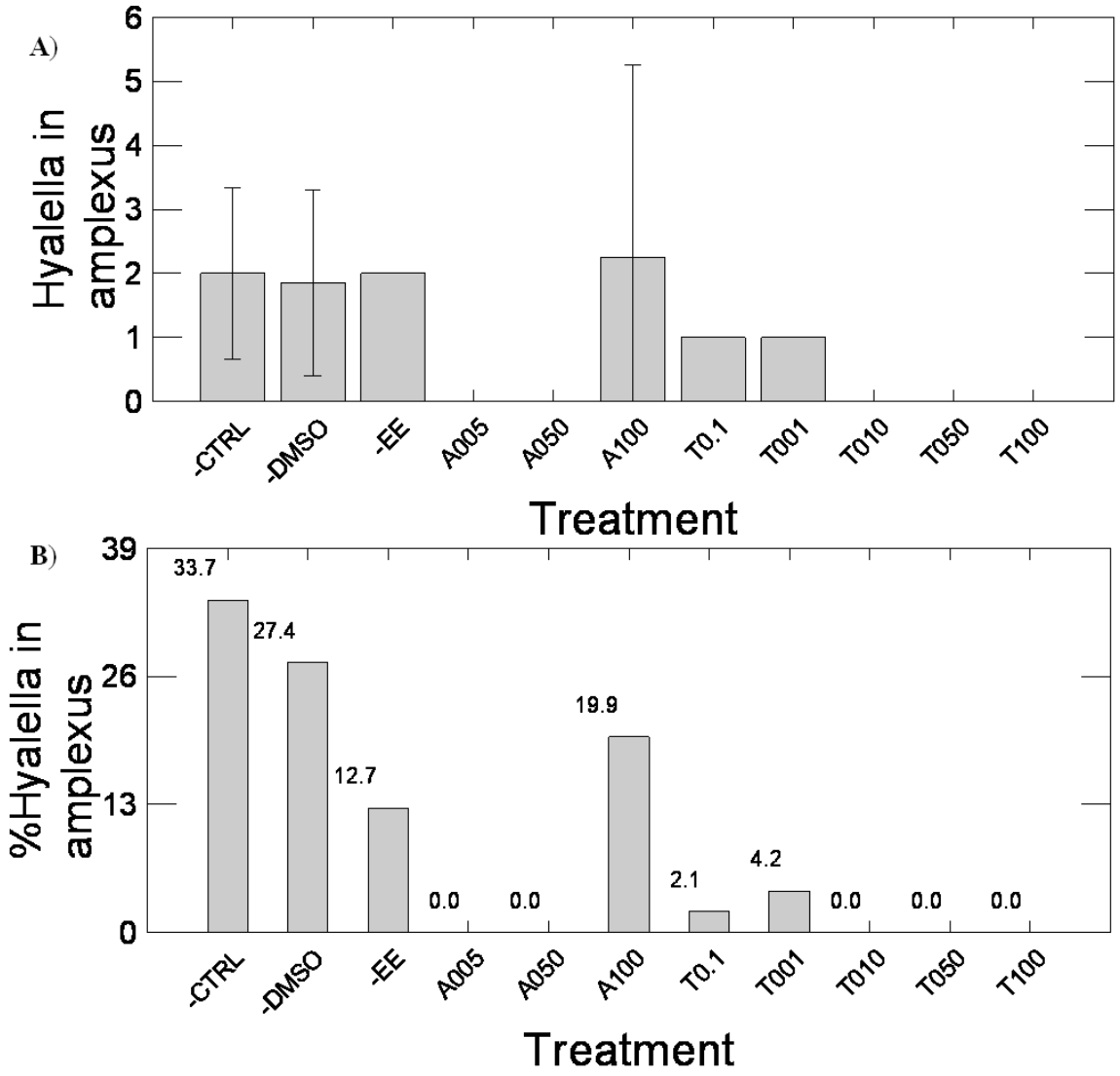

Figure 19: Amplex behaviour in *Hyalella* during treatment. A) Average amplex behaviour observed during bioassay by treatment . Error bars represent a 95% confidence interval. B) Percentage of total *Hyalella* observed in amplex behaviour by treatment. Values above bars indicate percentage of amplexes out of 100%. CTRL = No treatment control. A005, A050, A100 = Atrazine at 5, 50 and 100 µg/L, respectively. T0.1, T001, T010, T050 and T100 = TBT at 0.1, 1, 10, 50 and 100 µg/L, respectively.

Figure 19 B shows the percentage of observed amplex behaviours during each treatment out of a total of one hundred percent. The control treatments had the highest percentage of the total observed amplexes (33.7%), followed by DMSO (27.4%), then atrazine at 100 µg/L (19.9%), EE (12.7%), TBT at 1 µg/L (4.2%) then TBT at 0.1 µg/L (2.1%). The other concentrations had zero observed amplexes throughout the 42 day assay. Interestingly enough, atrazine at 100 µg/L has a higher rate of amplexus than lower concentrations of atrazine for reasons unknown. It appears that observed amplexus was rare in *Hyallela* exposed to even small doses of TBT. It is possible that tributyltin may have an effect on male libido, or female oestrous. It is more likely that tributyltin causes stress as shown in acute behavioural studies by Marshall (2009) and that while stressed, mating priority is significantly decreased.

There appears to be less precopulatory behaviour in all conditions where a chemical stressor is involved. There is also a significant increase in death rate in the chemical stressor treatment groups. These results have important ecological implications, as these chemicals are currently present in the environment and may be reducing wild populations of *Hyalella in vivo*. TBT especially, could have serious implications when you combine the amplex data with the data for death. Not only does TBT appear to interfere with mating behaviour, but it is also toxic and potentially lethal to *Hyalella* in a dose-dependent manner. Even small quantities of TBT in the environment could drastically reduce populations of *Hyalella* in a two-fold manner.

3.1.4 *Hyalella azteca* Male 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 when log-transformed (p-value = 0.393). The data were also checked for equality of variance (grouped both by day of bioassay initiation and by treatment) with Levene's test for length, area and the log-transformed ratio. Variances were equal among dates

of bioassay initiation with regard to length (p-value = 0.264), area (p-value = 0.181) and log-transformed ratio (p-value = 0.422). Similarly, variances were equal among treatments with regard to treatment (p-value = 0.448), area (p-value = 0.578) and log-transformed ratio (p-value = 0.820). Therefore, length, area and log-transformed ratio met the assumptions for parametric testing. When the ANOVA was run it was revealed that there was no significant difference in male lengths among the various treatments (p-value = 0.870). There were also no significant differences between area or log-transformed ratio between treatments (p-values = 0.882 and 0.917, respectively). To check if day of initiation had an effect on the outcome, a general linear model analysis was run in Systat accounting for day and treatment, which slightly improved the outcome for length (p-value = 0.684), area (p-value = 0.281) and log-transformed ratio (p-value =0.219). The day of initiation was found to have an effect, likely due to temperature differences in the laboratory between the first day of initiation and the last day of initiation. Overall it was found with the ANOVA that there was no significant difference among treatments on the length of males, or the area of their secondary gnathopods.

Figure 20 shows the average values for body length, secondary gnathopod area and the ratio of body length to secondary gnathopod area with 95% confidence intervals according to the various treatments. Although the ANOVA found that there were no significant differences among treatment groups, the graph below shows an interesting trend. A larger value for the ratio of length to secondary gnathopod area indicates a proportionally smaller secondary gnathopod with respect to length. Atrazine at 5 µg/L is found to have a slight inhibitory effect on body length and secondary gnathopod area. Atrazine at 50 µg/L also appears to have a slight inhibitory effect on secondary gnathopod area. There appears to be a slight dose-response effect if increasing body length with increasing concentrations of atrazine. When body length is factored into the area, atrazine appears to have a dose-response effect of decreasing the L/A ratio. Atrazine at 100 µg/L has a slightly enhanced average secondary gnathopod area. Tributyltin shows a slight trend of stimulating body length and secondary gnathopod area in male *Hyalella* above the average of the no treatment control. It is believed that TBT is an androgen inducing compound (Yamabe *et al.*, 2000; Santos *et al.*,2005), which would indicate

that body length is possibly under androgen control in addition to gnathopod development. The results show trends as a result of the chemicals; however, the trends are not statistically significant according to the ANOVA. Ethinylestradiol at 0.1 µg/L is found to have a slight stimulatory effect on body length and secondary gnathopod area. While body length is not known to be under endocrine control in invertebrates, secondary gnathopod area is (Vandenbergh *et al*., 2003). The reason for EE having a slight stimulatory effect on body length; however, is unknown. This indicates that EE may have an effect on the growth and development on tissues known to be under endocrine control.

In contradiction to Vandenbergh *et al*. (2003), EE in this study was not found to have an inhibitory effect on male gnathopod area. Although this experiment only studied F1 generation, it found a slight stimulatory effect on both body length and secondary gnathopod development instead. The results from this study appear to be closer to those of Dussault *et al.,* (2008), who found no significant effect of EE on secondary gnathopods and argued that what Vandenbergh *et al*. discovered in 2003 was actually due to chronic toxicity of EE and not mediated by disruption of endocrine pathways. Also interesting to note, is that male *Hyalella* appear to be more sensitive to atrazine at 5 µg/L than any other concentration. The reason for this is unknown.

It is plausible that androgens may enhance the growth of secondary gnathopods in *Hyalella azteca*, as indicated by the slightly higher average in the T10 treatment. However, it is likely that the toxicity of TBT overshadowed its endocrine disrupting effects. This is the likely reason behind the size of the error bars in Figure 20. Follow-up experiments could be conducted where testosterone or other androgen analogs are used as a positive control for androgen-disrupting compounds to determine the effect of xenoandrogens on secondary gnathopod development in *Hyalella azteca.*

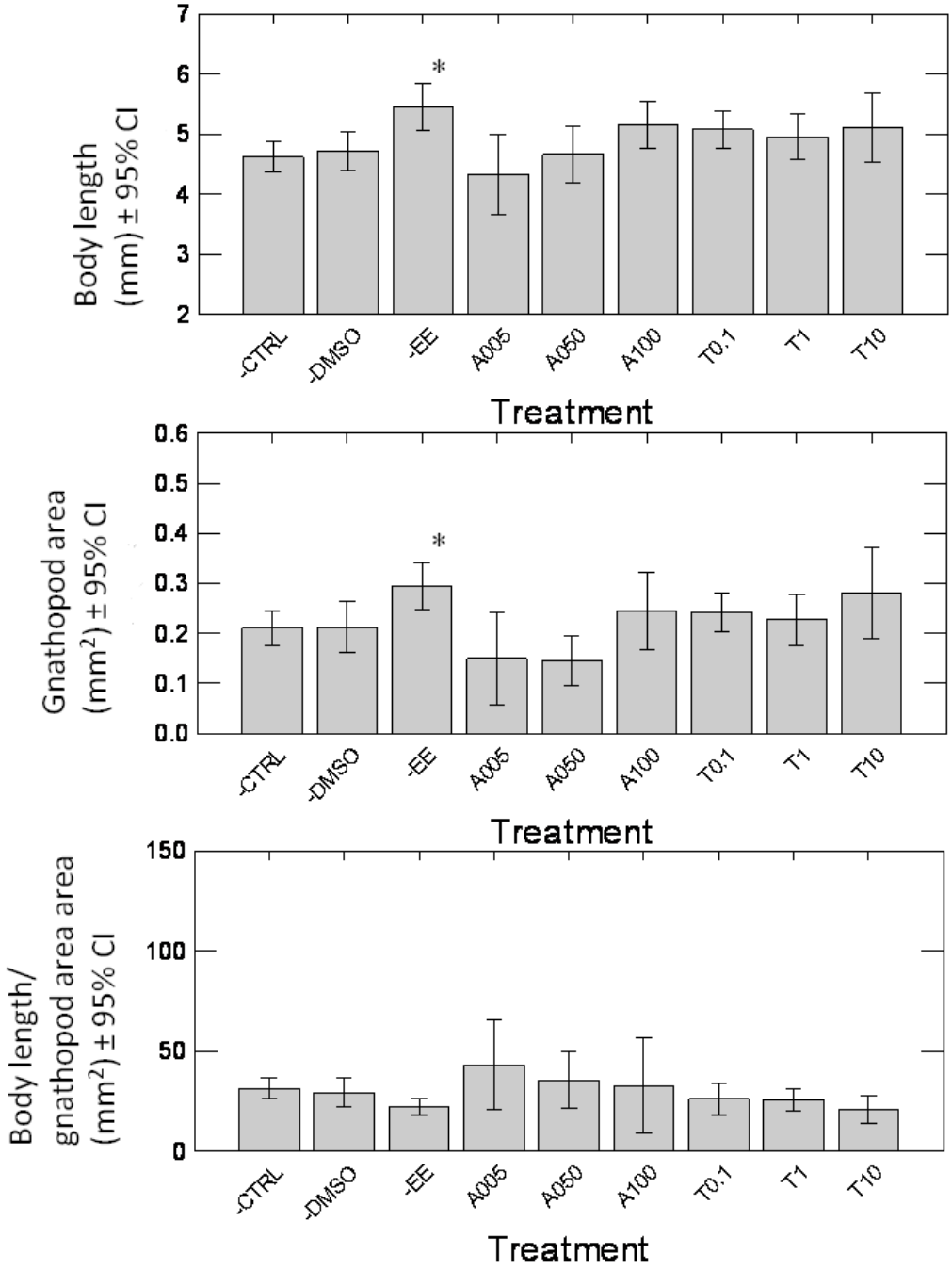

Figure 20: Male values. Error bars represent 95% Confidence Interval. CTRL = Control / No treatment; A005, A050 and A100 = Atrazine at 5, 50 and 100 µg/L respectively; T0.1, T1 and T10 = TBT at 0.1, 1 and 10 µg/L respectively. Length (L) represents body length in mm. Area (A) represents secondary gnathopod area in mm^2. Ratio represents L/A. Larger values mean proportionally smaller secondary gnathopods. An asterisk above the error bar indicates a significant difference from the control.

3.1.5 *Hyalella azteca* Female 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 0.667, 0.225; respectively). Ratio, however was not found to be normally distributed, but was found to be normally distributed when log-transformed (p-value = 0.454). The data were also checked for equality of variance (grouped both by day of bioassay initiation and by treatment) with Levene's test for length, area and the log-transformed ratio. Variances were relatively equal among dates of bioassay initiation with regard to length (p-value = 0.110), area (p-value = 0.130) and log-transformed ratio (p-value = 0.180). Similarly, variances were equal among treatments with regard to treatment (p-value = 0.862), area (p-value = 0.996) and log-transformed ratio (p-value = 0.898). Therefore, Length, area and log-transformed ratio met the assumptions for parametric testing. When the ANOVA was run it was revealed that there was no significant difference in female lengths among the various treatments (p-value = 0.854). There were also no significant differences between area or log-transformed ratio between treatments (p-values = 0.935 and 0.609, respectively). To check if day of initiation had an effect on the outcome, a general linear model analysis was run in Systat accounting for day and treatment, which slightly improved the outcome for length (p-value = 0.602); however, not for area (p-value = 0.966) and log-transformed ratio (p-value =0.687). The day of initiation was found to have an effect, likely due to temperature differences in the laboratory between the first day of initiation and the last day of initiation. Overall it was found with the ANOVA that there was no significant difference among treatments on the length of females, or the area of their brood pouches.

Figure 21 shows the average values for body length, brood pouch area and the ratio of body length to brood pouch area with 95% confidence intervals according to the various treatments. Although the ANOVA found that there were no significant differences among treatment groups, the graph below shows an interesting trend. A larger value for the ratio of length to brood pouch area indicates a proportionally smaller brood pouch with respect to length.

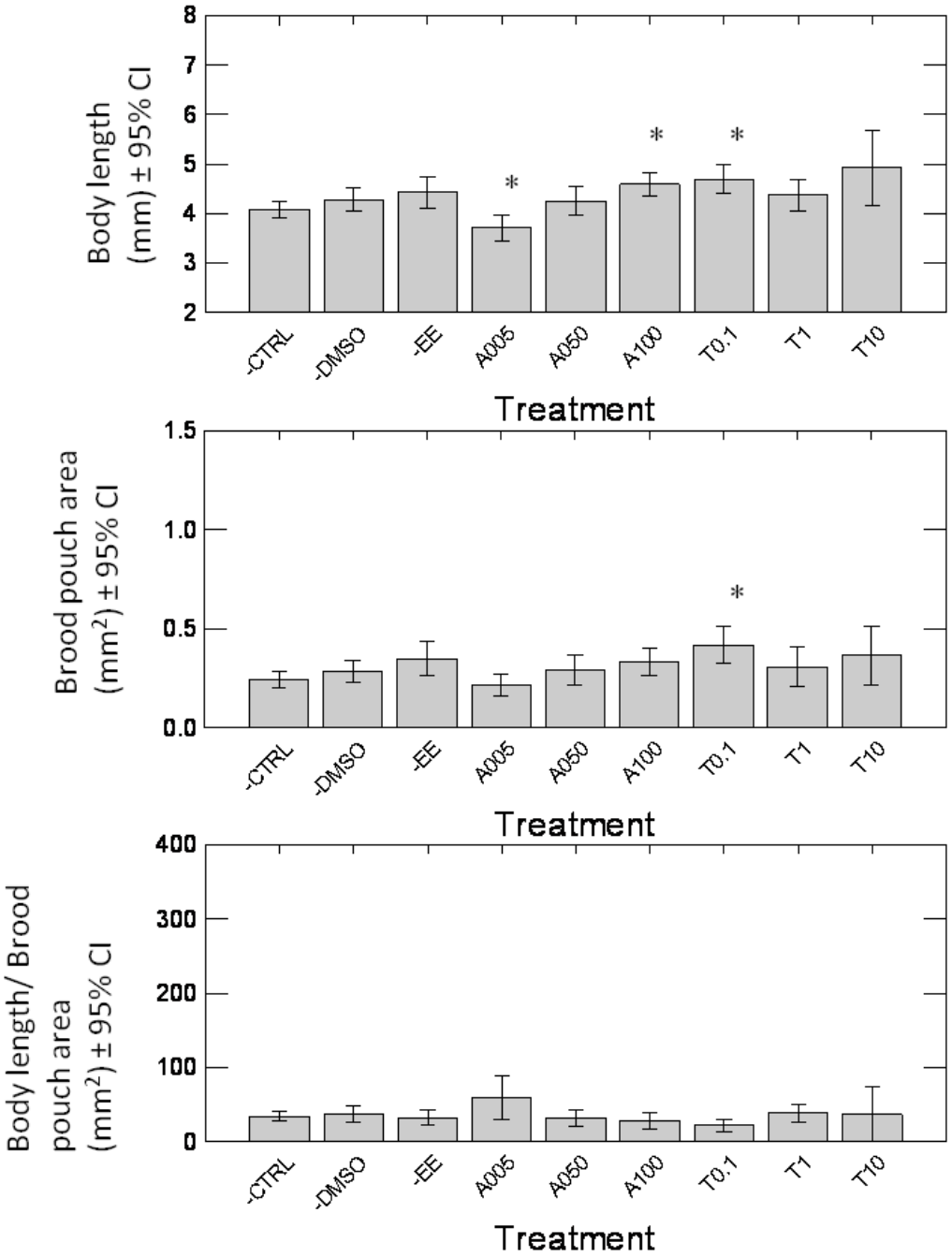

Figure 21: Female values. Error bars represent 95% Confidence Interval. CTRL = Control / No treatment; A005, A050 and A100 = Atrazine at 5, 50 and 100 µg/L respectively; T0.1, T1 and T10 = TBT at 0.1, 1 and 10 µg/L respectively. Length (L) represents body length in mm. Area (A) represents brood pouch area in mm^2. Ratio represents L/A. Larger values mean proportionally smaller brood pouches. An asterisk above the error bar indicates a significant difference from the control.

Atrazine follows a similar trend with females as it does for males. Increasing concentrations tend to have a dose-response stimulatory effect on length and brood pouch area. Atrazine at 5 µg/L also appears to have an effect opposite to that of atrazine at 100 µg/L. The reason for the disparity is unknown. Moore *et al.* (2006) found that *Hyalella* growth rates were significantly reduced when chronically exposed to a cocktail of pesticides including atrazine in the water column. Atrazine at 5 µg/L appears to be the only compound in this study that inhibits 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 skewing the data, or an alternate, unknown reason. Brood pouch area appears to be slightly affected by concentrations of EE at 0.1 µg/L overall and when body length is factored in. This could be due to a slight stimulatory response of the synthetic estrogen on egg development within the female *Hyalella* brood pouch, or because the results are within the margin of error, these results could simply be due to error. A similar response is shown by TBT at 0.1 µg/L. Despite being associated with increased androgen levels (Yamabe *et al.*, 2000; Santos *et al.*,2005), the stimulatory effects witnessed on female *Hyalella* could be due to the aromatization of the excess free testosterone induced by the TBT. However, there is so little known about the hormonal control of brood pouch development in *Hyalella azteca*, that it is impossible to decipher a cause. Higher concentrations of TBT have a slight stimulatory effect on body length and brood pouch area, but when body length is factored in; the effect appears to be slightly inhibitory.

TBT and Estradiol have similar average values, which could be due to an increase in free testosterone from the TBT, which then becomes aromatized in the females. Since TBT appears to have no effect on gender ratios (see below); 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. What can be determined from these results is that the utilization of this novel endpoint of measurement may be a useful tool in detecting endocrine disruption, if endocrine disruption were to occur. Lutz *et al*. (2006) and Köhler *et al.*, (2007) argued 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*. This could be a potential reason why EE did not appear to play a significant role in altering secondary gnathopod development or brood pouch development, for that matter, because the receptor for it simply may not exist. 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 *Hyalella azteca* during a 42-day chronic assay.

3.1.6 *Hyalella azteca* Gender Ratio

Before testing the data using an ANOVA, the data were checked for normality with One-sample KS Lilliefors tests. The percentage of males versus females, and the percentage of males versus females and juveniles were both found to be normally distributed (p-values of 0.709, 0.452; respectively). The data were also checked for equality of variance with Levene's test. The values for percentage of males versus females, and the percentage of males versus females and juveniles, were found to have equal variances (p = 0.3 and 0.551, respectively). Therefore, the data met the assumptions for parametric testing.

After running ANOVAs, it was found that there was no significant difference among treatments for the percentage of males versus females, and the percentage of males versus females and juveniles (p=0.392 and 0.609, respectively).This indicates that atrazine, TBT and EE may not have a significant effect on gender ratios after 42 days of exposure. These results do not disagree with those of Vandenbergh *et al*. (2003), as they found a slight shift after two generations of exposure.

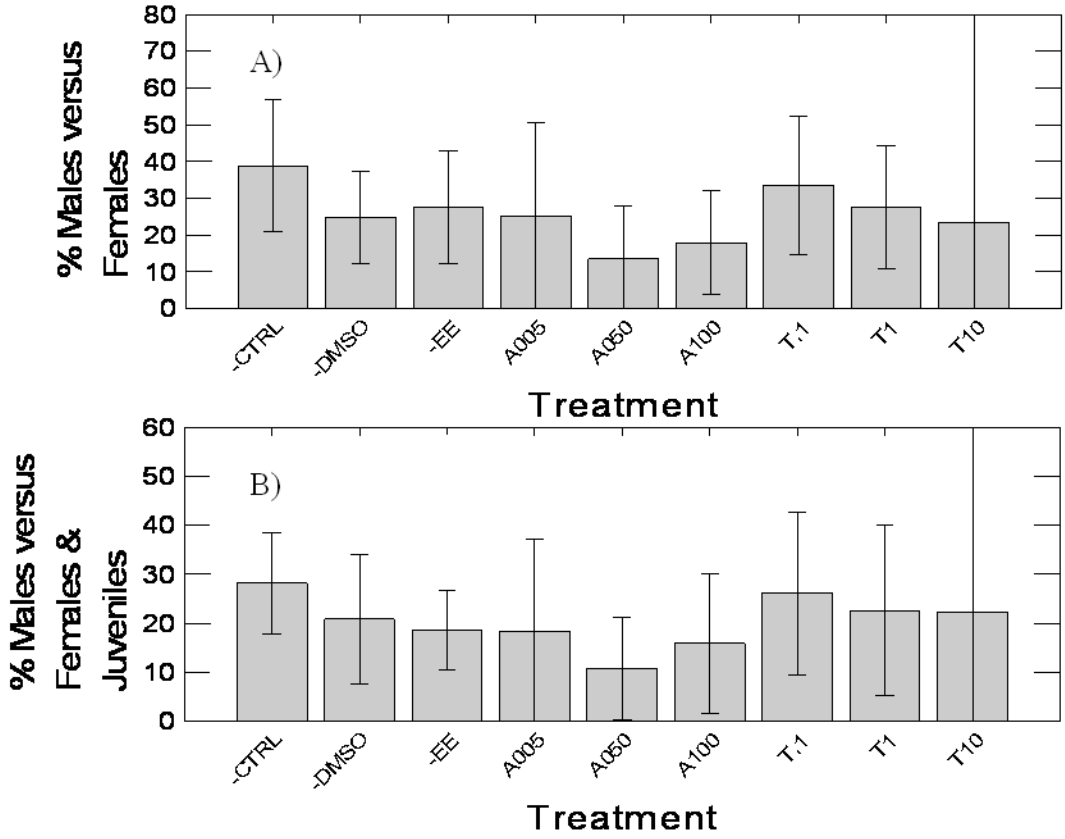

Figure 22: Gender ratios. Error bars represent 95% Confidence Interval. CTRL = Control / No treatment; A005, A050 and A100 = Atrazine at 5, 50 and 100 µg/L respectively; T0.1, T1 and T10 = TBT at 0.1, 1 and 10 µg/L respectively. **A**) shows the proportion of juveniles versus adults according to treatment. **B**) shows the proportion of males in the population versus females according to treatment.

Figure 22 shows the values for gender ratios as a percentage of males versus (A) females and (B) females + juveniles. The error bars represent 95% confidence intervals. According to these results there appears to be no significant differences between treatments on the gender ratios of *Hyalella* populations. When comparing the ratio adult males to adult females (Figure 22 A), atrazine at 50 and 100 µg/L and tend to slightly favour a larger female population. However, the results are within the 95% confidence interval and these findings are not significant. When juveniles are factored into the equation (Figure 22 B), atrazine at 50 and 100 µg/L as well as EE at 0.1 µg/L, tend to slightly favour an increased female population. 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 50 µg/L. The reason for this is unknown.

Flick *et al.* (2001) found that EDCs EE, BPA and octyphenol had little effect on *Hyalella* at environmentally relevant concentrations during a partial life cycle test, but had significant effects in the second generation. Vandenbergh *et al*. (2003) found a statistically insignificant shift in the population favouring females in the second generation exposure to EE. Watts *et al*. (2001) utilized a 100 day assay for *Gammarus pulex* and found an increase in the female population after exposure to EE. Despite being only a single-generation, 42-day assay, it appears that this experiment has generated results similar to Vandenbergh *et al*. (2003) with respect to gender ratios. However, it may be possible for future studies to double the length of the assay, allowing for a second generation in order to produce more significant results. It may be possible to generate results similar to Flick *et al.* (2001) and Watts *et al*. (2001).

When the results of this graph are compared to those of Figure 20 and Figure 21, where atrazine shows a slight tendency to slightly reduce body lengths and secondary gnathopod sizes in males subjected to a concentration under 100 µg/L, decrease brood pouch area in females subjected to 5 µg/L, and increase body length and brood pouch area in females subjected to 100 µg/L; it appears that atrazine may have significant population impacts on *Hyalella azteca*. However, using the most conservative statistical analysis, there appears to be no statistically significant difference between control and atrazine 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*. Future studies on these effects may be able to find more significant effects.

3.1.7 *Hyalella azteca* Juvenile Data

Data were checked for normality using a One-sample KS Lilliefors test and the percentage of juveniles versus adults was found to be normally distributed (p-value = 0.163) but the length of juveniles was found to not be normally distributed (p-value < 0.001). A log transformation was

performed in attempt to normally distribute the data, but this failed to work (p-value = 0.001). The data for percentage of juveniles versus adults were checked for equality of variance using Levene's test and it was found that variances among treatments were not statistically different (p-value = 0.691). Thus, the data for percentage of juveniles followed the assumptions for parametric testing, whereas the data for length of juveniles did not. An ANOVA was performed on the percentage of juveniles and it was found that there was no significant difference among treatments on the percentage of juveniles in the population (p-value = 0.499). A Kruskal-Wallis test was utilized to determine if there was a significant difference among treatments with respect to juvenile length. It was determined that there was a significant difference among treatments (p-value = 0.000). However, the Kruskal-Wallis test does not allow for pairwise comparisons. The differences among treatments were estimated from figure 23 A and attributed to EE at 0.1 µg/L and TBT at 1 µg/L.

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 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 by treatment. The error bars represent 95% confidence intervals. Ethinylestradiol at 0.1 µg/L and TBT at 1 and 10 µg/L show a significant

inhibitory effect on the length of juveniles. TBT at 10 µg/L only had one juvenile in the whole experiment which is not enough information to make a statistical conclusion. If other juveniles were found at this length, it could be said that TBT has an inhibitory effect on growth. It is also possible that since TBT is bioaccumulative, there is potentially a heavier toxic load on older (therefore longer) juveniles. For now, all that can be said is that TBT reduces the survivorship of neonates (more detail below). Atrazine at 100 µg/L also shows a slight decrease in the average juvenile length. A decrease in average length would indicate a stimulatory effect on sexual differentiation at a younger age. Tributyltin was not found to have any significant effect on gnathopod or brood pouch development at these concentrations; it is not conclusive that these effects are due to any endocrinological reason. However, EE and atrazine at 100 µg/L were associated with larger secondary gnathopod areas and slightly larger brood pouch areas, so it is possible that the effects of these chemicals on juveniles could be due to endocrine disruption. A decrease in juvenile length could also mean that these compounds are growth inhibitors, but this cannot be true of EE, as it was associated with longer adults on average in both males and females. Atrazine at 5 µg/L was found to have a slight stimulatory effect on the average length of juveniles. This would indicate that sexual differentiation becomes prolonged when juveniles are found to be longer on average.

Figure 23 B) shows the average percentage of juveniles versus adults in populations of *Hyalella azteca* according to treatment. The error bars represent 95% confidence intervals. TBT at 10 µg/L has a decreased proportion of juveniles, likely due to its toxicity, not necessarily for any endocrine disruption events. Atrazine at 100 µg/L also appears to have a decreased proportion of juveniles. There appears to be a slight dose-response effect of atrazine and TBT on juvenile development. As the concentrations increase, the average length of juveniles and the percentage of juveniles in the population appear to both decrease. Further studies are needed to truly understand what is going on, but the effects could be due to toxicity, endocrine disruption, or both.

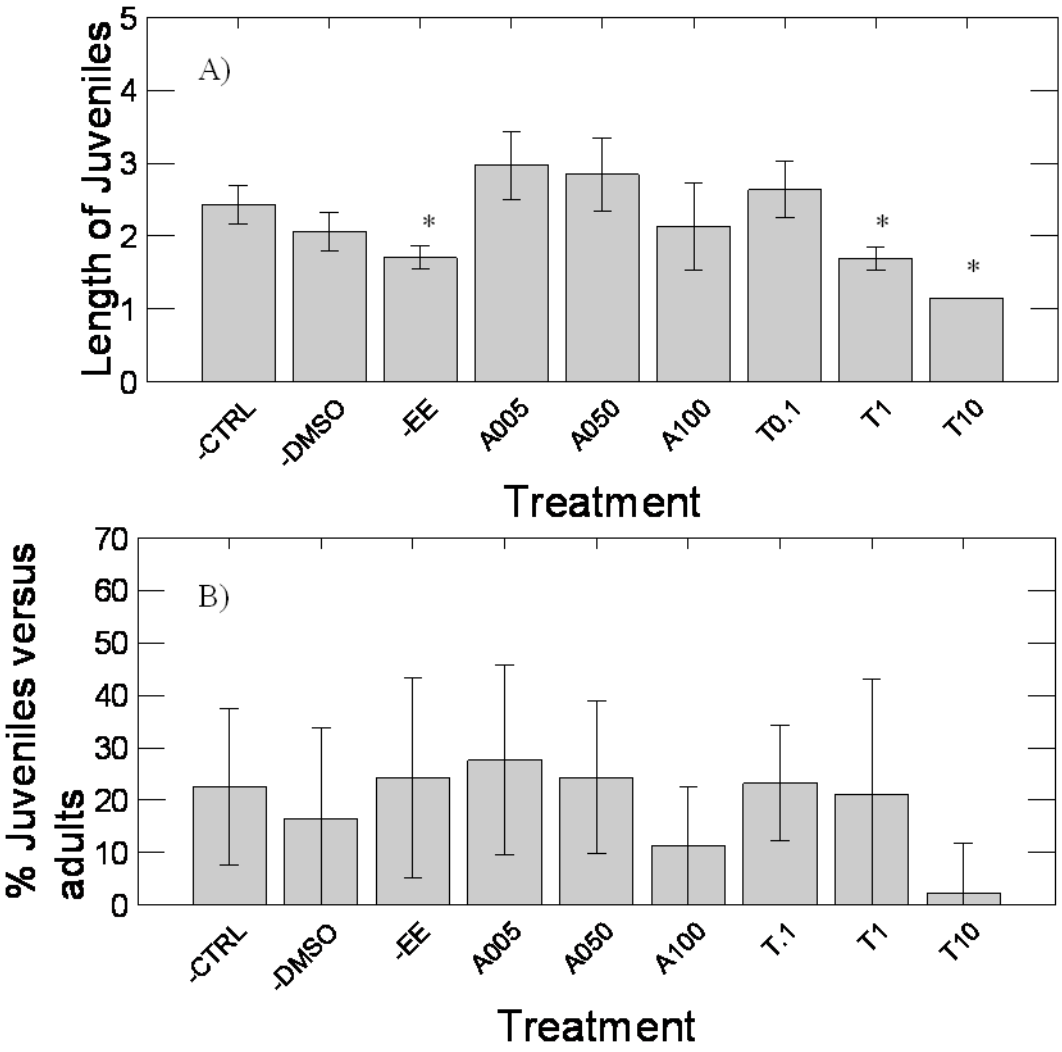

Figure 23: Juvenile values. Error bars represent 95% Confidence Interval. CTRL = Control / No treatment; A005, A050 and A100 = Atrazine at 5, 50 and 100 µg/L respectively; T0.1, T1 and T10 = TBT at 0.1, 1 and 10 µg/L respectively. **A**) shows the length of juveniles in mm according to treatment. **B**) shows the proportion of juveniles versus adults according to treatment. An asterisk above the error bar indicates a significant difference from the control.

Summary

Despite the significant difference in juvenile length among treatments based on the Kruskal-Wallis test, there appears to be no significant reasoning to suggest endocrine disruption is occurring with respect to juvenile length and proportions of juveniles in the population. There appears to be slight trends of a dose-response effect which could be due to endocrine disruption, but are just as likely due to toxicity.

3.1.8 *Hyalella azteca* Grouping Behaviour and Sediment Association

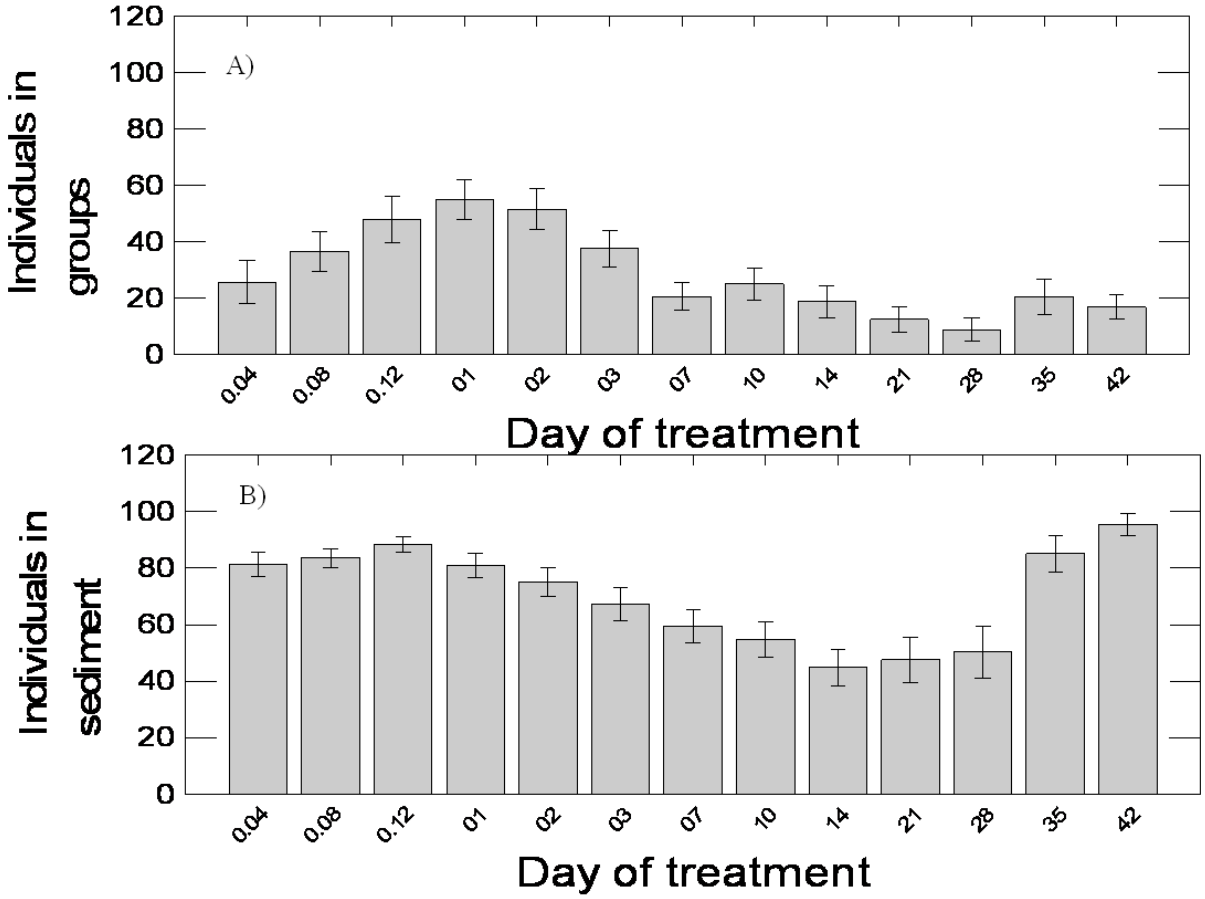

Figure 24: Number of *Hyalella* within **A**) groups by day of treatment. **B**) sediment by day of treatment. Error bars represent 95% confidence interval.

Figure 24 shows A) grouping behaviour over time and B) 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. A likely explanation 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 25 A) shows grouping behaviour as a function of treatment. It appears that there is no significant difference among any treatments. Treatments do not appear to be associated with an increased tendency to form groups. One exception may be TBT at 100 µg/L which has a higher proportion of *Hyalella* in groups. However, the value is within a large margin of error and may not be significant. This particular concentration of TBT is the most toxic used in this bioassay, so grouping behaviour could be induced by toxic stress. Hatch and Burton (1999) and Marshall (2009) 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.

The results from this study do not appear to correlate with other studies that have looked at grouping behaviour as an endpoint. 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. Interestingly enough, Marshall (2009) found that increasing concentrations of atrazine appeared to have no time- or concentration-dependent relationship with grouping, which appears to agree with the results of this study with respect to atrazine.

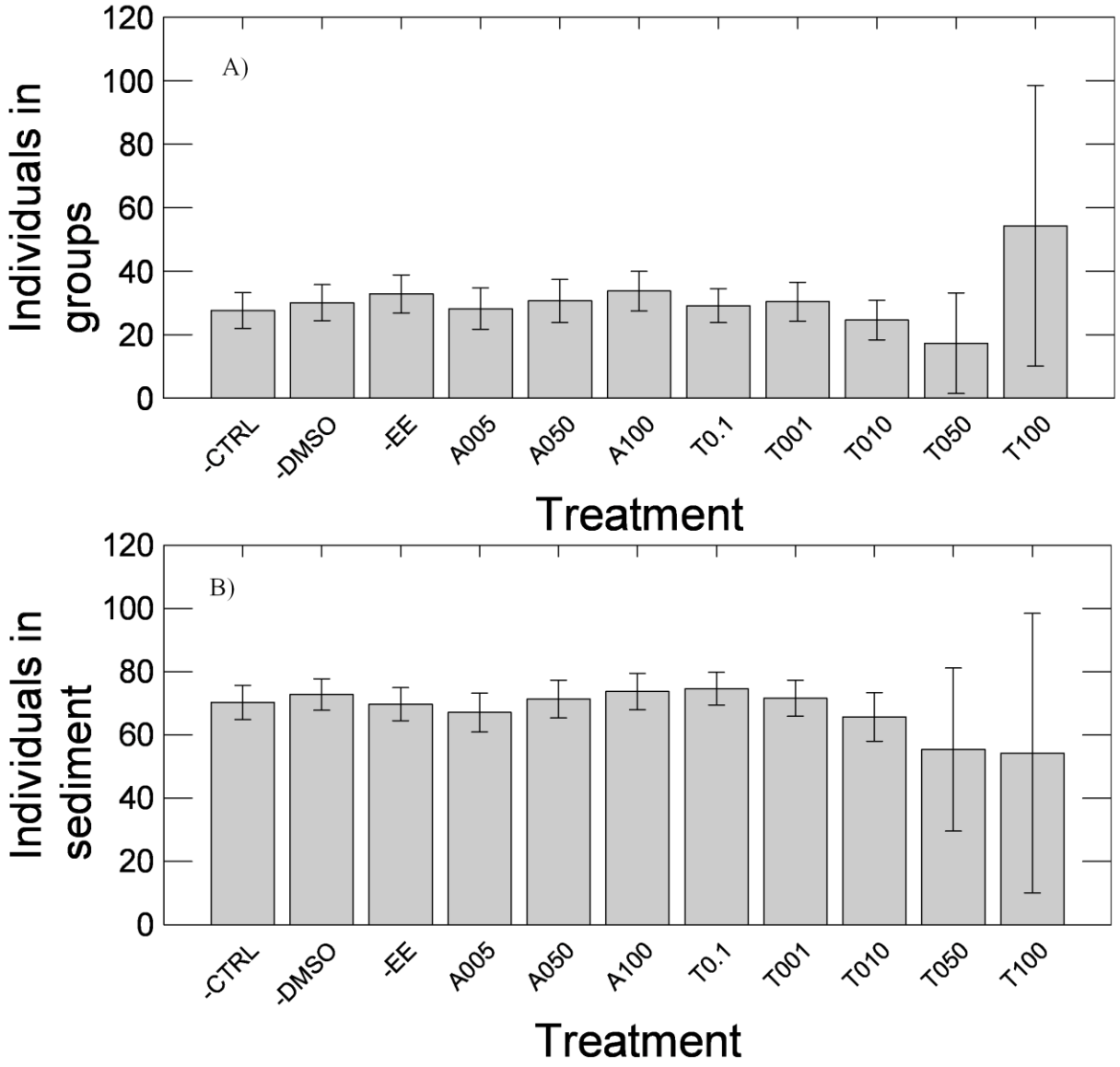

Figure 25: Number of *Hyalella* within **A**) groups by treatment . **B**) sediment by treatment. Error bars represent 95% confidence interval.

Figure 25 B) 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.

It may be the case that there is a concentration-dependent increase in variation for TBT. As the concentrations of the toxin increase, the error bars increase as well, indicating a decrease in predictability of behaviour. Higher levels of toxins appear to cause a greater tendency for individuals within the population to behave in an aberrant way. This applies to individuals in the sediment as well as in groups.

3.2 Preliminary Experiments Using *Daphnia magna*

Several researchers have found that atrazine causes the production of male *Daphnia* (Macek et al., 1976; Dodson et al., 1999a; 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 exerted 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*.

Early experiments in this study utilized *Daphnia magna* to assess the potential impact of EDCs. Attempts were made to culture *Daphnia magna* under conditions which allowed for development of males and ephippial females. While the results from these preliminary experiments were too marginal to be given serious consideration in the body of this thesis, it was still felt to be useful to document observations to assist future researchers who may wish to try to replicate these bioassays. With that in mind, two separate laboratory setups were used

in these preliminary experiments: 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 that could control temperature exactly. 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 high rates in all ten test vessels. For example, during Week Two of testing, 150mL of the 300 mL of overlying solution had evaporated in four days resulting in many deaths. During Weeks 3 and 4 of testing, the temperature was reduced to 12 degrees Celsius, yet the evaporation 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. However, 100 percent lethality within 72 hours under these conditions still occurred, without any resulting 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 survivorship either. Therefore, the experiment had to be terminated in the trial phase after sixteen weeks before chemicals could be tested.

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.

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.

***Daphnia magna* 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.

It is possible that the cause of the evaporation within the vessels was due to low ambient humidity. In the future, it could be advantageous when using air pumps to pump the air through water before being pumped into the bioassay vessel, which may reduce the moisture loss to the environment. It might be worthwhile to investigate, as this could prove a useful addition to *Daphnia* protocols.

Explanations in the literature regarding explanations for die-offs within populations of *Daphnia magna* are lacking. The reasons for *Daphnia* dying without producing any ephippia is a poorly-understood phenomenon, 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. It must be noted here that many researchers in this laboratory (Marshall 2009; Fleet 2010; Gebert 2010 amongst others) have had success culturing and utilizing *Daphnia magna* in bioassays. However, manipulating the reproductive strategy of daphnids has been relatively uncommon. Additionally, in the literature success in manipulating the reproductive strategy of *Daphnia* 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). Thus, 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.

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 requires further refinement.

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.

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 treatment effects. The acute results taken during the course of the assay show that TBT is both acutely and chronically toxic (Figure 22 B). However, there appears to be no concrete evidence that it affects the brood pouch or secondary gnathopod of female and male *Hyalella*, respectively.

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, *Daphnia* downstream of the runoff produced males. Dodson *et al.*, (1999a) 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 an associated condition requires time to manifest. Small quantities of a toxin at particular life stages may have an astronomical effect on the adult organism. 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 disorder, and toxicity testing becomes even more confusing and frustrating. For this reason, it is important to determine endpoints rapidly and to reduce as many confounding factors as possible. Also, because toxicity tests that include the entire life cycle of an organism are difficult and costly to be applied, several short-term tests would be ideal to estimate potential chronic toxicity in a short term. A test with speed, sensitivity and cost would benefit all in order to gauge the safety of potential products before being released to the environment, or even the public. If a problem was found with the chemical of interest, its release could be limited or aborted with minimal exposure.

As 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 an androgen-inhibiting compound, such as atrazine; 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. Therefore, there is a need to learn much more about 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, in many cases unknown. The detection of an endocrine disruptor is best conducted with more than one species, so that the results may complement each other.

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.

Additionally, in the literature success in manipulating the reproductive strategy of *Daphnia* 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). Thus, 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 pH, ammonia, nitrite, nitrate and hardness, would have to be monitored stringently over time, as these factors may unknowingly contribute to stress and/or death. They are a useful organism to study because of their sensitivity, but this sensitivity can make them difficult to culture under some conditions.

Multigenerational studies in *Hyalella azteca* may provide a more accurate depiction of events that occur in the environment. Larsen *et al*. (2009) studied the reversibility of female-biased sex ratios in zebrafish after exposure to EE. It would be interesting to study if this phenomenon is possible, in a multigenerational assay with *Hyalella azteca*. If it was determined that their genders can be modified by xenohormones, would the effect be reversible? It would also be interesting to study the fecundity of the F2 and possibly the F3 generations of that study to test for reproductive deficiency.

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.

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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 the 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 Hr	2 Hr	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	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 Hr	2 Hr	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	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 Hr	2 Hr	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	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												
Day	1 Hr	2 Hr	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	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 Hr	2 Hr	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	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 Hr	2 Hr	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	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 Hr	2 Hr	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	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 Hr	2 Hr	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	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 Hr	2 Hr	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	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 Hr	2 Hr	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	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 Hr	2 Hr	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			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 Hr	2 Hr	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			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 Hr	2 Hr	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			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 Hr	2 Hr	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	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 Hr	2 Hr	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			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 (Hours)	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)
Day = Day the bioassay was initiated

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	3.227	0.091	Apr-20	EE

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
3.741	May-07	CTRL
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
6.284	0.694	May-31	EE

2.849	May-07	CTRL	4.992	0.562	May-31	EE
2.919	May-07	CTRL	6.221	0.549	May-31	EE
1.699	May-07	CTRL	6.375	0.868	May-31	EE
1.28	May-07	CTRL	6.461	1.117	May-31	EE
1.275	May-07	CTRL	5.883	0.411	May-31	EE
1.596	May-07	CTRL	5.352	0.439	May-31	EE
1.444	May-07	CTRL	5.648	0.463	May-31	EE
1.343	May-07	CTRL	4.559	0.230	Apr-23	A100
1.364	May-07	CTRL	3.463	0.149	Apr-23	A100
1.033	May-07	CTRL	2.485	0.032	Apr-23	A100
1.289	May-07	CTRL	2.694	0.053	Apr-23	A100
0.929	May-07	CTRL	3.792	0.071	Apr-24	A100
1.105	May-07	CTRL	3.818	0.251	Apr-24	A100
1.269	May-07	CTRL	3.732	0.206	Apr-24	A100
1.166	May-07	CTRL	4.212	0.263	Apr-24	A100
1.306	May-07	CTRL	5.256	0.465	Apr-24	A100
3.091	May-31	CTRL	5.251	0.385	Apr-24	A100
2.586	May-31	CTRL	4.203	0.235	Apr-24	A100
3.526	May-31	CTRL	4.493	0.246	Apr-24	A100
3.144	May-31	CTRL	4.390	0.117	Apr-24	A100
2.565	May-31	CTRL	4.506	0.256	Apr-24	A100
2.236	May-31	CTRL	3.730	0.162	Apr-25	A100
3.03	May-31	CTRL	5.230	0.309	Apr-25	A100
2.575	Apr-22	DMSO 1	3.575	0.021	Apr-25	A100
3.170	Apr-22	DMSO 1	3.543	0.059	Apr-25	A100
3.080	Apr-22	DMSO 2	3.126	0.023	Apr-25	A100
2.355	Apr-22	DMSO 2	4.323	0.214	Apr-27	A100
2.964	Apr-22	DMSO 2	4.357	0.191	Apr-27	A100
2.233	Apr-22	DMSO 2	4.750	0.362	Apr-27	A100
2.174	Apr-22	DMSO 2	5.516	0.798	May-07	A100
3.119	Apr-22	DMSO 2	5.377	0.747	May-07	A100
2.275	Apr-22	DMSO 2	5.277	0.669	May-07	A100
3.496	Apr-27	DMSO	5.116	0.294	May-31	A100
3.205	Apr-27	DMSO	5.346	0.438	May-31	A100
3.207	May-07	DMSO	5.303	1.005	May-31	A100
1.663	May-31	DMSO	4.377	0.179	May-31	A100
1.524	May-31	DMSO	5.449	0.252	May-31	A100
1.651	May-31	DMSO	5.134	0.634	May-31	A100
1.643	May-31	DMSO	4.399	0.307	May-31	A100
1.509	May-31	DMSO	4.915	0.460	May-31	A100
1.643	May-31	DMSO	4.863	0.290	May-31	A100
1.898	May-31	DMSO	5.932	0.373	May-31	A100
1.228	May-31	DMSO	5.058	0.371	May-31	A100
1.514	May-31	DMSO	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	4.625	0.173	May-31	A50

1.222	May-31	EE	3.487	0.085	May-31	A50
1.541	May-31	EE	3.939	0.148	Apr-20	A5
1.503	May-31	EE	3.409	0.018	Apr-20	A5
1.375	May-31	EE	2.708	0.018	Apr-20	A5
1.522	May-31	EE	2.596	0.010	Apr-20	A5
1.312	May-31	EE	2.809	0.021	Apr-20	A5
1.355	May-31	EE	2.287	0.014	Apr-20	A5
1.436	May-31	EE	2.687	0.009	Apr-20	A5
1.536	May-31	EE	3.916	0.363	Apr-20	A5
1.403	May-31	EE	3.567	0.238	Apr-20	A5
1.547	May-31	EE	4.379	0.254	Apr-20	A5
1.339	May-31	EE	3.981	0.312	Apr-20	A5
1.193	May-31	EE	3.114	0.092	Apr-20	A5
1.211	May-31	EE	4.484	0.342	Apr-24	A5
1.167	May-31	EE	3.680	0.203	Apr-24	A5
1.297	May-31	EE	3.688	0.171	Apr-24	A5
1.223	May-31	EE	3.785	0.340	Apr-27	A5
1.306	May-31	EE	4.196	0.331	Apr-27	A5
1.315	May-31	EE	3.490	0.444	Apr-27	A5
1.281	May-31	EE	4.277	0.513	Apr-28	A5
1.229	May-31	EE	2.893	0.075	Apr-29	A5
2.709	Apr-23	A100	3.556	0.040	Apr-29	A5
2.473	Apr-23	A100	3.553	0.119	Apr-29	A5
2.827	Apr-24	A100	3.867	0.231	Apr-29	A5
2.117	Apr-24	A100	3.870	0.247	Apr-29	A5
2.931	Apr-25	A100	4.030	0.337	Apr-29	A5
1.400	May-31	A100	4.033	0.424	May-07	A5
1.312	May-31	A100	4.218	0.358	May-31	A5
1.267	May-31	A100	5.512	0.409	May-31	A5
2.379	Apr-21	A50	4.709	0.338	May-31	A5
2.707	Apr-21	A50	4.122	0.033	May-31	A5
3.166	Apr-22	A50	3.562	0.225	Apr-21	T10
3.933	Apr-22	A50	2.595	0.083	Apr-21	T10
3.425	Apr-22	A50	4.621	0.397	May-07	T10
2.530	Apr-24	A50	5.602	0.466	May-07	T10
2.492	Apr-24	A50	3.546	0.015	May-07	T10
2.892	Apr-26	A50 1	5.464	0.538	May-07	T10
2.76	Apr-26	A50 1	5.029	0.631	May-07	T10
3.953	Apr-26	A50 1	4.986	0.065	May-07	T10
3.998	Apr-26	A50 1	5.779	0.491	May-07	T10
3.105	Apr-26	A50 2	7.628	0.825	May-31	T10
1.238	May-31	A50	5.347	0.512	May-31	T10
1.239	May-31	A50	4.526	0.183	May-31	T10
2.922	Apr-24	A5	5.317	0.300	May-31	T10
2.432	Apr-24	A5	4.070	0.038	Apr-24	T1 1

3.037	Apr-27	A5	4.788	0.339	Apr-24	T1 1
3.253	Apr-27	A5	3.305	0.281	Apr-24	T1 1
3.382	Apr-27	A5	2.789	0.077	Apr-24	T1 2
2.882	Apr-28	A5	3.204	0.097	Apr-24	T1 2
3.530	Apr-29	A5	3.163	0.068	Apr-24	T1 2
1.622	May-07	A5	3.480	0.025	Apr-24	T1 2
2.681	May-07	A5	4.931	0.569	Apr-27	T1
4	May-31	A5	4.317	0.228	Apr-27	T1
1.15	May-07	T10	4.190	0.413	Apr-27	T1
1.675	Apr-24	T1 2	3.710	0.060	Apr-27	T1
2.375	Apr-24	T1 2	4.338	0.062	Apr-27	T1
2.233	Apr-27	T1	3.151	0.021	Apr-27	T1
2.462	Apr-27	T1	3.791	0.196	Apr-28	T1
2.443	Apr-27	T1	3.554	0.031	Apr-28	T1
2.252	May-07	T1	6.002	0.370	Apr-29	T1
1.349	May-07	T1	4.083	0.073	Apr-29	T1
1.474	May-07	T1	4.160	0.056	Apr-29	T1
1.492	May-07	T1	3.487	0.166	Apr-29	T1
1.297	May-07	T1	3.570	0.076	Apr-29	T1
1.379	May-07	T1	3.548	0.123	Apr-29	T1
1.266	May-07	T1	4.198	0.097	Apr-29	T1
2.314	May-07	T1	4.039	0.091	Apr-29	T1
2.302	May-07	T1	4.026	0.131	Apr-29	T1
1.68	May-07	T1	4.805	0.416	May-07	T1
1.325	May-07	T1	5.828	0.700	May-07	T1
1.311	May-07	T1	5.418	0.628	May-07	T1
1.439	May-07	T1	5.213	0.832	May-07	T1
1.191	May-07	T1	5.936	0.896	May-07	T1
1.187	May-07	T1	5.553	0.916	May-07	T1
1.786	May-07	T1	6.508	1.276	May-07	T1
1.725	May-07	T1	6.382	0.854	May-07	T1
1.508	May-07	T1	4.268	0.317	May-31	T1
1.624	May-07	T1	5.124	0.314	May-31	T1
2.045	May-07	T1	3.955	0.368	May-31	T1
1.599	May-07	T1	4.354	0.052	May-31	T1
1.209	May-07	T1	4.449	0.085	May-31	T1
1.21	May-07	T1	4.324	0.361	May-31	T1
1.955	May-07	T1	4.469	0.330	Apr-25	T0.1 1
2.431	Apr-25	T0.1 1	4.880	0.400	Apr-25	T0.1 1
2.384	Apr-25	T0.1 2	5.354	0.411	Apr-25	T0.1 1
3.144	Apr-25	T0.1 2	4.169	0.336	Apr-25	T0.1 1
2.722	Apr-25	T0.1 2	4.355	0.240	Apr-25	T0.1 2
2.799	Apr-25	T0.1 2	4.933	1.041	Apr-25	T0.1 2
3.431	Apr-25	T0.1 2	3.541	0.245	Apr-25	T0.1 2
4.373	Apr-26	T0.1	3.241	0.069	Apr-25	T0.1 2

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

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		
Replicate	M	F	J	M	F	J	M	F	J
1	3	15	1	3	6	0	2	4	1
2	5	12	8	2	12	3	1	7	1
3	8	16	4	3	15	6	2	5	6
4	4	14	2	6	7	0	1	9	0
5	10	4	7	3	9	2	4	5	4
6	10	8	18	1	15	1	1	7	1
7	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		
Replicate	M	F	J	M	F	J	M	F	J
1	1	4	2	0	6	2	0	11	1
2	1	10	2	2	3	3	3	3	2
3	1	5	1	0	3	2	2	3	3
4	0	3	0	0	6	4	2	1	1
5	2	3	0	2	6	1	0	6	1
6	6	22	3	1	4	0	0	1	2
7				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		
Replicate	M	F	J	M	F	J	M	F	J
1	0	2	0	0	3	1	1	4	1
2	0	0	0	1	4	2	2	4	5
3	0	0	0	1	6	3	1	3	1
4	7	7	1	2	2	0	6	7	6
5	1	4	0	3	9	0	0	6	2
6				5	8	24	9	6	2
7				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