CHRONIC TOXICITY OF PHTHALATES, BISPHENOL A AND A CANADIAN BOTTLED WATER STORED UNDER DIFFERENT LIGHT REGIMES USING THE CNIDARIAN *HYDRA VIRIDISSIMA*©

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Abstract:

There is general concern whether human exposure to selected Canadian bottled waters and the chemicals which may leach from them, could potentially cause deleterious effects. This research was designed to determine whether exposures to bottled water and plastic leachates caused toxicity to the freshwater Cnidarian *Hydra viridissima* (green hydra). Three chemicals used in the production of polycarbonate and polyethylene plastics, bisphenol A (BPA) and two phthalate esters: dibutyl phthalate (DBP) and bis (2-ethylhexyl) phthalate (DEHP) along with one type of commercial bottled water were investigated. One brand of bottled water was analyzed over four months (stored in light and dark conditions) along with lab water similarly stored in glass, polycarbonate and polyethylene bottles. Following 2, 4, 8 and 16 weeks in each of the two treatments, hydra bioassays were conducted. Chronic toxicity tests were also conducted on the two phthalates and BPA. The chronic toxicity tests showed that BPA caused effects on hydra morphology and population at low doses and DBP and DEHP both showed signs of hormesis.

Key words: Bottled water, dibutyl phthalate, bisphenol A, bis(2-ethylhexyl) phthalate, hydra

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Table of Contents:

List of Tables:

List of Figures:

List of Abbreviations:

BCF - Bioconcentration factor

bw/day – body weight per day

BPA – Bisphenol A

CEPA - *Canadian Environmental Protection Act*

DBP – Dibutyl Phthalate

DEHP – Bis (2-ethylhexyl) phthalate

DEP – Diethyl phthalate

EC_{50} – Concentration affecting 50% of the test organisms relative to the control

GC-MS – Gas chromatography – mass spectrometry

HDPE – High density polyethylene

K_{oc} – Soil Organic Carbon

K_{ow} – Octanol-water partitioning coefficient

LC_{50} – Concentration killing 50% of the test organisms relative to the control

LDPE - Low density polyethylene

LOEC – Lowest observed effect concentration

MAC - Maximum acceptable concentration

MBP - Monobutyl phthalate

MEHP - Mono(2-ethylhexyl) phthalate

mg/L – milligrams per litre

ng/mL – nanograms per millilitre

NOEC – No observed effect concentration

NTP-CERHR - The National Toxicology Program - Center for the Evaluation of Risks to Human Reproduction

PC – Polycarbonate

PETE – Polyethylene terephthalate

PP – Polypropylene

ppb – parts per billion - (µg/L)

ppm – parts per million - (mg/L)

PS - Polystyrene

PVC – Polyvinyl chloride

SD – Standard Deviation

SE – Standard Error

SPE – Solid phase extraction

µg/L – micrograms per litre

VCM – Vinyl chloride monomer

1.0 - Introduction:

Bottled water is a growing concern for consumers for many reasons which include potential chemicals that could be leaching from the bottles in addition to environmental problems and many other issues. Within the last decade, bottled water has become an essential commodity to the international community (Liu & Mou, 2004; Wilk, 2006). Many consumers believe that bottled water is a healthier alternative to tap water and many do not like the taste of chlorinated tap water (Liu & Mou, 2004). However bottled water is not tested as regularly as tap water and has fewer regulations (Rosenberg, 2003). It has not been proven that bottled water is safer, purer or healthier than tap water (Wilk, 2006). Bottled water labels can also be deceiving and in most cases the water in the plastic bottle is just tap water, although other sources which include distilled water, carbonated water, ground water and others may be used too (Foltz, 1999). Many bottled water companies obtain their water from nearby municipalities (Foltz, 1999).

The manufacturing and delivery of bottled water causes many environmental problems (Wilk, 2006). Producing these plastic water bottles and shipping them requires energy, which thereby pollutes the environment and contributes to global warming (Wilk, 2006). Recently there has been a large increase in the concern of the quality of bottled water and what inorganic and organic chemicals are present in the water (Saleh *et al.,* 2008). One recent issue of concern is the possibility of chemicals leaching from the plastic water bottles. Bottled waters held in storage under varying conditions, for months could potentially be leaching chemicals into the water. The leaching could potentially become worse when the bottled water is stored outside in direct sunlight (Casajuana &

Lacorte, 2003). The chemicals that could potentially be leaching from the plastic into the water include a range of phthalate esters, bisphenol A (BPA) and various elements such as antimony (Monarca *et al.,* 1994, Shotyk *et al.,* 2005). Serious issues are being raised concerning the possible migration of chemicals from plastic water bottles into the water. For example recent public concern over increased migration levels of BPA from polycarbonate baby bottles is one major issue. Increased levels of BPA have been found in liquids held in polycarbonate bottles following repeated dishwashing, boiling and brushing (Brede *et al.,* 2003).

Baby bottles are not the only plastic bottles that are of concern. Many people use polyethylene terephthalate (PETE) bottles, which include most bottled waters including the brands of Dasani, Aquafina, and Nestlé Pure Life. PETE bottles are advantageous due their stability, light weight, easily recycled and easily moulded into various shapes (Wilk, 2006). Not a great deal of research has been done on PETE bottles in comparison to polycarbonate bottles and this should be resolved because many consumers drink PETE bottled water on a daily basis. Focusing on the issue of bottled water and the potential chemicals that could be leaching from the plastic will allow more information to be discovered on what effect these chemicals could have on organisms.

2.0 - Comprehensive Literature Review:

2.1 - Issue of Bottled Water:

Recently there has been an increase in the purchasing of bottled water (Wilk, 2006). There are various reasons why people may prefer bottled water over tap water which include the claim that it is healthy, the taste and portability. People claim to taste a variation between tap and bottled waters but often fail to do so in blind tests likely due to the fact that the water sold in bottled water is often tap water (Wilk, 2006). There are many important issues associated with drinking bottled water. Companies use illustrations from nature such as mountains, describe it as having a long list of minerals, and use words such as 'pure' and 'pristine' (Wilk, 2006). There are many brands of bottled water offered for sale and they include a variety of domestic and imported spring and mineral waters, tap waters treated by filtration, reverse osmosis or distillation (Pip, 2000). These descriptions are not valid the majority of the time (Pip, 2000, Wilk, 2006). Canadian bottled water companies may obtain water from one province and then bottle it in another province (Pip, 2000). The chemical analysis may not be supplied or may be offered for specific parameters (Pip, 2000). Water quality may be good at the specific source, but the quality may decline through handling, shipping and storage (Pip, 2000). There is also the issue of the development of microorganisms in bottled water which can occur for example by contamination with flakes of human skin (Pip, 2000).

One recent issue is the problem of organic compounds migrating from plastic into liquid. It is thought that food may become contaminated with organic compounds from plastic by the diffusion process identified as leaching (Monarca *et al.,* 1994). Several

studies have found that there is an increase in leaching of organic compounds depending on the type of plastic bottle that was being analyzed (Fayad *et al.,* 1997, Pip, 2000, Brede *et al.,* 2003). One study looked at the leaching of a vinyl chloride monomer (VCM) and adipate and phthalate ester plasticizers from polyvinyl chloride (PVC) material into bottled drinking water (Fayad *et al.,* 1997). The study found that the concentration of VCM did not result in a quantifiable increase but that various volatile and semi-volatile organic compounds were detected by gas chromatography mass spectrometry (GC-MS) after exposure to sunlight (Fayad *et al.,* 1997). Some of the semi-volatile compounds that were identified included di-n-octyl adipate and bis(2-ethylhexyl) phthalate (DEHP) (Fayad *et al.,* 1997). The leaching of these organic compounds seems to depend on the storage time, temperature and exposure to sunlight (Fayad *et al.,* 1997).

The leaching of organic compounds from plastic is not the only problem that is affecting bottled water. There is also the issue of contamination from various inorganic chemicals such as antimony. Shotyk & Krachler (2007) looked at the contamination of bottled waters with antimony leaching from the polyethylene terephthalate (PETE) bottles and showed that it increased upon storage (Shotyk & Krachler, 2007). Dabeka *et al.,*(2002), looked at samples of bottled water of mineral, spring and other types of bottled waters (Dabeka *et al.,* 2002). Of the 199 samples that were studied, 22% surpassed the Canadian or World Health Organization (WHO) guidelines for one or more of the elements: boron, manganese, chromium, nickel, arsenic, selenium and lead (Dabeka *et al.,* 2002). There is also the issue of unintentional or intentional contamination by microorganisms. Microorganisms are already present naturally in water but the multiplication of bacteria following bottling depends on the dissolved organic substances

that are in the water and physical characteristics of the water like temperature which can increase growth (Ferretti *et al.,* 2007). For example natural mineral water is not sterilised, pasteurised or treated to eliminate microorganisms (Armas & Sutherland, 1999). It has been seen that the number of bacteria in bottled water is usually low, but in uncarbonated water the microorganism count increases greatly after 1 to 3 weeks of storage (Armas & Sutherland, 1999).

Different types of plastic have a specific resin code associated with it. The Society of Plastic Industry introduced the resin coding system in 1988 (Environment Canada, 2002). These codes can be seen underneath plastic bottles, containers and packaging (Environment Canada, 2002). A code of 1 indicates the plastic is made up of polyethylene terephthalate (PETE) and this type of plastic is used for most bottled waters and two litre soft drinks (Environment Canada, 2002). Resin Code 2 indicates high density polyethylene (HDPE), resin code 3 indicates polyvinyl chloride (PVC), resin code 4 indicates low density polyethylene (LDPE), resin code 5 indicates polypropylene (PP), resin code 6 indicates polystyrene (PS) and resin code 7 indicates any other type of plastic which includes polycarbonate (PC) (Environment Canada, 2002). The two resin codes that were investigated in this experiment are resin code 1 (PETE) and resin code 7 (PC).

Plastic bottles such as PETE may be leaching various organic compounds, more specifically phthalates, when exposed to sunlight for a duration of time. PETE packaging is mainly used for carbonated soft drinks and mineral waters (Monarca *et al.,* 1994). It is also used for packaging of beers and wines and edible foods (Monarca *et al.,* 1994). Various studies have shown leaching of organic chemicals from PETE plastic into

drinking water. For example Casajuana & Lacorte, (2003), looked at the occurrence and release of phthalic esters and other endocrine disrupting chemicals in drinking water (Casajuana & Lacorte, 2003). Plastic bottled waters were bought directly from the bottled water companies and they were analyzed using solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC-MS) before and after 10 weeks of storage outdoors where temperature could reach up to 30°C (Casajuana & Lacorte, 2003). The study found an increase in various organic compounds after the 10 weeks of storage. Specifically diethyl phthalate (DEP) and bis(2-ethylhexyl) phthalate (DEHP) were found with the highest mean concentrations of 0.214 and 0.314 µg/L after the 10 weeks of storage (Casajuana & Lacorte, 2003).

Biscardi *et al.,* (2003) also looked at the potential migration of compounds in PETE bottles (Biscardi *et al.,* 2003). PETE bottles were obtained from a factory that produced naturally carbonated mineral water and natural mineral water (Biscardi *et al.,* 2003). They sampled monthly for 12 months and when GC-MS was conducted they discovered DEHP was present in PETE bottles with natural water at 9 months and in carbonated water at 10 months (Biscardi *et al.,* 2003. They were not looking for plasticizers but did find DEHP present in both types of waters within 9 months to the end of the sampling period (Biscardi *et al.,* 2003). It was concluded that after 9 to 10 months of storage in a PETE bottle the concentration of DEHP may correlate with storage time (Biscardi *et al.,* 2003).

Other plastic bottles include polycarbonate (PC) bottles which could potentially be leaching BPA from the plastic when exposed to sunlight for a duration of time. The most recent media concerns about BPA involved baby bottles and sports bottles leaching

BPA from the plastic. Various studies have found high concentrations of BPA in samples of water after putting hot water in the bottles and after repeated dishwashing (Brede *et al.,* 2003; Hoa *et al.,* 2008). The manufacture of polycarbonate plastic uses BPA as a monomer and it is used to make baby bottles, sports bottles, epoxy resins etc. (Sajiki & Yonekubo, 2004). Following simulated use of the PC bottles by boiling, brushing and dishwashing, there was a considerable increase in leaching of BPA (Brede *et al.,* 2003). The mean BPA level in new bottles was 0.23 μg/L, while the mean levels in bottles subjected to simulated use was 8.4 μg/L (dishwashed 51 times) and 6.7 μg/L (dishwashed 169 times) (Brede *et al.,* 2003). A recent study of BPA leaching involved measuring BPA levels following exposure of PC bottles to boiling water (Hoa *et al.,* 2008). Exposure to boiling water was found to amplify the rate of BPA leaching by up to 55 fold (Hoa *et al.,* 2008). Many other studies have come across the same conclusion of BPA leaching from PC bottles after repeated and heavy use (Sajiki & Yonekubo, 2004, Vandenberg *et al.,* 2007, Hoa *et al.,* 2008).

2.2 - Phthalates:

Phthalic acid esters were used as plasticizers for the first time in 1920 and they remain the largest class of plasticizers in the 21st century (Rahman & Brazel, 2004). They are one of the most frequently used plasticizers and account for 92% of the plasticizers created worldwide (Rahman & Brazel, 2004). Phthalates have many advantageous characteristics when used as plasticizers including: good fusion characteristics, they form

highly elastic compounds, they are relatively non-volatile at ambient conditions, and their low expense (Rahman & Brazel, 2004).

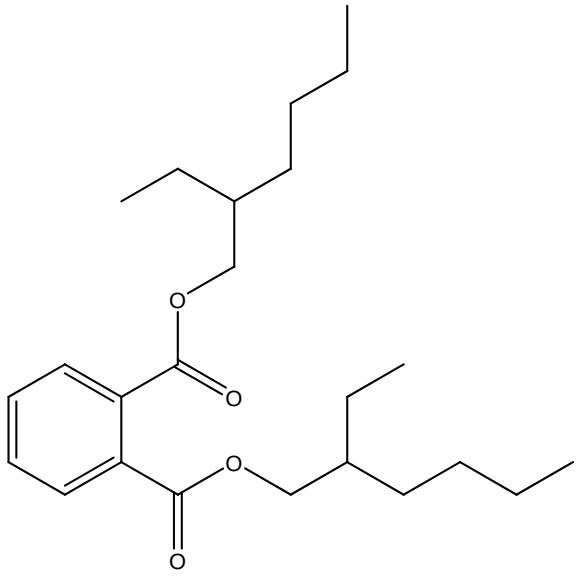
Phthalates are a group of endocrine disruptors that are used worldwide and to which humans are exposed to on a daily basis (Foster *et al.,* 2001). They are esters of phthalic acid (1,2-benzenedicarboxylic acid) and they include dimethyl phthalate, dibutyl phthalate, bis(2-ethylhexyl) phthalate, diisooctyl phthalate to name a few (Adams *et al.,* 1995). Phthalates are used ubiquitously including in construction, automotive, household products, apparel, toys, packaging and medical products (DeFoe *et al.,* 1990). They are present in food wraps, plastic tubing, floor tiles, furniture, automobile upholstery, shower curtains and in lesser amounts in insect repellents, cosmetics and perfumes (Adams *et al.,* 1995). Phthalate esters are extensively used in the formation of plastics due to their ability to enhance the flexibility and durability of high molecular weight polymers (Adams *et al.,* 1995).

DEHP is one of the most significant plasticizers utilized in Canada and accounts for 51% of the phthalates produced as plasticizers (Environment Canada & Health Canada, 2004, Rahman & Brazel, 2004). In 1991 the manufacture of DEHP in Canada amounted to 5 kilotonnes (kt) and an extra 5 kt were imported into Canada in plasticized PVC and in other various plastic products (Environment Canada & Health Canada, 1994a). Dibutyl phthalate (DBP) is not produced in Canada and about 540 tonnes/year are imported into the country for use primarily as a plasticizer (Environment Canada & Health Canada, 1994b). DBP can also be imported into Canada in other plastic products (Environment Canada & Health Canada, 1994b).

2.2.1 - Toxicity of Bis(2-ethylhexyl) phthalate (DEHP):

DEHP is one of the most prevalent phthalate plasticizers used in various consumer products and building materials and is still used as a plasticizer in medical instruments (Jahnke *et al.,* 2005; Heudorf *et al.,* 2007) (Figure 1). There are various routes of exposure for phthalates which include leaching from consumer products and through direct contact (Schettler, 2006). The emissions of phthalates into the atmosphere are thought to be the main entry into the environment (Environment Canada & Health Canada, 1994a). Processes that have an effect on the dispersal and transformation of DEHP in the environment include: atmospheric photo-oxidation, partitioning into soil, sediment and biota, and aerobic degradation (Howard *et al.,* 1991; Staples *et al.,* 1997). Gaseous DEHP has an approximate photo-oxidation half life of 2.9 to 29 hours and its estimated photolysis half-life is no longer than 144 days (Howard *et al.,* 1991). It was also estimated that the photolysis half-life of DEHP in water is 144 days or longer (Howard *et al.,* 1991).

Bioconcentration is defined as the build-up of contaminants due to aqueous exposure solely (Staples *et al.,* 1997). For several aquatic algae and invertebrates the bioconcentration factor for DEHP was 6.9 for the oyster (*Crassostrea virginica*) and 5400 for alga (*Chlorella fusca*) (Wofford *et al.,* 1981; Geyer *et al.,* 1982). Bioconcentration factors for fish exposed to waterborne DEHP ranged from 42 for rainbow trout (*Oncorhynchus mIykiss*) to 304 for the fathead minnow (*Pimpephales promelas*) (Staples *et al.,* 1997). If bioconcentration factors are greater than 1000 there is a high potential to bioaccumulate (Staples *et al.,* 1997). Bioconcentration factors seem to



Bis(2-ethylhexyl) Phthalate

Figure 1. Molecular structure of Bis (2-ethylhexyl) phthalate (DEHP)

(Created Using ChemBioDraw Ultra 11.0)

be much higher in aquatic invertebrates and algae rather than fish, which appear to readily metabolize DEHP.

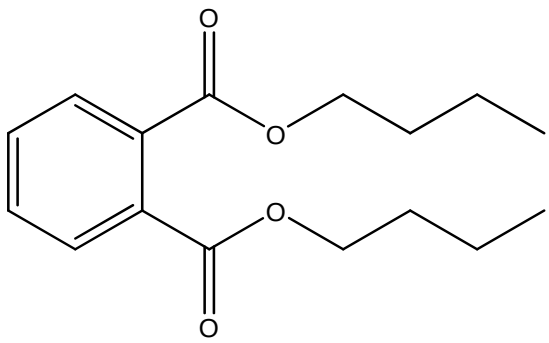
When mammals ingest DEHP, the phthalate is first hydrolyzed by a nonspecific lipase in the gastrointestinal tract to form mono(2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol and the MEHP is readily absorbed (Environment Canada & Health Canada, 1994; Koch *et al.,* 2006). DEHP is significantly metabolized irrespective of the route of uptake (Koch *et al.,* 2006). Five major metabolites are formed which include MEHP, mono(2-ethyl-5-hydroxylhexyl) phthalate (5OH-MEHP), mono(2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP), mono(2-ethyl-5-carboxypentyl) phthalate (5cx-MEPP), mono[2(carboxymethyl)hexyl)hexyl] phthalate (2cx-MMHP) (Koch *et al.,* 2006; Wittassek & Angerer, 2008). For example in adults it is known that majority of a DEHP dose is excreted in urine (Koch *et al.,* 2006). One study found that after oral doses of isotopically labelled DEHP were given to humans, about 74% was excreted via the kidneys in urine (Wittassek & Angerer, 2008). The five oxidized metabolites are the main urinary DEHP metabolites (Koch *et al.,* 2006). Due to slow removal, DEHP and its oxidized metabolites could potentially be accumulating in the human body (Koch *et al.,* 2006). The latest studies have shown that DEHP may not be the main toxicant but rather its oxidized metabolites specifically MEHP (Koch *et al.,* 2006).

DEHP is toxic to humans and animals, and is considered to be the phthalate of greatest toxicological risk in the phthalates group (Heudorf *et al.,* 2007). The major groups at greatest risk from DEHP exposure include children younger than 1 year of age, critically ill children and pregnant women going through therapies or medical care using medical tools that contain DEHP (Heudorf *et al.,* 2007). The tolerable daily intake values

(TDI) for phthalates were calculated and DEHP has one of the smallest calculated TDI values of 0.044 mg/kg bw/day (Environment Canada & Health Canada, 1994a, Heudorf *et al.,* 2007). In one study, DEHP was found to have a LD_{50} value of 38.35 ml/kg in rats following a 1 week observation period (acute) but the LD_{50} value decreased greatly for the chronic toxicity test (up to 10 weeks) in rats to 6.40 ml/kg, which is a 599% increase in toxicity (Lawrence *et al.,* 1973). High dose exposure of DEHP to rats and mice caused higher lung, liver and kidney weights in the high dose group in both rats and mice (David *et al.,* 2000). The National Toxicology Program (NTP) Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR) stated that there should be concern over the developmental effects that increased levels of DEHP may have on the reproductive tract of male infants specifically; levels that are higher than what the general population are exposed to (Jahnke *et al.,* 2005).

2.2.2 – Toxicity of Dibutyl Phthalate (DBP):

Dibutyl phthalate (DBP) is used as a part of latex adhesives and is also used in cosmetics and various other personal care products, insecticides and pharmaceuticals (Jahnke *et al.,* 2005, Schettler, 2006) (Figure 2). Humans can be exposed to DBP in various circumstances. Food is one of the largest sources of phthalate exposure but DBP is also present in pharmaceuticals, where it is used in coatings of pills such as antibiotics, antihistamines and laxatives (Environment Canada & Health Canada, 1994b; Schettler, 2006). Humans can also be exposed to DBP through topically applied insect repellents, hairspray, perfume solvent and nail polish (Schettler, 2006). Dibutyl phthalate can be



Dibutyl Phthalate

Figure 2. Molecular structure of Dibutyl phthalate (DBP)

(Created Using ChemBioDraw Ultra 11.0)

released into the atmosphere through emissions from the production and use of DBP and from the partial combustion of plastic (Environment Canada & Health Canada, 1994b).Effluents from Canadian textile mills contain DBP levels up to 158 µg/L and in Ontario municipality sewage effluents, DBP has reached concentrations of up to 3 µg/L (Environment Canada & Health Canada, 1994b). DBP's half life in surface or ground water ranges from 1 to 23 days and the rate of atmospheric photolysis is high (Howard *et al.,* 1991; Staples *et al.,* 1997). DBP's photo-oxidation is very slow in water with a half of 2.4 to 12.2 years (Howard *et al.,* 1991; Staples *et al.,* 1997). If DBP is discharged into water it will adsorb to sediment and particulates (Howard *et al.,* 1989). DBP is relatively poorly bioconcentrated in fish where it is rapidly metabolized (Howard *et al.,* 1989). In other aquatic organisms such as *Daphnia*, DBP's bioconcentration factor is 403 which means it moderately accumulates (Staples *et al.,* 1997).

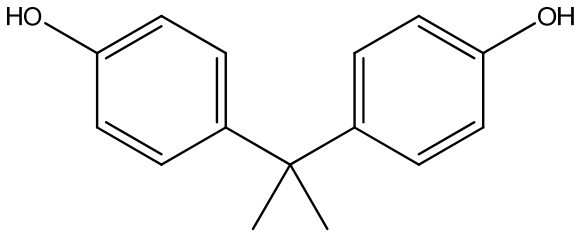
Similar to DEHP, DBP metabolites are formed when it is ingested. DBP is reported to have low toxicity when ingested orally, but has some toxicity (Williams & Blanchfield, 1975). When rats were given DBP orally, the major metabolites formed included monobutyl phthalate (MBP) and phthalic acid in the urine (Williams & Blanchfield, 1975; Ema & Miyawaki, 2001; Swan *et al.,* 2005). MBP and phthalic acid only accounted for 2 to 3% of the ingested dose (Williams & Blanchfield, 1975). After further analysis, more metabolites were discovered and consisted of mono(3-hydroxybutyl) phthalate and mono(4-hydroxybutyl) phthalate (Williams & Blanchfield, 1975). DBP is metabolized and released into the urine within 48 hours after ingestion (Williams & Blanchfield, 1975). MBP was shown to interfere with testosterone and dihydrotestosterone-dependent events such as decrease in anogential distance (AGD), and

undescended testes (Ema & Miyawaki, 2001). MBP is responsible for the anti-androgenic effects resulting from DBP exposure (Ema & Miyawaki, 2001). Neither DBP nor its metabolites accumulate in the tissues or organs of rats (Williams & Blanchfield, 1975).

DBP exposure at high levels will cause adverse reproductive effects (Foster *et al.,* 2001; Jahnke *et al.,* 2005; Foster, 2006; Howdeshell *et al.,* 2008). Rodents exposed to DBP during the postnatal (after birth) or prenatal stages were much more sensitive to developing reproductive effects compared to DBP exposed adult animals (Jahnke *et al.,* 2005). Foster *et al.,* (2001), exposed rats to DBP during a critical stage of male reproductive development, then observed the animals until adulthood (Foster *et al.,* 2001). DBP caused reproductive system effects which included malformations of the epididymis, vas deferens and hypospadias, and a decrease in anogenital distance (Foster *et al.,* 2001). Rats exposed to 500 mg/kg or above had major decreases in body weight gain and food consumption and decreased fertility (Ema *et al.,* 2000). In humans, a correlation between natural phthalate exposure through the environment and male genital development was observed in Swan *et al.,* (2005), supporting the hypothesis that prenatal phthalate exposure at environmental levels can affect male reproductive development in humans (Swan *et al.,* 2005). However the NTP-CERHR stated that there should be minimal concern over the developmental effects that increased levels of DBP may have on the male reproductive tract when pregnant women are exposed to levels of DBP that range from 2 to 10 µg/kg bw/day (Jahnke *et al.,* 2005).

2.3 - Toxicity of Bisphenol A (BPA):

Bisphenol A (BPA) is an industrial chemical used in the production of polycarbonate and various plastic products and it is widely used to make polycarbonate feeding bottles, epoxy food can linings for many food and beverage cans, dental sealants and as an additive (Lyons, 2000; Vom Saal & Hughes, 2005; Dekant & Völkel, 2008). Since BPA is used in a variety of products it is thought that human exposure to BPA is widespread and it has been demonstrated that these exposures may reach elevated levels (Yamamoto & Yasuhara, 1999; Kang *et al.,* 2006; Dekant & Völkel, 2008). BPA is one the most produced chemicals worldwide and is among the highest manufactured chemicals and in 2003 the global production of BPA exceeded 6.4 billion pounds (2.9 billion kg) (Staples *et al.,* 1998). BPA is a known endocrine disruptor made up of two phenol rings linked with a methyl bridge and with two methyl functional groups connected to the bridge (Kang *et al.,* 2006) (Figure 3). BPA has many different routes of exposure in humans. The first one is through the aquatic environment contaminated with wastewater (Kang *et al.,* 2006). BPA's half life in river water averages 3 to 5 days (Kang *et al.,* 2006). A half life of 3 to 5 days is long enough to have a drastic effect on aquatic organisms (Kang *et al.,* 2006). BPA is still found in wastewater even after treatment because it is not totally removed from the water (Kang *et al.,* 2006). BPA has also been detected in leachates from a waste landfill and ranged in concentration from 0.5 to 5.1 ng/mL in effluents following treatment treated sewage is known to be a main source of BPA contamination in the aquatic environment (Yamamoto & Yasuhara, 1999; Fürhacker *et al.,* 2000; Kang *et al.,* 2006). BPA is also found in various food products due to leaching from plastic food can linings or plastic water bottles. BPA content in



Bisphenol A

Figure 3. Molecular structure of Bisphenol A (BPA)

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Singapore seafood including prawns, crabs, squid and fish ranged from 13.3 to 213.1 ng/g wet weight and it is thought that seafood contaminated by BPA is the main route of contamination for humans (Kang *et al.,* 2006). The photo-oxidation half life for airborne BPA is 0.74 to 7.4 hours, while the photo-oxidation of BPA in water ranged from 66 hours to 160 days (Howard, 1989; Staples *et al.,* 1998; Kang *et al.,* 2006). The calculated K_{ow} value for BPA is 3.40 and the K_{oc} value is in a range of 314 to 1524 which indicates that soil and sediment are moderate sinks for BPA discharged into ground or surface water (Staples *et al.,* 1998; Kang *et al.,* 2006). BPA goes through biodegradation, adsorption to suspended solids and sediments and possible photodegradation (Staples *et al.,* 1998). BPA's potential to accumulate in the environment is considered low and its bioconcentration factor (BCF) is estimated to be below 200 with, calculated BCF's ranging from 42 to 196 (Howard, 1989; Staples *et al.,* 1998). BCF's that are below 1000 are classified as 'not a bioaccumulative chemical of concern' (Staples *et al.,* 1998).

The biodegradation pathway of BPA was studied using a gram negative bacterium, aerobic bacillus and one major and one minor pathway of degradation (Lobos *et al.,* 1992; Staples *et al.,* 1998). The major pathway formed two main metabolites, 4-hydroxyacetephenone and 4-hydroxybenzoic acid which quickly degraded to CO_2 and water or were assimilated into bacterial cells (Lobos *et al.,* 1992; Staples *et al.,* 1998). The minor pathway also formed two main metabolites: 2,2-bis(4-hydroxyphtenyl)-1-propanol which converts to 2,3,-bis(4-hydroxyphenyl)-1-2-propanediol (Lobos *et al.,* 1992; Staples *et al.,* 1998). It was also found that 60% of the carbon went to CO_2, 20% went to bacterial cell growth and another 20% went to various soluble organic compounds (Lobos *et al.,* 1992). Following oral administration BPA in rodents and in

primates (consisting of humans too), BPA is quickly and effectively (>95% of the dose) absorbed from the gastrointestinal tract and is metabolized in the gut wall and liver (Dekant & Völkel, 2008). There is little indication of significant bioaccumulation in the body due to rapid biotransformation and excretion (Dekant & Völkel, 2008).

Human exposure to BPA can occur from a variety of sources consisting of direct contact from food with BPA-containing plastics, BPA leaching from the plastic used to line food and drink cans, and the potential migration from plastic water bottles into bottled water (Lyons, 2000). It has been known for many years that bisphenol A is capable of mimicking the female hormone estrogen but only in the year 1990 did scientists start to worry about low levels of exposure of BPA (Lyons, 2000; Vom Saal & Hughes, 2005). Studies have found that BPA can easily pass through the placenta following oral intake to pregnant rats (Schönfelder *et al.,* 2002; Kang *et al.,* 2006). The mean concentration of BPA reported was 3.1 ng/mL in maternal plasma, 2.3 ng/mL in fetal plasma and 12.7 ng/g in placental tissue which indicated that BPA accumulated in the placenta (Schönfelder *et al.,* 2002; Kang *et al.,* 2006). This study showed that a fetus is at more risk from BPA exposure than an adult (Schönfelder *et al.,* 2002; Kang *et al.,* 2006). Vom Saal *et al.,* 1998 discovered that male mice exposed to low concentrations of BPA in the womb (gestation day 11 to 17) displayed increased prostate weight, lower sperm formation, lower size of seminal vesicles and larger size of preputial glands (Vom Saal *et al.,* 1998; Lyons 2000). Female pups of mice exposed to BPA in the womb (gestation day 11 to 17) experienced early puberty and were much heavier than the control female pups (Howdeshell *et al.,* 1999; Lyons, 2000). BPA decreased the number

of days between vaginal opening and initial vaginal oestrus ovulation (Howdeshell *et al.,* 1999; Lyons, 2000).

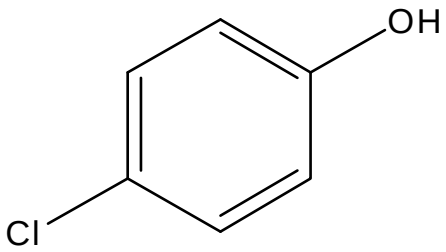
The toxicity of BPA to various species of hydra have also been studied (Fukuhori *et al.,* 2005). Exposure of brown hydra (*Hydra littoralis*) to 2-4 mg/L had toxic effects on both sexual and asexual reproduction (Fukuhori *et al.,* 2005). Testis formation and egg production were both affected by BPA (Fukuhori *et al.,* 2005). Another study found that the 96 hour LC_{50} value for BPA was 6.9 mg/L for pink hydra (*Hydra vulgaris*) (Pascoe *et al.,* 2002). At concentrations higher than 42 µg/L of BPA, the structure and physiology of hydra were significantly affected and at 500 µg/L, sexual reproduction failed to occur (Pascoe *et al.,* 2002). *Marisa cornuarietis* (snail) that were exposed to BPA concentrations between 0.1 to 640 µg/L had no effect on egg hatchability, nor on the time of hatching when compared to controls (Forbes *et al.,* 2008). The only significant effect BPA had on the snail was a decrease in female growth and wet weight in the 640 µg/L treatment while there was a major increase in male growth rate and wet weight in the 1 µg/L treatment compared with controls (Forbes *et al.,* 2008).

2.4 - Toxicity of 4-chlorophenol:

Reference toxicants are standard toxicants used to measure changes in sensitivity of populations of organisms in a laboratory over time and to provide a measure of intra-laboratory precision (Alexander and Clarke, 1978; Environment Canada, 2005). Reference toxicant tests use a standard toxic chemical at established concentrations to measure effects on organisms (Environment Canada, 2005). The two main goals of

conducting a reference toxicant test includes firstly to identify any variation in sensitivity of organisms over time and secondly to measure any variations in the measuring method of the laboratory (Environment Canada, 2005). They are also done to assess repetition of toxicity test techniques (Alexander & Clarke, 1978). The chemical 4-chlorophenol (para-chlorophenol) was used as a reference toxicant in this study and was chosen because it is a recognized reference toxicant, it is an organic chemical and it has high stability, purity and solubility in water (Kuiper & Hanstveit, 1984; Environment Canada, 2005) (Figure 4). The reference toxicant test is also known as a *positive control* and other common reference toxicants include phenols, sodium chloride, sodium pentachlorophenate, dodecylsodium sulphate or metals such as cadmium chloride (Alexander & Clarke, 1978; Environment Canada, 2005).

The chemical 4-chlorophenol is a toxic pollutant and is used in the chemical industry as an intermediate in the degradation of phenoxyacetic acid, several carbamates, and other biocides (Kuiper & Hanstveit, 1984). Chlorophenol contamination may be occurring from discharge of industrial effluents containing organic contaminants into coastal inlets or estuaries (Petroutsos *et al.,* 2007). The concentration of chlorophenols in effluent varies between 0.5 and 10,000 µg/L but the concentration in marine environments is around 10 µg/L (Petroutsos *et al.,* 2007). Residues of all chlorophenol isomers have been discovered in fresh and marine waters which include coastal sea waters (Petroutsos *et al.,* 2007). Chlorophenolic compounds have been described as having lethal effects on marine organism's especially marine phytoplankton at low concentrations (Petroutsos *et al.,* 2007) (Table 1).



4-chlorophenol

Figure 4. Molecular structure of 4-chlorophenol

(Created Using ChemBioDraw Ultra 11.0)

Table 1. The acute toxicity of 4-chlorophenol (mg/L) to various aquatic organisms

Species: (Common Name)	**Duration: (Hours)**	**LC_{50} Value: (mg/L)**	**Reference:**
Hydra Vulgaris (Pink Hydra)	96	32	(Pollino & Holdway, 1999)
Hydra viridissima (Green Hydra)	96	45	(Pollino & Holdway, 1999)
Daphnia Magna (Water flea)	48	4.1	(LeBlanc, 1990)
Pimephales promelas (Fathead minnow)	96	12.84	(Janardan *et al.*, 1984)
Lepomis macrochirus (Bluegill)	96	8.33	(Janardan *et al.*, 1984)

2.5 - Regulation of DEHP, DBP & BPA in Canada:

In Canada there are three categories of drinking water – the first is tap water, the second is bottled water such as mineral or spring water and the last category is other bottled water such as flavoured waters, distilled waters and reverse osmosis water (Dabeka *et al.,* 2002). Bottled water falls under the pre-packaged category in agreement with the Canadian Food and Drug Regulations (Dabeka *et al.,* 2002). All pre-packaged water is considered food in Canada and because of this the sale of bottled water must follow all rules and regulations set up by the Canadian Food and Drugs Act and Regulations (Dabeka *et al.,* 2002). There are various guidelines set up for various inorganic chemicals such as arsenic and lead. Arsenic's and lead's maximum acceptable concentration (MAC) is 0.01 mg/L (Environment Canada, 2008).

Phthalates have not had the same level of regulatory concern compared to BPA. The most recent regulation by the Government of Canada involves collaboration with the plastic industry to have some phthalates eliminated from the formulation of children's soft vinyl merchandise sold in Canada (Environment Canada, 2007b). Phthalates are no longer used in soft vinyl teethers and baby products (Environment Canada, 2007b). Also the Medical Devices Bureau of Health Canada is writing a Clinical Practice Guidelines to help in the appropriate administration of tools plasticized with phthalates (Environment Canada, 2007b). Environment Canada has indicated that phthalate research and evaluation is still ongoing (Environment Canada, 2007b).

Recently the Canadian government has taken steps to identify whether various chemicals such as BPA and phthalates pose any toxic health effects. The *Canadian Environmental Protection Act,* 1999 (CEPA, 199) is an act that regards pollution

prevention and the safeguarding of the environment and human health thereby contributing to sustainable development (Environment Canada, 2007a). The act requires the removal of any emissions to the environment from substances found toxic under the Act that have three types of characteristics: bioaccumulative, persistent and anthropogenic (Environment Canada, 2007a).

DEHP has been placed on Schedule 1 of the CEPA 1999 Act (Environment Canada, 2007a). After evaluation of how toxic DEHP was, it was determined that there was no detectable link between human exposure and the formation and/or use of DEHP containing plastics (Environment Canada, 2007a). It was concluded that Environment Canada and Health Canada should not continue with additional risk management actions and Health Canada indicated that they would keep on monitoring levels of DEHP in foods and conduct other studies involving plasticizers (Environment Canada, 2007a).

Both DEHP and DBP were placed on the first Priority Substances List (PSL1) which was available to the public in 1989 published in the Canadian Gazette and it was determined whether they posed a risk to human health or the environment along with 42 other substances (Health Canada, 2008). The PSL1 classifies substances to be considered on a priority basis to establish whether they are toxic and may pose a threat to humans or to the environment (Health Canada, 2008). It was discovered that DEHP may enter the environment in a quantity or concentration that may pose a threat to human health and the environment. After evaluating DBP, it was determined that it was not being released into the environment at a concentration or quantity that would pose a threat to human life or the environment (Health Canada, 2008). Health Canada concluded that for both DEHP

and DBP more research would have to be done to make final conclusions (Health Canada, 2008). Currently both DBP and DEHP remain on the PSL1.

On December 8, 2006 the Government of Canada launched the Chemical Management Plan with the goal of increasing the quality of protection from hazardous chemicals (Canada Gazette, 2009). Substances that are deemed toxic are added to Schedule 1 of the Act to allow Ministers to form risk management tools such as regulations and guidelines (Canada Gazette, 2009). In 2007 under the Chemical Management Plan the government requested the industry inform them on how to manage BPA, which resulted on October 17, 2008, BPA being added to the list of toxic substances (Environment Canada, 2007a). BPA was one of the first chemicals where direct action was taken to reduce exposure to humans and the environment. Canada was the first country to set forth regulations on BPA (Environment Canada, 2007b).

Currently research is being conducted to see what steps need to be taken. Health Canada is moving forward to ban the importation, selling and marketing of polycarbonate baby bottles (Environment Canada, 2007b). They are also working on forming strict migration targets for BPA in infant formula (Environment Canada, 2007b). Environment Canada is thinking of setting forth a regulation that would restrict the maximum concentration of BPA that can be discharged into the environment (Environment Canada, 2007b). This would allow BPA to be released into the environment at safe concentrations for fish and various other aquatic life (Environment Canada, 2007b). The government will also begin a research plan centered on mothers, the fetus, newborns and infants and other damaging effects such as prostate or breast cancer to understand the basis of exposure and effects can occur (Environment Canada, 2007b). Various stores have also

phased out any type of PC bottles such as Nalgene sports bottles or various brands of PC baby bottles after the news of BPA being added to the toxic substances list.

2.6 – An Introduction to Hydra:

2.6.1 - Hydra Characteristics and Physiology:

Hydra (Cnidaria-Hydrozoa) are micro-invertebrate multicellular organisms that are about 2 to 3 mm wide and 5 to 20 mm long and are present in many freshwater environments (Holdway, 2005). Hydra acquire their name from the nine-headed sea snake from Greek mythology. They are more than 500 million years old and are freshwater relatives of the corals, sea anemones and jellyfish which are also part of the phylum Cnidaria. Cnidarians fit into the simplest metazoans group and diverged from the metazoan family before the development of Bilaterians (Steele, 2002; Böttger & Alexandrova, 2007; Hoffmeister-Ullerich, 2007). The phylum Cnidaria also refers to the stinging cells which hydra and other animals in this phylum possess (Böttger & Alexandrova, 2007). Hydra are typically found in fast moving waters rather than slow (Karntanut & Pascoe, 2000). Green hydra (*Hydra viridissima*) are usually found in clear waters while pink hydra (*Hydra vulgaris*) are usually found in turbid waters (Holdway *et al.*, 2001).

Hydra typically reproduce asexually by budding which results in the rapid production of a large number of genetically identical organisms (Pollino & Holdway, 1999). Usually budding will take place anywhere on the hydra column, where on a small

region of the parent body a bud will form a tiny but whole hydra (Hyman, 1928; Otto & Campbell, 2005). During the process of budding, cells arise from the proliferation region on the body column of the parent body and are deposited into the bud (Böttger & Alexandrova, 2007). The first stage that occurs is a tiny bud will emerge on the side of the adult; it will grow in length and then form a hypostome and tentacles (Böttger & Alexandrova, 2007). Finally a peduncle forms close to the parent body and the bud detaches (Böttger & Alexandrova, 2007).

The process of the bud detaching from the parent body lasts approximately 3 days or less (Otto & Campbell, 2005). These organisms have a high asexual reproductive rate which allows large numbers of hydra to be cultured in a short period of time (Holdway, 2005; Böttger & Alexandrova, 2007). The rapid asexual reproductive rate will allow reproduction effects of a possible toxicant to be determined (Mitchell & Holdway, 2000). Hydra can also go through sexual reproduction, where hydra will make male and/or female gonads and stimulate a sexual cycle (Böttger & Alexandrova, 2007). They tend to reproduce sexually under stressful conditions such as variations in water temperature or other environmental stimuli (Holdway, 2005; Böttger & Alexandrova, 2007).

Hydra are diploblastic which allows toxic substances to be exposed to all body surfaces of the hydra (Karntanut & Pascoe, 2000, Quinn *et al.,* 2008). Hydra have a two tissue layer body composition which includes an ectoderm and endoderm (Holdway, 2005, Hoffmeister-Ullerich, 2007). Instead of the mesoderm layer they possess a fine acellular mesoglea layer found in-between the ectoderm and endoderm (Steele, 2002, Holdway, 2005, Hoffmeister-Ullerich, 2007). Hydra possess a simple nervous system which include a nerve net that stretches throughout the hydra's body (Sakaguchi *et al.,*

1996). Neurons are found close to the basal sides of the two epithelial cell layers of the hydra (Sakaguchi *et al.,* 1996). Hydra possess sensory cells, nerve cells and muscle cells which have the form and role of epithelial cell layers (Muller, 1996, Benson & Boush, 1983).

Hydra consist of a tube made up for two connected epithelial cell layers (Muller, 1996, Steele, 2002) (Figure 5). The tube includes approximately 100,000 cells (Muller, 1996). At the top end of the tube there is an opening, the mouth (hypostome), enclosed by tentacles (Muller, 1996, Steele, 2002). The tentacles have stinging cells attached to it and this allows the hydra to catch prey easily (Muller, 1996, Steele, 2002). The mouth and tentacles are called the hydranth (Holdway, 2005). The rest of this organism is known as the column (Holdway, 2005). The column has four distinctive sections: the gastric section located between the tentacles and the first (apical) bud, the budding section which produces the buds, the peduncle which is located between the lowest bud and basal disc and the basal disc which is the foot-like formation (Holdway, 2005). The foot consists of an adhesive gland allowing the hydra to stick on to the medium (Muller, 1996). Hydra that are well fed transport cells by forming buds in the middle of their body (Muller, 1996). The buds expand to genetically related offspring (Muller, 1996). Hydra also have the ability to regenerate. When hydra polyps are cut into pieces they are able to regenerate the absent structures entirely (Böttger & Alexandrova, 2007, Hoffmeister-Ullerich, 2007).

Hydra need to be fed a large amount of food to encourage asexual reproduction and they eat live organisms such as brine shrimp nauplii (*Artemia salina*) or water fleas (*Daphnia magna*) as food. Hydra go through a series of steps when feeding on prey.

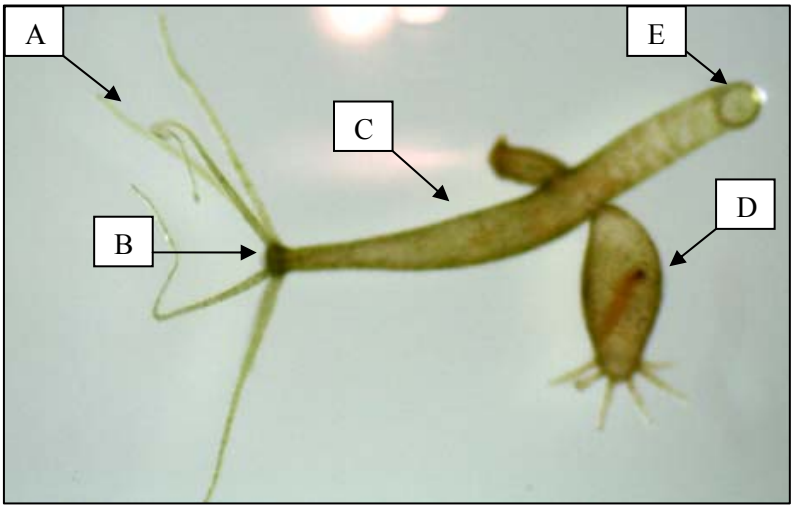


Figure 5. Body parts of a green hydra (A – tentacles, B – mouth, C – body column, D – bud, E – base)

Feeding in hydra starts off with the prey unintentionally bumping into an extended tentacle of the hydra (Lenhoff, 1968). The prey is caught, wounded and poisoned through stinging cells that line the tentacles of the hydra (Lenhoff, 1968). These stinging cells have a nematocyst capsule (cnidocyst) that ejects toxins into the prey (Böttger & Alexandrova, 2007, Lenhoff, 1968). Once the prey has been secured the tentacles contract in the direction of the hydra's mouth and the mouth opens (Lenhoff, 1968). The contraction of the tentacles towards the hydra's mouth is controlled by a chemical (Lenhoff, 1968). The feeding response is activated by reduced amounts of glutathione (Lenhoff, 1968). Once the prey has made contact with the mouth the food is consumed (Lenhoff, 1968).

The hydra species used in this study was *Hydra viridissima* (green hydra) (Figure 6). The green colour is provided by several intracellularly located algae and most of these symbionts are found inside the upper region of the body column, which causes the upper region of the hydra to be greener than the lower region (Habetha *et al.,* 2003). Green hydra contain stable algal symbiotes and tend to be smaller than other hydra such as *Hydra littoralis* (Slobodkin *et al.,* 1991; Pollino & Holdway, 1999, Holdway *et al.,* 2001,) (Figure 7). Green hydra have both female and male reproductive units and use carbohydrate metabolism for energy (Holdway *et al.,* 2001).

Carbohydrate metabolism needs oxygen and this is made available to green hydra via their symbiotic photosynthetic algae (zoochlorellae) (Holdway *et al.,* 2001; Kovacević *et al.,* 2007). The zoochlorellae are found in the endodermal cells of the hydra (Blank & Muscatine, 1987, Holdway *et al.,* 2001, Habetha *et al.,* 2003). The green hydra

Figure 6. Green hydra (*Hydra viridissima)*

Figure 7. Brown hydra (*Hydra littoralis)*

holds many unicellular *Chlorella* algae and these endodermal cells are within vacuoles, which allow them to be shielded from the host's digestive enzymes (Slobodkin *et al.,*1991; Habetha *et al.,* 2003). The symbionts of green hydra supply nutrients to the hydra polyps in the form of maltose or glucose-6-phosphate (Habetha *et al.,* 2003). The nutrients provided by the symbionts allow green hydra to last for long periods of time even through stages of starvation (Slobodkin *et al.,* 1991; Habetha *et al.,* 2003). If starvation conditions occur green hydra can obtain nourishment directly from their symbionts (Slobodkin *et al.,* 1991).

2.6.2 - Hydra Classification:

The first *Hydra* species was discovered in 1758 by Carl Linné and he taxonomically characterized the hydra as *Hydra polypus* (Hemmrich *et al.,* 2007). In the following years more hydra species were discovered and were put into one genus (Hemmrich *et al.,* 2007). Another method of classifying hydra was developed at the start of the 20th century in which hydra species were separated into three genera *Hydra, Pelmatohydra* and *Chlorohydra*, based on morphological variations in the body plan (body shape, stalk, symbiotic algae), various modes of tentacle development during the process of budding and differences in certain types of nematocytes (Hemmrich *et al.,* 2007). In 1987 all hydra species were placed under one genus but into four separate groups (Hemmrich *et al.,* 2007). These groups are the viridissima group (green hydra), which consists of a single species, the oligactis group (large stalked hydra), which consists of 3 to 5 species, the braueri group (tiny hermaphroditic hydra) which also consists of 3 to 5 species and the vulgaris group (common hydra) which consists of 4 to 6

species (Hemmrich *et al.,* 2007, Jankowski *et al.,* 2008). There are about 80 different species of hydra but only about 15 species are well defined (Jankowski *et al.,* 2008). Various species of hydra are used for different analytical reasons. For example *Hydra viridissima* are used to learn about symbiosis and *Hydra vulgaris* are used for developmental studies (Hemmrich *et al.,* 2007).

2.6.3 - Hydra Culture Methods:

Hydra are a relatively easy organism to culture most of the time. To culture large amounts of hydra, an abundance of food, clean culture solution and daily care is required (Lenhoff & Brown, 1970). They are a fairly easy organism to take care of and can double in population every 1 to 4 days as long as the culture is being raised in the proper conditions (Loomis, 1953; Lenhoff & Brown, 1970) Some of the requirements for a healthy stock culture include feeding of live brine shrimp nauplii (*Artemia salina*) with all salt washed out or any other appropriate food such as daphnia, cleaning of the stock culture after feeding to prevent contamination of the stock culture by fungi and bacteria and daily care (Loomis, 1953, Lenhoff & Brown, 1970). *Artemia* cysts are the most suitable and least costly food for starting and maintaining a stock culture (Lenhoff & Brown, 1970).

Hydra have to be fed plenty of food for a minimum of 30 minutes daily, so the stock culture can increase dramatically (Lehnoff & Brown, 1970, Holdway *et al.,* 2001). Once hydra have been fed the water must be replaced with fresh new water or medium (Lenhoff & Brown, 1970). Green hydra can survive at a wide range of temperature but are healthier and have a higher reproductive rate at a higher temperatures such as 25°C

(Holdway, 2005). Various factors can contribute to the growth of hydra and they include the water, temperature, pH, dissolved oxygen, amount of food and ionic maintenance. Studies have shown that calcium ions and potassium ions are required for hydra stock cultures, especially *Hydra littoralis* and *Hydra viridissima* species (Lenhoff & Brown, 1970). Even with sufficient food hydra stock cultures can be hard to maintain in some cases because of the occurrence of "depression" (Loomis, 1953). Hydra can be cared for daily but they can still pass into this stage and if actions are not taken the stock population will die out (Loomis, 1953). When the depression stage occurs the hydra's stalk and tentacles shorten and the hydra does not eat and shortens into a stumpy form and followed by disintegration (Loomis, 1953).

2.6.4 - The Advantages of Using Hydra in Ecotoxicology:

Hydra are interesting model organisms for biotesting objectives for various reasons which include: morphological changes can be easily identified; their widespread prevalence in freshwater ecosystems makes it a demonstrative bio-indicator, their fast reproductive rate, cost-effective and are easily cultured and cared for in the laboratory (Arkhipchuk *et al.*, 2006). Another advantage is the ability of hydra to regenerate due to constant proliferating epithelial and interstitial cells in the body column of the hydra which allows the hydra to bud at a rapid rate (Hoffmeister-Ullerich, 2007). Since hydra are diploblastic, this allows for simple interaction with the toxicant (Beach & Pascoe, 1998). The structure of the hydra and its simple anatomy allows it to be a valuable indicator of pollution or other pressures in the outside environment (Beach & Pascoe,

1998; Holdway *et al.,* 2001). Another advantage hydra have when used for toxicity testing is that hydra reproduce asexually the majority of the time and produce other hydra that are genetically similar (Beach & Pascoe, 1998). Since there is minor genetic variation, this allows experimental results to be replicated with less difficulty and allows for a decreased coefficient of variation (Beach & Pascoe, 1998).

Hydra have been used in a variety of ecotoxicological studies; for example hydra were used to assess the acute and chronic effects of 11 compounds found in primary treated effluent from a Montreal wastewater treatment plant (WWTP) (Quinn *et al.,* 2008). Hydra have also been used to study heavy metals, estrogenic compounds, pesticides and pharmaceuticals (Benson & Boush, 1983, Holdway *et al.,* 2001, Pascoe *et al.,* 2003, Karntanut & Pascoe, 2007). The effects of organic compounds such as BPA and 17α-Ethinylestradiol have also been investigated using hydra along with the effects of various pesticides and PCBs (Benson & Boush, 1983, Pascoe *et al.,* 2002) Metal toxicity has also been assessed using hydra including cadmium, zinc and copper (Pollino & Holdway 1999, Holdway *et al.,* 2001). Hydra are generally more sensitive to metals than organic compounds (Pollino & Holdway, 1999, Holdway, 2005).

3.0 - Research Objectives:

The null hypothesis of this study is that potential chemicals that are leaching from Canadian bottled water have no effect on the survival and reproduction of *Hydra viridissima* (green hydra).

The first research objective is to use the Cnidaria *Hydra viridissima* (green hydra) to determine if Canadian bottled water has an effect on these organisms. The effect of external light exposed versus internal dark stored bottles on chemical leaching and potential toxicity had to be assessed. The second research objective involved conducting range finders using dibutyl phthalate (DBP), bis(2-ethylhexyl) phthalate (DEHP) and bisphenol A (BPA) to establish suitable concentrations for the chronic toxicity tests. The third objective involved conducting chronic toxicity tests using dibutyl phthalate (DBP), bis-2-ethylhexyl phthalate (DEHP) and bisphenol A (BPA). The next objective involved testing the sensitivity of green hydra using the reference toxicant, 4-chlorophenol. The last objective was to conduct chemical analysis by solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC-MS), part of which was conducted by an external lab.

4.0 - Methods:

4.1 - Laboratory Culturing of Hydra:

One culture of hydra species (Green hydra - *Hydra viridissima*) were reared throughout the year. Green hydra were purchased from Ward's Natural Science Establishment (St. Catherines, Ontario, Canada). Stock cultures were maintained at room temperature (21°C) and in a warm temperature control room (25°C). Glass jars and bowls were used and hydra were moved from these jars and glass bowls weekly to minimize bacteria and fungi. Bowls and jars were rinsed with ethanol and lab water and then left to dry. Hydra were fed brine shrimp nauplii four times a week and then everyday when experiments were to be conducted. The hydra were given 30 minutes to eat, and then stock culture jars were rinsed and a new solution of lab water was added to each jar or bowl. Hydra were stock cultured with ultra pure lab water to reduce the amount of fungus and bacterial infection. The ultra pure lab water goes through several processes. When it comes in it is filtered through charcoal, then through resin and brine to soften the water, then through a reverse osmosis filter which removes all of the dissolved ions and salts. Magnesium and calcium is then added to bring the pH back to about 7.5.

4.2 – Field Experiment:

The field experiment consisted of investigating two types of environments, sunlight and darkness and four types of treatments. The different treatments were Lab Water-PETE, Brand A-PETE, Lab Water-Glass and Lab Water-Polycarbonate (PC). The

volume of water contained in the bottles was 500 mL of sample. There were five different sampling periods, weeks 0, 2, 4, 6, 11. On week 0 only two treatments were analyzed, Lab Water and Brand A water. In weeks 2, 4, 6 and 11 all four treatments were looked at including Lab Water which was used as a control. The sunlight environment bottles were put into a transparent Rubbermaid container with holes drilled on the bottom of the container to let water drain out. The bottles were put on top of grass and no lid was placed on top of the container. The darkness environment had bottles stored in a cabinet in random order under complete darkness.

4.2.1 - Field Experiment Bioassays:

Field experiment bioassays were run from August 7th to October 31st at specific sampling times which included weeks 0, 2, 4, 6 and 11. Pyrex Petri dishes (150 mm X 20 mm) were labelled and set up on the lab bench at room temperature (21°C). Samples were collected from each of the two environments. Two bottles of each treatment were collected from each environment. Each bottle contained 500 mL of sample. Three Petri dishes for each treatment was set up. The treatments included Lab Water-PETE, Brand A-PETE, Lab Water-Glass, Lab Water-PC and Lab Water as a control. Altogether 27 Petri dishes were set up on the lab bench. In each treatment 250 mL of sample was used for the bioassay, while the remaining 750 mL was moved into a separate aluminum covered glass bottle for solid phase extraction. Using 5 mL pipettor, 10 mL of each sample was put into each Petri dish. The remaining sample was moved into an aluminum foil covered clean glass flask for when renewals would be done.

Five hydroids were placed in each Petri dish and lids were placed on top of each dish to avoid evaporation. The glass Petri dishes were put on the lab bench in a randomized order. The hydroids were observed at 0 hours and then daily for seven days (168 hours). Survival and reproductive observations were recorded. A scoring technique obtained from Wilby, (1988), was used to record morphological changes exhibited by the hydra (Wilby, 1988). A score of 10 indicated a normal hydra with extended tentacles, body reactive, 9 indicated partially contracted and slow reactions, 8 indicated clubbed tentacles and body slightly contracted, 7 indicated shortened tentacles, body slightly contracted, 6 indicated tentacles and body shortened, 5 indicated totally contracted, tentacles visible, 4 indicated totally contracted, no visible tentacles, 3 indicated expanded, tentacles visible, 2 indicated expanded, no visible tentacles, 1 indicated dead but intact and 0 indicated a disintegrated organism (Wilby, 1988) (Table 2) (Figure 8). After survival and reproductive observations were recorded the hydra are fed brine shrimp nauplii. Test solutions were changed after 30 minutes of feeding. This was completed by doing a 50% partial replacement which means half the sample was removed. Out of the 10 mL of sample in the Petri dish, 5 mL of sample was removed and a fresh 5 mL of sample was added to each Petri dish. All brine shrimp were removed from the Petri dishes to avoid any fungus from growing in the dishes. Observations were recorded before and after renewal. The above procedure was repeated daily for seven days. During the bioassay the temperature, pH, water hardness and alkalinity (Jungle Quick Dip Test Strips) was recorded daily. The dissolved oxygen (La Motte) was measured for all test solutions at time zero and afterwards the dissolved oxygen was recorded for lab water daily. Dissolved oxygen was measured using a kit and titration method.

4.2.2 – Field Experiment Chemical Analysis – Solid Phase Extraction (SPE) (Part External Lab)

For each sampling period (Week 0, 2, 4, 6, & 11) 2 bottles were collected of each sample. So out of a total of 1000 mL, 750 mL of sample was moved into a glass bottle covered in aluminum and stored in a 4°C fridge for 1 week. Solid phase extraction cartridges were purchased from the Waters Corporation. 60 µm Oasis hydrophilic-lipophilic-balanced (HLB) Plus Extraction Cartridges were purchased. The procedure used for solid phase extraction (SPE) was adapted from Casajuana & Lacorte, 2003. Cartridges were first conditioned by passing 5 mL of methanol and 5 mL of Milli Q water through the cartridge. 750 mL of water samples were extracted in 225 mg Oasis cartridges. The sample was loaded at a flow rate of 5 mL/min. After the sample had run through the cartridges each cartridge was put into labelled tin foil pouches and stored in a 4°C fridge. All samples were loaded onto the cartridges for all sampling periods and then stored in the fridge for an external lab (York-Durham Regional Environment Laboratory, Pickering, Ontario, Canada) to analyze. The external lab performed the two step elution procedure.

Table 2. Scoring key of assessing progressive toxic effects in *hydra* (Wilby, 1988)

Score:	Morphology of Hydra:
10	Extended tentacles and body reactive
9	Partially contracted, slow reactions
8	Clubbed Tentacles, body slightly contracted
7	Shortened tentacles, body slightly contracted
6	Tentacles and body shortened
5	Totally contracted, tentacles visible
4	Totally contracted, no visible tentacles
3	Expanded, tentacles visible
2	Expanded, no visible tentacles
1	Dead but intact
0	Disintegrated

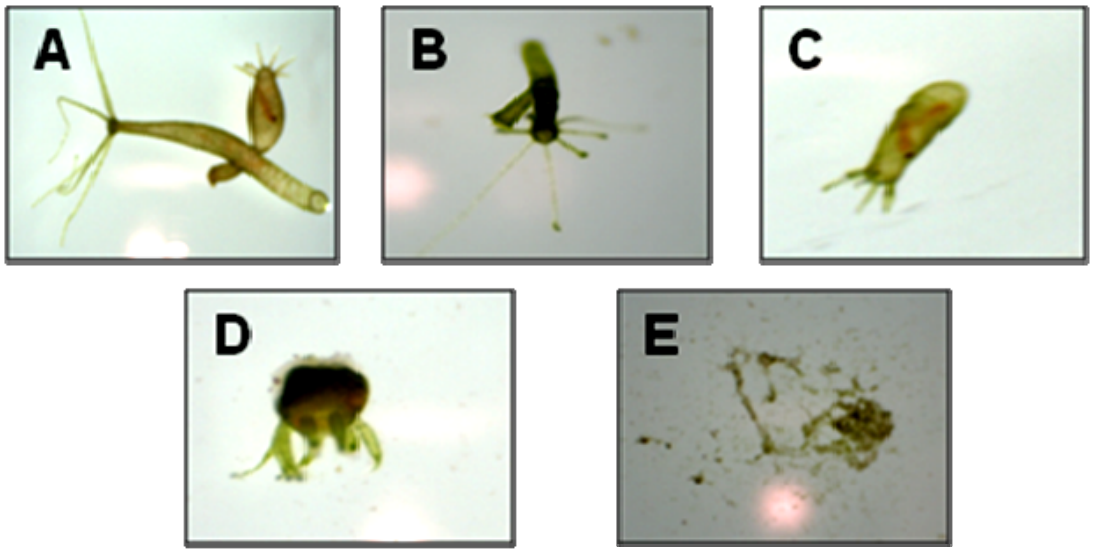


Figure 8. Stages of toxic effects in *Hydra viridissima* (Green Hydra) (A) normal hydra (score 10), (B) clubbed tentacles (Score 8), (C) shortened tentacles (Score 7), (D) tulip Stage (Score 5), (E) Disintegration (Score 0)

4.23 - Chemical Analysis – Gas Chromatography-Mass Spectrometry (External Lab):

An external lab (York-Durham Regional Environment Laboratory, Pickering, Ontario, Canada) conducted gas-chromatography-mass spectrometry (GC-MS) analysis on the various samples. Samples that were analyzed included Week 0 samples (Baseline) of lab water and Brand A water as baseline controls. The remaining samples were all Week 11 samples consisting of lab water in PETE bottles, Brand A water in PETE bottles, lab water in PC bottles and lab water in glass bottles for each the sunlight and dark environments. Altogether ten samples were analyzed. The external lab looked to see if DBP, DEHP and BPA were present in the samples.

4.3 – In-Lab Experiments:

A general range finder was conducted to see what concentrations of each chemical were suitable for the chronic toxicity tests. Another three separate range finders were run to see what concentrations were appropriate. These range finders were done with the three chemicals: dibutyl phthalate (DBP) (Sigma Aldrich), bis(2-ethylhexyl) phthalate (DEHP) (Sigma Aldrich) and bisphenol A (BPA) (Sigma Aldrich). All three chemicals were 99% pure.

4.3.1 - Range Finders for Bis(2-ethylhexyl) phthalate (DEHP), Dibutyl phthalate (DBP) & Bisphenol A (BPA):

A range finder was initially conducted with DBP, DEHP & BPA. The range finder was a 96 hour test that used a range of concentrations that may affect hydra survival. This test was run on the lab bench at room temperature (21°C). The concentrations that were used for the first range finder consisted of nominal concentrations: 0 µg/L (Lab Water), 0.1 µg/L, 1.0 µg/L, 10 µg/L, 100 µg/L for each of the three chemicals (DEHP, DBP & BPA). This test was run without replicates to establish what concentrations were appropriate to use. The number of hydroids in each Petri dish was recorded. Survival and reproductive observations were recorded. A scoring technique was used to record morphological changes exhibited by hydra (Wilby, 1988) (Table 2).

Another set of range finders were conducted except this time each chemical range finder was conducted separately and in triplicates. These range finders were conducted in the warm room under a temperature of 25°C. The concentrations varied for each chemical. For DEHP the nominal concentrations that were used were 0 µg/L (lab water – control), 1.0 µg/L, 3.2 µg/L, 10 µg/L, 32 µg/L and 100 µg/mL. Range finders for DBP and BPA consisted of nominal concentrations of 0 µg/L (lab water – control), 10 µg/L, 32 µg/L, 100 µg/L, 320 µg/L and 1000 µg/L. In the dry lab stock solutions were pre-made and the amount of stock solution was gathered from the original stock solution bottle and transferred into another labelled bottle. Safety gloves, glasses were worn during the bioassay. The stock solution bottle was left in the warm room to equilibrate with warm room temperature. Appropriate volume of lab water was added to each Petri

dish. Following which the calculated volume of stock solution of each chemical was transferred. This was repeated for each replicate.

Five non-budding hydra were placed into each of the Petri dishes using a Pasteur pipette. Hydra were picked out of random stock cultures. It was ensured that no extra liquid was placed in Petri dishes when moving hydra. The hydroids were observed under a microscope at 0 hours and then daily (every 24 hours) for 4 days (96 hours). Petri dishes were placed in random order on lab bench. All materials were cleaned up and gloves were disposed into waste bucket along with plastic tips. The number of hydroids in each Petri dish was recorded. Survival and reproductive observations were also recorded. A scoring technique was used to record morphological changes exhibited by the hydra (Wilby, 1988) (Table 2). Hydra were not fed for 24 hours before the bioassay and during the bioassay and test solutions were not changed or renewed. During this bioassay the pH, temperature, conductivity, alkalinity and dissolved oxygen (La Motte) on lab water was evaluated and recorded each day. The pH, conductivity and alkalinity were measured using test strips (Jungle Quick Dip Test Strips).

4.3.2 - Reference Toxicant Test using 4-chlorophenol:

A reference toxicant test was conducted every two months on the green hydra. The reference toxicant used was 4-chlorphenol (Sigma Aldrich) with 95% purity. The test was a 96 hour static exposure test. A stock solution of 4-chlorophenol was prepared at a concentration of 100 mg/L with the chemical being diluted in ultra pure lab water. Six nominal concentrations were used which were 0 mg/L, 1.0 mg/L, 3.4 mg/L, 10 mg/L, 34

mg/L and 100 mg/L. The Petri dishes were set up and labelled. The appropriate volume of water was added to each Petri dish. The various test concentrations were prepared and added to the Petri dishes. Proper precautions were used to ensure no extra liquid was added to each Petri dish. Each Petri dish had a total of 10 mL in it. The test was run in duplicate.

Twenty non-budding hydra were put into each Petri dish. The test was run for five days including time zero, with no feeding and no replacement of water. The highest concentration would have to have 100% mortality for the test to be considered valid. Lab water was used as control (0 μg/mL) and Petri dishes were placed in a random order to ensure randomization. The hydroids were observed under a microscope at 0 hours and then daily for 4 days. Survival and reproductive observations were recorded. Obvious changes in morphology were recorded with a scoring technique provided by Wilby, 1988 was used (Table 2)**.** The toxicity endpoints for this test were the tulip and disintegrated stages (Table 2). Also the number of hydroids in each Petri dish was recorded. . During the reference toxicant test the pH, temperature, conductivity, alkalinity and dissolved oxygen (La Motte) on lab water was evaluated and recorded each day. The pH, conductivity and alkalinity were measured using test strips (Jungle Quick Dip Test Strips).

4.3.3 - Chronic Toxicity Tests for Dibutyl phthalate (DBP), Bis (2-ethylhexyl) phthalate (DEHP) and Bisphenol A (BPA):

In the wet lab all Petri dishes were set up on the lab bench in the warm room (25°C). In the dry lab stock solutions were prepared for each chemical. Safety glasses, gloves and a respirator were used to make each stock solution. Stock solutions were made at concentrations of: 0.25 µg/L for DEHP and 1.0 µg/mL for DBP and BPA. All chemicals were purchased from Sigma Aldrich. Stock solution bottle was left in warm room to equilibrate with warm room temperature. Calculated lab water solution was added to each Petri dish. Nominal concentrations that were used for each chemical remained the same for all chronic toxicity tests that were conducted and consisted of: 0 µg/L (lab water – control), 0.1 µg/L, 1.0 µg/L. 10 µg/L, and 100 µg/L. Appropriate amount of stock solutions were added into Petri dish.

Five budding hydra were added into each Petri dish using a Pasteur pipette. Hydra with one healthy bud were added to Petri dishes and were picked from random stock cultures. It was ensured that no extra liquid was added to each of the Petri dishes when hydra were added. They hydroids were observed after addition to Petri dishes (0 hours) and daily for seven days. Any hydra that were not healthy were replaced with healthy hydra at 0 hours only. Petri dishes were then placed in a random order. The number of hydroids in each Petri dish was recorded. Survival and reproductive observations were recorded. A scoring technique was used to record morphological changes exhibited by hydra (Wilby, 1988) (Table 2). Hydra were fed daily for this test. Brine shrimp was fed to hydra for 30 minutes. One drop of diluted brine shrimp was added to each Petri dish. A 95% renewal of solution was done for each Petri dish. Before and after feeding

observations were recorded. During this bioassay the pH, temperature, conductivity, alkalinity and dissolved oxygen (La Motte) on lab water was evaluated and recorded each day. The pH, conductivity and alkalinity were measured using test strips (Jungle Quick Dip Test Strips).

4.4 – Statistical Analysis of Results:

A standard curve analysis was used to calculate the 96 hour LC_{50} values for the reference toxicant tests using 4-chlorophenol. A linear equation was used to calculate LC_{50} values. A one way analysis of variance (ANOVA) was used to detect significance between LC50 values between the various reference toxicant test that were conducted ($p \leq 0.05$).

For the field experiment bioassays, the data was tested for normality using the Shapiro-Wilks Test. A one-way analysis of variance (ANOVA) was used to detect significant differences between treatments and between hydra numbers on day 7 for the various treatments. If significant differences were seen ($p \leq 0.05$) then a post hoc Tukey test determined differences between treatments ($p \leq 0.05$).

For the chronic toxicity tests the Shapiro Wilks Test was conducted to test for normality and a one-way ANOVA was used to detect significance ($p \leq 0.05$). A post hoc Tukey test was done to see where significance was occurring ($p \leq 0.05$).

The final day (day seven) mean relative population growth rate (K value) was calculated for the chronic exposures to all toxicants, including DEHP, DBP & BPA (Holdway, 2005). The mean relative growth rate is classified as:

$$K = \frac{\ln (ny) - \ln (nx)}{ty-tx}$$

where nx is the initial number of hydra (day 0), ny is the total number of hydra on day 7 and ty-tx is the experiment length in days (7 days) (Holdway, 2005).

Graphs and tables were created using Sigmaplot, Microsoft Excel and Microsoft Word. Chemical structures were created using ChemBioDraw Ultra 11.0.

5.0 Results:

5.1 – Hydra Culture Establishment:

Stock cultures of green hydra were effectively maintained throughout the year using reverse osmosis filtrated lab water and daily feeding with brine shrimp (Appendix 1). During the year various hydra toxicity tests were conducted with a 16 hour light and 8 hour dark photoperiod using hydra from stock cultures.

5.2 – Water Characteristics:

Abiotic factors including pH, temperature, alkalinity and dissolved oxygen were recorded. Total water hardness measurements were recorded as well. All abiotic factors that were measured for both the field experiments and in-lab experiment are detailed in Tables 3 to 6. Water hardness was the only abiotic factor that was different between treatments. Brand A bottled water was 25 ppm higher in water hardness than all the other treatments. Temperature information which includes the temperature, precipitation and relative humidity for the field site was obtained from a weather station located at the Crime Scene House on 275 Conlin Road East, Oshawa, Ontario. The measurements for the period of August 7th to August 28th were not available due to the malfunctioning of the weather station. Weather station temperatures are listed in the Appendix 11 and The in-lab temperatures are listed in Appendix 12.

Table 3. Mean ± Standard deviation values for water characteristics measured for field experiment for Week 0.

Treatment	**pH**	**Temperature (°C)**	**Water Hardness (ppm as $CaCO_3$)**	**Alkalinity (ppm as $CaCO_3$)**	**Dissolved Oxygen (ppm)**
Control	6.80 ± 0.00	21.33 ± 0.05	0 ± 0.00	80 ± 0.00	7.66 ± 0.09
Brand A - PETE	6.20 ± 0.00	21.31 ± 0.04	25 ± 0.00	80 ± 0.00	8.40[a]

[a] – Only one measurement was taken due to limited amount of sample

Table 4. Mean ± Standard deviation values for water characteristics measured for field experiment – sunlight environment.

Treatment	Week:	pH	Temperature (°C)	Water Hardness (ppm as $CaCO_3$)	Alkalinity (ppm as $CaCO_3$)	Dissolved Oxygen (ppm)
Control	2	6.80 ± 0.00	20.40 ± 0.00	0 ± 0.00	80 ± 0.00	7.70 ± 0.09
Brand A - PETE	2	6.20 ± 0.00	20.33 ± 0.05	25 ± 0.00	80 ± 0.00	8.40[a]
Lab Water - PETE	2	6.80 ± 0.00	20.33 ± 0.08	0 ± 0.00	80 ± 0.00	7.90[a]
Lab Water - Glass	2	6.80 ± 0.00	20.38 ± 0.07	0 ± 0.00	80 ± 0.00	7.60[a]
Lab Water - PC	2	6.80 ± 0.00	20.36 ± 0.07	0 ± 0.00	80 ± 0.00	8.00[a]
Control	4	6.80 ± 0.00	21.36 ± 0.05	0 ± 0.00	80 ± 0.00	7.69 ± 0.05
Brand A - PETE	4	6.20 ± 0.00	21.34 ± 0.05	25 ± 0.00	80 ± 0.00	8.20[a]
Lab Water - PETE	4	6.80 ± 0.00	21.30 ± 0.00	0 ± 0.00	80 ± 0.00	8.00[a]
Lab Water - Glass	4	6.80 ± 0.00	21.31 ± 0.04	0 ± 0.00	80 ± 0.00	7.80[a]
Lab Water - PC	4	6.80 ± 0.00	21.31 ± 0.04	0 ± 0.00	80 ± 0.00	8.20[a]
Control	6	6.80 ± 0.00	21.41 ± 0.04	0 ± 0.00	80 ± 0.00	7.70 ± 0.09
Brand A - PETE	6	6.20 ± 0.00	21.40 ± 0.05	25 ± 0.00	80 ± 0.00	8.30[a]
Lab Water - PETE	6	6.80 ± 0.00	21.39 ± 0.04	0 ± 0.00	80 ± 0.00	8.00[a]

Table 4. Mean ± Standard deviation values for water characteristics measured for field experiment – sunlight environment **continued...**

Treatment:	Week:	pH	Temperature (°C)	Water Hardness (ppm as $CaCO_3$)	Alkalinity (ppm as $CaCO_3$)	Dissolved Oxygen (ppm)
Lab Water – Glass	6	6.80 ± 0.00	21.40 ± 0.05	0 ± 0.00	80 ± 0.00	7.80[a]
Lab Water – PC	6	6.80 ± 0.00	21.38 ± 0.07	0 ± 0.00	80 ± 0.00	8.20[a]
Control	11	6.80 ± 0.00	21.51 ± 0.06	0 ± 0.00	80 ± 0.00	7.76 ± 0.06
Brand A – PETE	11	6.20 ± 0.00	21.51 ± 0.06	25 ± 0.00	80 ± 0.00	8.00[a]
Lab Water – PETE	11	6.80 ± 0.00	21.52 ± 0.07	0 ± 0.00	80 ± 0.00	7.90[a]
Lab Water – Glass	11	6.80 ± 0.00	21.50 ± 0.07	0 ± 0.00	80 ± 0.00	7.80[a]
Lab Water – PC	11	6.80 ± 0.00	21.53 ± 0.05	0 ± 0.00	80 ± 0.00	7.60[a]

[a] – Only one measurement was taken due to limited amount of sample

Table 5. Mean ± standard deviation values for water characteristics measured for field experiment – dark environment.

Treatment:	Week:	pH	Temperature (°C)	Water Hardness (ppm as $CaCO_3$)	Alkalinity (ppm as $CaCO_3$)	Dissolved Oxygen (ppm)
Control	2	6.80 ± 0.00	20.40 ± 0.00	0 ± 0.00	80 ± 0.00	7.70 ± 0.09
Brand A - PETE	2	6.20 ± 0.00	20.34 ± 0.07	25 ± 0.00	80 ± 0.00	8.20[a]
Lab Water - PETE	2	6.80 ± 0.00	20.36 ± 0.07	0 ± 0.00	80 ± 0.00	7.70[a]
Lab Water - Glass	2	6.80 ± 0.00	20.36 ± 0.07	0 ± 0.00	80 ± 0.00	7.60[a]
Lab Water - PC	2	6.80 ± 0.00	20.38 ± 0.07	0 ± 0.00	80 ± 0.00	8.20[a]
Control	4	6.80 ± 0.00	21.36 ± 0.05	0 ± 0.00	80 ± 0.00	7.69 ± 0.05
Brand A - PETE	4	6.20 ± 0.00	21.31 ± 0.04	25 ± 0.00	80 ± 0.00	8.40[a]
Lab Water - PETE	4	6.80 ± 0.00	21.35 ± 0.05	0 ± 0.00	80 ± 0.00	7.90[a]
Lab Water - Glass	4	6.80 ± 0.00	21.30 ± 0.00	0 ± 0.00	80 ± 0.00	8.20[a]
Lab Water - PC	4	6.80 ± 0.00	21.40 ± 0.00	0 ± 0.00	80 ± 0.00	8.00[a]
Control	6	6.80 ± 0.00	21.41 ± 0.04	0 ± 0.00	80 ± 0.00	7.70 ± 0.09
Brand A - PETE	6	6.20 ± 0.00	21.40 ± 0.07	25 ± 0.00	80 ± 0.00	8.40[a]
Lab Water - PETE	6	6.80 ± 0.00	21.43 ± 0.07	0 ± 0.00	80 ± 0.00	8.30[a]

Table 5. Mean ± standard deviation values for water characteristics measured for field experiment – dark environment **continued...**

Treatment:	Week:	pH	Temperature (°C)	Water Hardness (ppm as $CaCO_3$)	Alkalinity (ppm as $CaCO_3$)	Dissolved Oxygen (ppm)
Lab Water - Glass	6	6.80 ± 0.00	21.41 ± 0.06	0 ± 0.00	80 ± 0.00	8.00[a]
Lab Water – PC	6	6.80 ± 0.00	21.38 ± 0.07	0 ± 0.00	80 ± 0.00	8.20[a]
Control	11	6.80 ± 0.00	21.51 ± 1000	0 ± 0.00	80 ± 0.00	7.76 ± 0.06
Brand A – PETE	11	6.20 ± 0.00	21.52 ± 0.07	25 ± 0.00	80 ± 0.00	8.00[a]
Lab Water – PETE	11	6.80 ± 0.00	21.53 ± 0.07	0 ± 0.00	80 ± 0.00	7.90[a]
Lab Water – Glass	11	6.80 ± 0.00	21.51 ± 0.06	0 ± 0.00	80 ± 0.00	7.80[a]
Lab Water – PC	11	6.80 ± 0.00	21.51 ± 0.06	0 ± 0.00	80 ± 0.00	7.80[a]

[a] – Only one measurement was taken due to limited amount of sample

Table 6. Mean ± standard deviation values for water characteristics measured for chronic toxicity test.

Test Compound:	Chronic Test:	pH	Temperature (°C)	Water Hardness (ppm)	Alkalinity (ppm)	Dissolved Oxygen (ppm)
Lab Water	1	6.80 ± 0.00	26.0 ± 0.04	0.00 ± 0.00	80.0 ± 0.00	7.73 ± 0.07
DBP Stock	1	6.80 ± 0.00	26.0 ± 0.12	0.00 ± 0.00	80.0 ± 0.00	-
Lab Water	2	7.00 ± 0.00	26.1 ± 0.12	0.00 ± 0.00	80.0 ± 0.00	8.95 ± 0.26
DEHP Stock	2	7.00 ± 0.00	26.1 ± 0.11	0.00 ± 0.00	80.0 ± 0.00	-
Lab Water	3	6.98 ± 0.00	26.0 ± 0.04	0.00 ± 0.00	80.0 ± 0.00	8.88 ± 0.15
BPA Stock	3	6.98 ± 0.00	26.1 ± 0.07	0.00 ± 0.00	80.0 ± 0.00	-

5.3 – Field Experiment Results:

5.3.1 – Field Experiment Bioassays:

Figure 9 shows the Week 0 graph between the two treatments lab water and Brand. All seven time periods were graphed from 0 hours to 168 hours (seven days) and compared against the number of hydra (Figure 9). There were no statistical differences between the treatments at each of the time periods (Figure 9 & Appendix 3). Figure 10 shows a comparison between Brand A and Lab Water on the final day of the toxicity test (168 hours). Brand A contained more hydra at the end of the bioassay but it was not statistically significant from the lab water treatment (Figure 10). Brand A on average had 12 hydra in each Petri dish at 168 hours while Lab Water had close to 11 hydra in each Petri dish (Table 8). Figure 11 shows the final morphological scores of all hydra ranging from score 10 to score 1 at 168 hours. Both treatments at Week 0 did not cause harmful morphological effects to hydra (Figure 11).

The week 2 bioassay exposed the hydra to more treatments which included lab water as a control, Brand A, Lab Water – PETE, Lab Water – Glass, & Lab Water – PC. The hydra were also exposed to two environmental treatments involving storage of bottled water in sunlight and darkness. Green hydra were exposed to samples for seven days. There were no significant differences in hydra numbers between replicates (Appendix 4) No significant differences were seen between the sunlight and darkness environment in treatments after 2 weeks of storage, when comparing the hydra numbers (Table 8 & Appendix 4). At 120 hours the Control and Lab Water-PC were significantly different from each other when looking at the Week 2 – sunlight

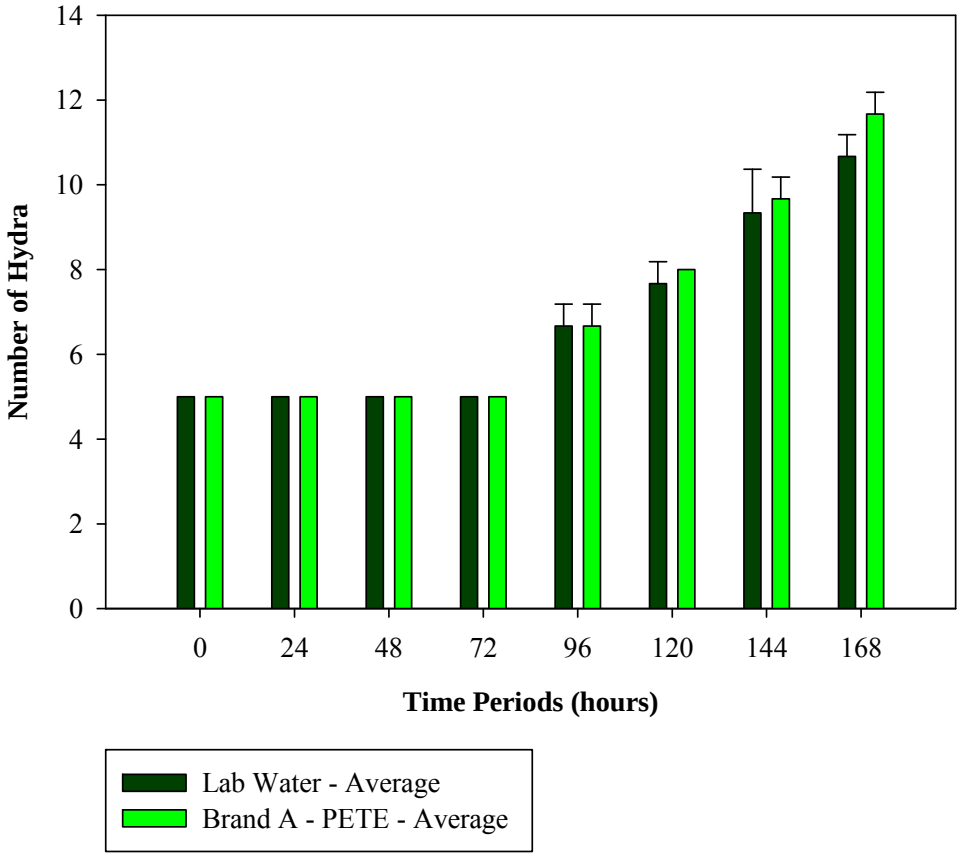


Figure 9. Effect of Brand A and Lab Water on the number of green hydra at week 0 Values are displayed as mean number of hydra ± standard deviation. If no superscripts were indicated at specific time periods there were no significant differences.

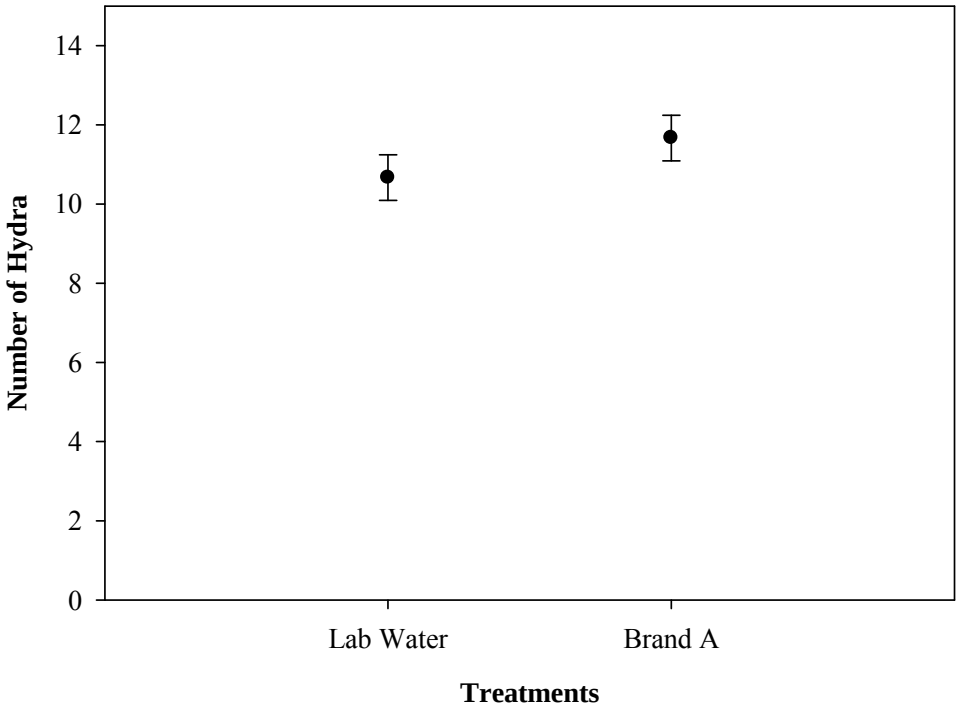


Figure 10. Effect of Brand A and Lab Water on the number of green hydra at week 0 on the final day (168 Hours). Values are displayed as mean number of hydra ± standard deviation. If no superscripts were indicated at specific treatments there were no significant differences.

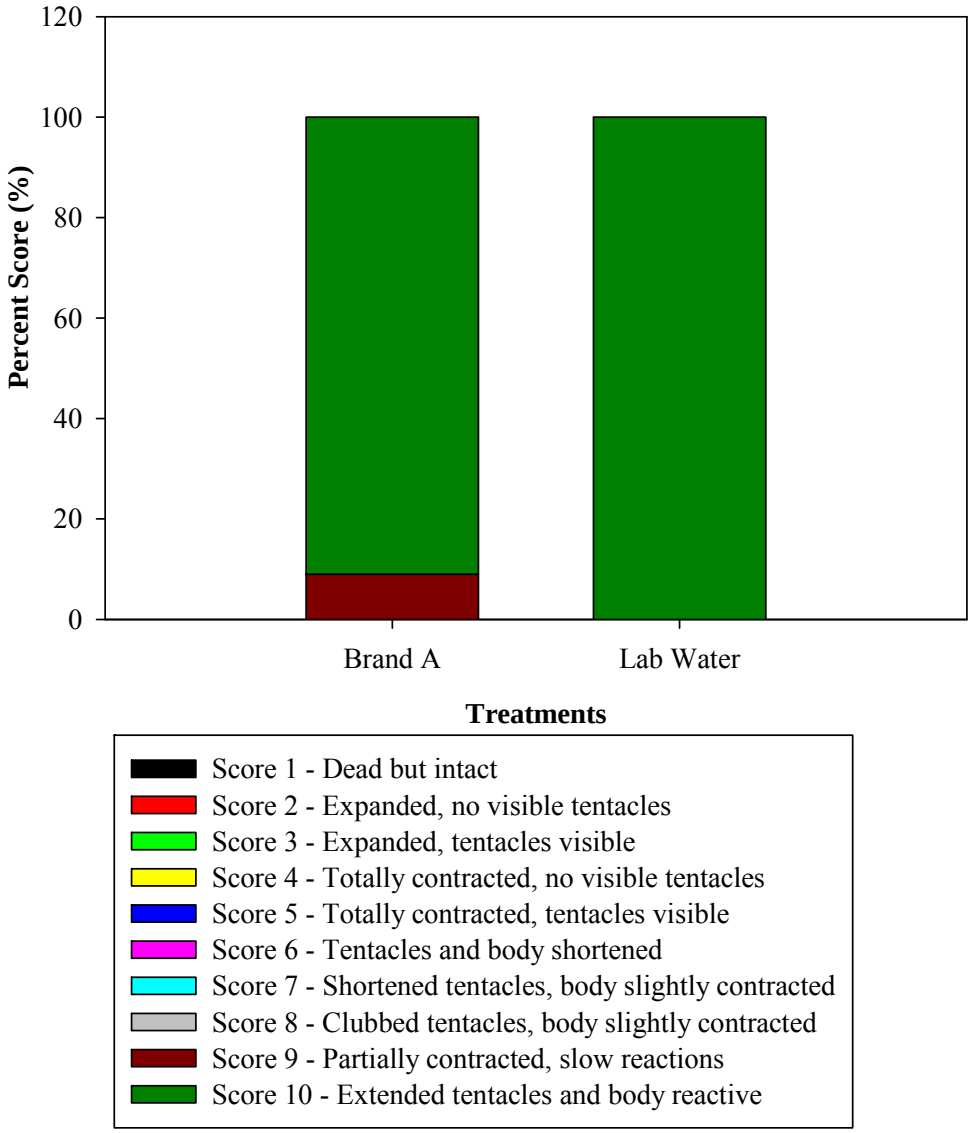


Figure 11. Effect of Brand A & Lab Water on the morphology of green hydra at week 0 at 168 hours.

environment graph (Figure 12 & Appendix 4). At 168 hours the control was significantly different from three treatments which included Lab Water-PETE, Lab Water-Glass & Lab Water-PC (Figure 12, 14 & Appendix 4). Lab Water-Glass & Lab Water-PC affected hydra morphology compared to the other treatments (Figure 16). The polycarbonate treatment resulted in 35% of the hydra being at a score of 6 and the glass resulted in 6% of the hydra being at a score of 6 on the final day of the bioassay (Figure 16).

The Week 2 – Darkness graph showed significant differences from treatments Lab Water-Glass & Lab Water PC from the control at 120 hours and 144 hours (Figure 13 & Appendix 4). At 168 hours the treatments Lab Water-Glass & Lab Water-PC were significantly different from the control (Figure 13, Figure 15 & Appendix 4). Also the Lab Water-Glass & Lab Water-PETE were significantly different from each other at 168 hours in the darkness environment (Figure 13, Figure 15 & Appendix 4). The percentage impact on green hydra morphology after a 7 day continuous exposure to all treatments, showed the Lab Water-Glass as having the most effect, with 11% of the green hydra being at a Score of 6 (Figure 17). For Week 2 no scores fell below 6. A score of 6 indicates shortened tentacles and body.

The Week 4 bioassay had no significance between replicates and no significance between the two environments, sunlight and darkness (Appendix 5). For both the sunlight and darkness environment there were no differences between treatments for any of the time periods (Appendix 5). The overall pattern that can be observed when looking at the

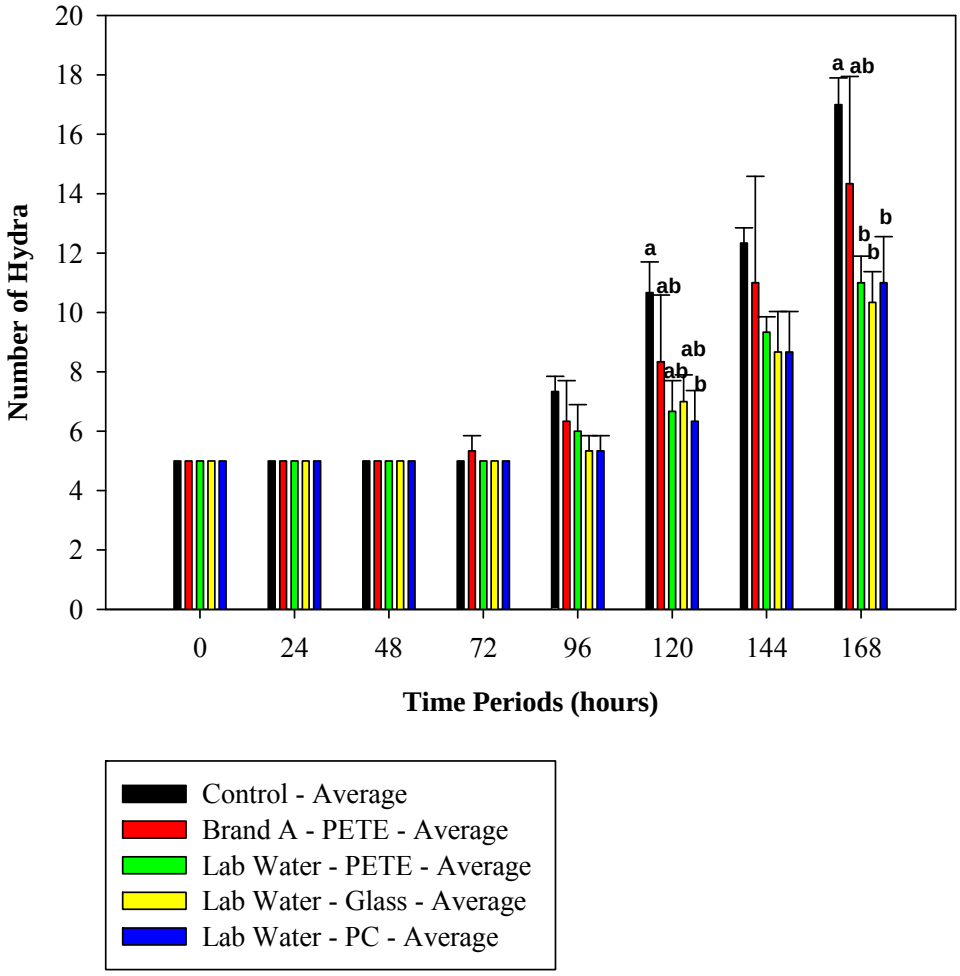


Figure 12. Effect of various treatments on the number of green hydra at week 2 in the sunlight environment. Values are displayed as mean number of hydra ± standard deviation. Number of hydra (within time periods) with alphabetical superscripts in common were not significantly different from each other. If no superscripts were indicated at specific time periods there were no significant differences.

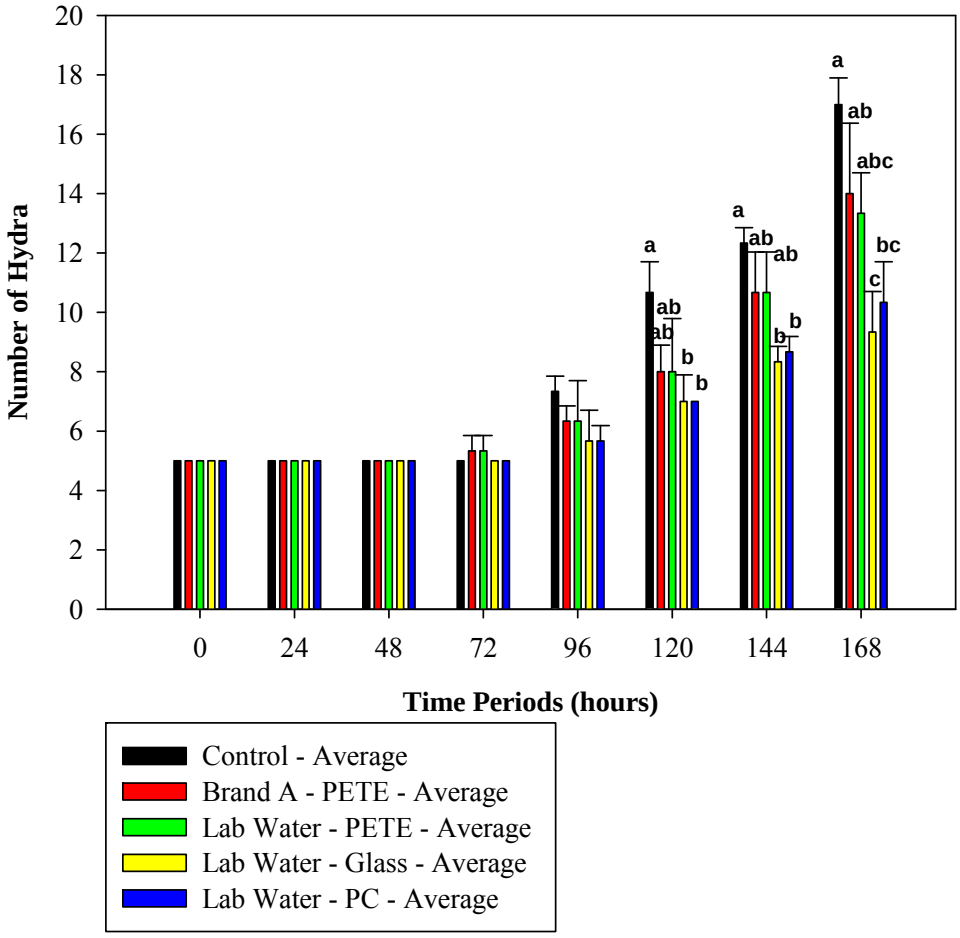


Figure 13. Effect of various treatments on the number of green hydra at week 2 in the dark environment. Values are displayed as mean number of hydra ± standard deviation. Number of hydra (within time periods) with alphabetical superscripts in common were not significantly different from each other. If no superscripts were indicated at specific time periods there were no significant differences.

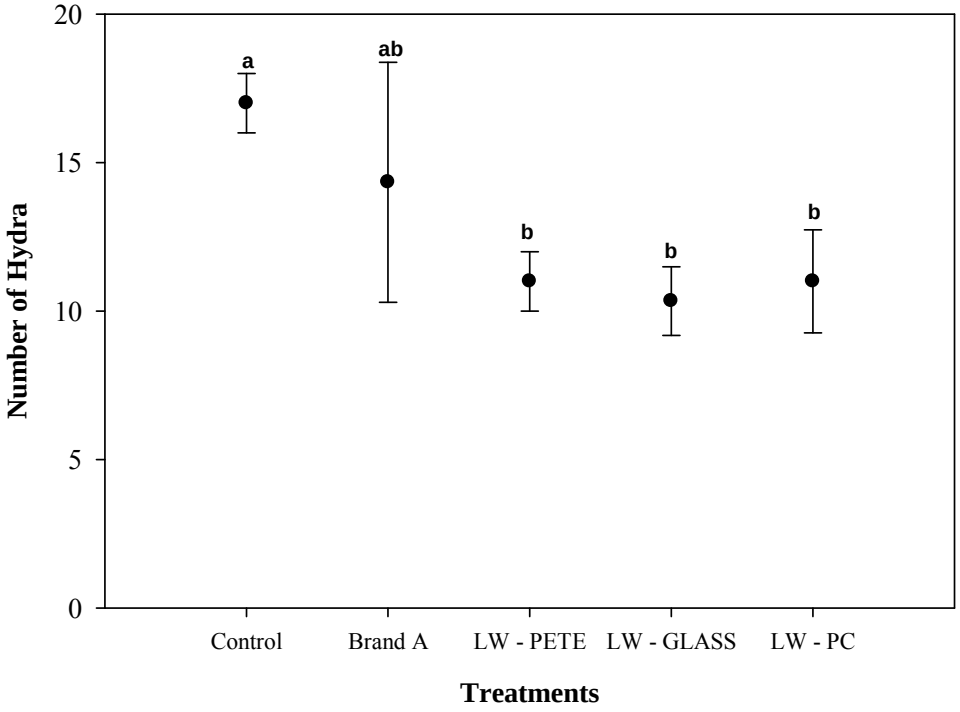


Figure 14. Effect of various treatments on the number of green hydra at week 2 in the sunlight environment on the final day (168 hours). Values are displayed as mean number of hydra ± standard deviation. Number of hydra with alphabetical superscripts in common were not significantly different from each other.

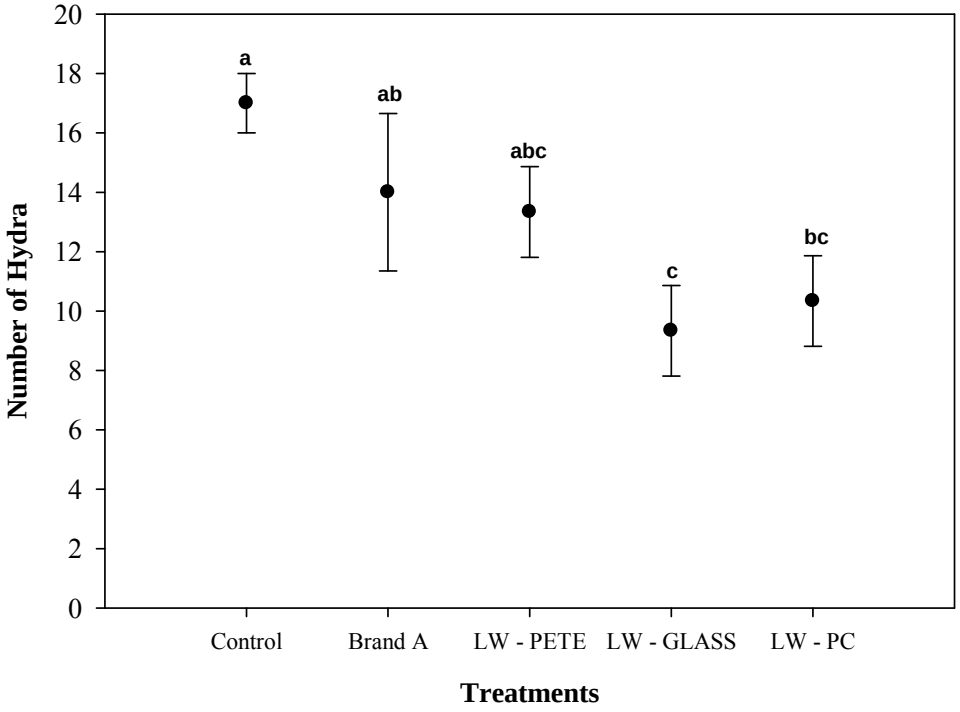


Figure 15. Effect of various treatments on the number of green hydra at week 2 in the darkness environment on the final day (168 hours). Values are displayed as mean number of hydra ± standard deviation. Number of hydra with alphabetical superscripts in common were not significantly different from each other.

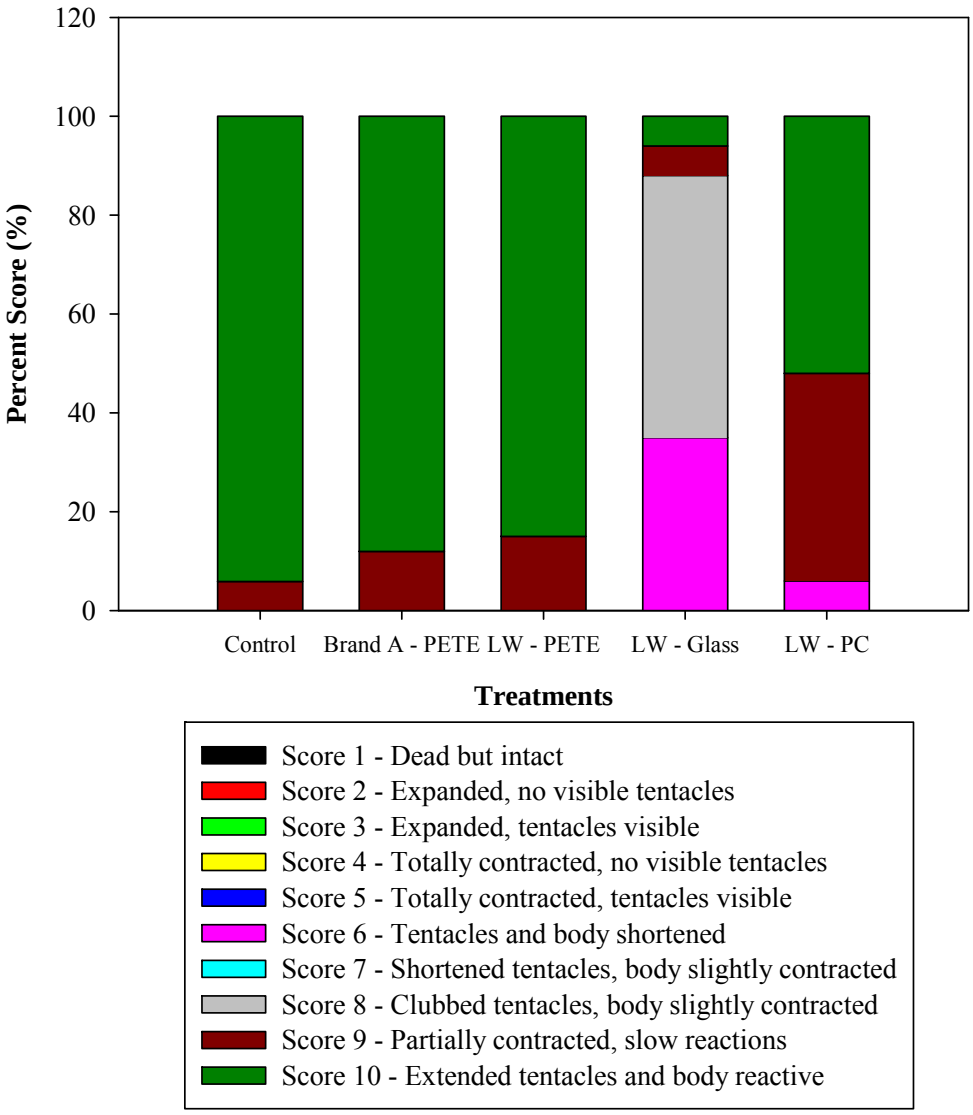


Figure 16. Effect of various treatments on the morphology of green hydra at week 2 at 168 hours in the sunlight environment.

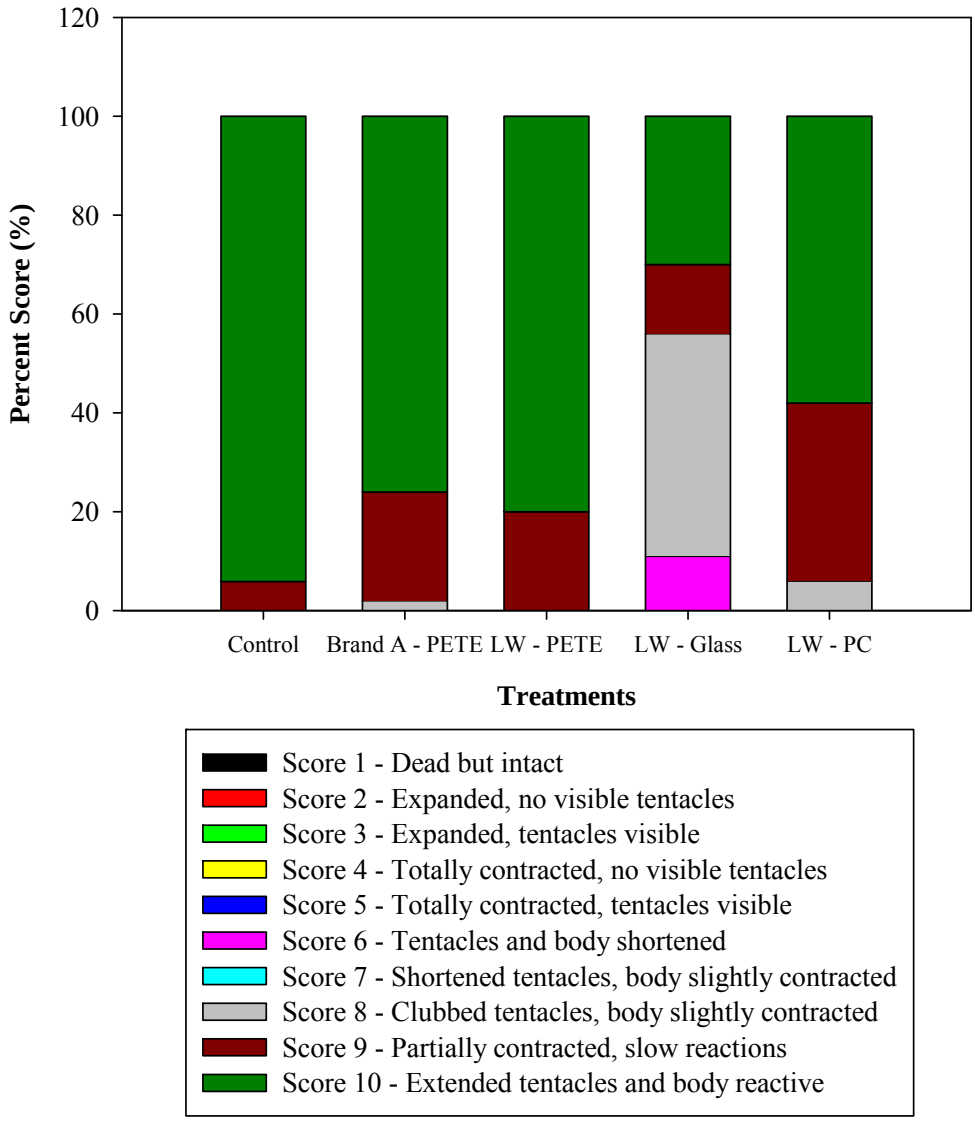


Figure 17. Effect of various treatments on the morphology of green hydra at week 2 at 168 hours in the darkness environment.

sunlight graph for Week 4 shows the lab water control as having the highest number of hydra at the end of the bioassay when compared with the other treatments (Figure 18 & Figure 19). Brand A and the polycarbonate (PC) treatment had the least amount of hydra on average at 168 hours with an average of 14 to 15 hydra per Petri dish for both environments (Figure 20 & Table 8). The sunlight environment at Week 4 had an effect on the morphology of the green hydra (Figure 22). The glass and polycarbonate treatments posed the highest threat to the green hydra. The glass treatment had 4% of the hydra at a score of 6, while the polycarbonate had about 14% of the hydra at a Score 5 (Figure 22).

The Week 4 – darkness graph consisted of a similar pattern as the sunlight environment. At 168 hours the control had the highest number of hydra with an average of 18 hydra in each Petri dish (Figure 19 & Table 8). The Brand A & Polycarbonate treatments had the least amount of hydra with an average of 15 hydra per Petri dish (Figure 21 & Table 8). Three treatments that had an effect on the morphology of the green hydra included Lab Water-PETE, Glass & Polycarbonate (Figure 23). Glass caused 4% of the hydra population to be at a score of 5 on the final day of the bioassay (Figure 23). The Lab Water-PETE & Polycarbonate treatments had approximately 7 to 8% of the hydra being at score 6 (Figure 23). The opposite effects were seen for the control treatment with majority of the hydra being at a Score of 10 (Figure 23).

No significant differences were found between replicates and between the two environments for Week 6 (Appendix 6). Significant difference between treatments for the

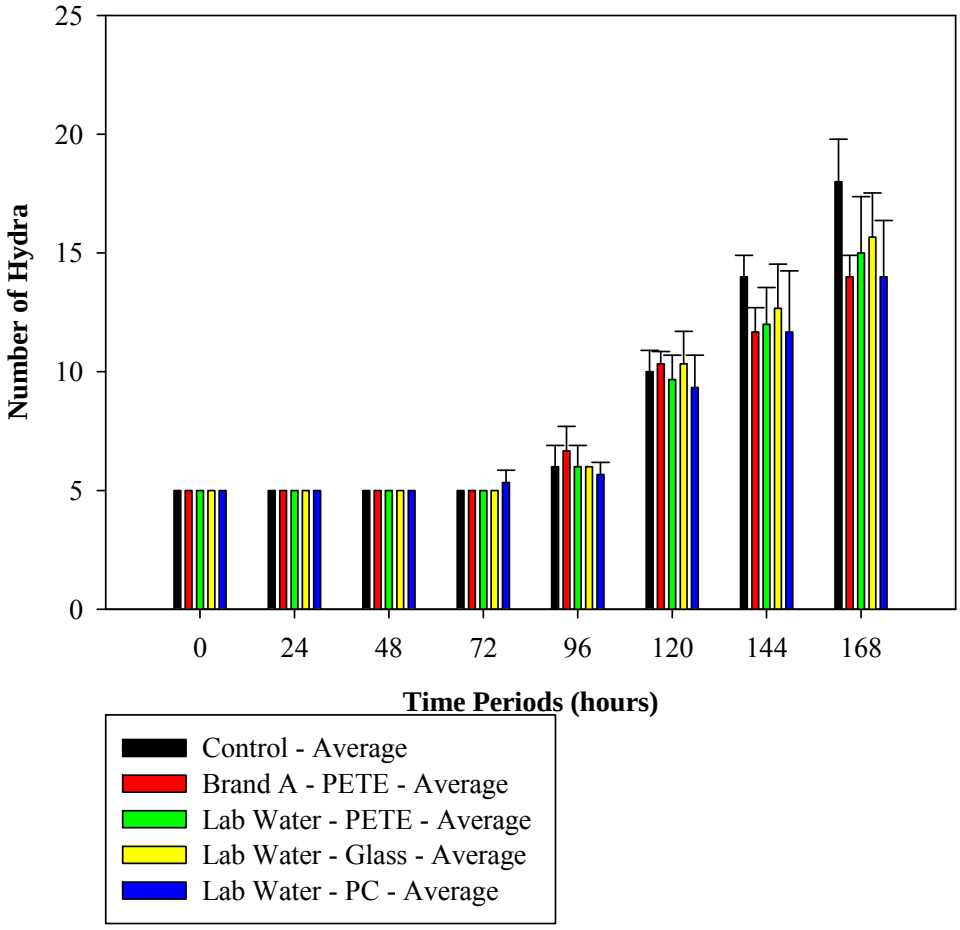


Figure 18. Effect of various treatments on the number of green hydra at week 4 in the sunlight environment. Values are displayed as mean number of hydra ± standard deviation. If no superscripts were indicated at specific time periods there were no significant differences.

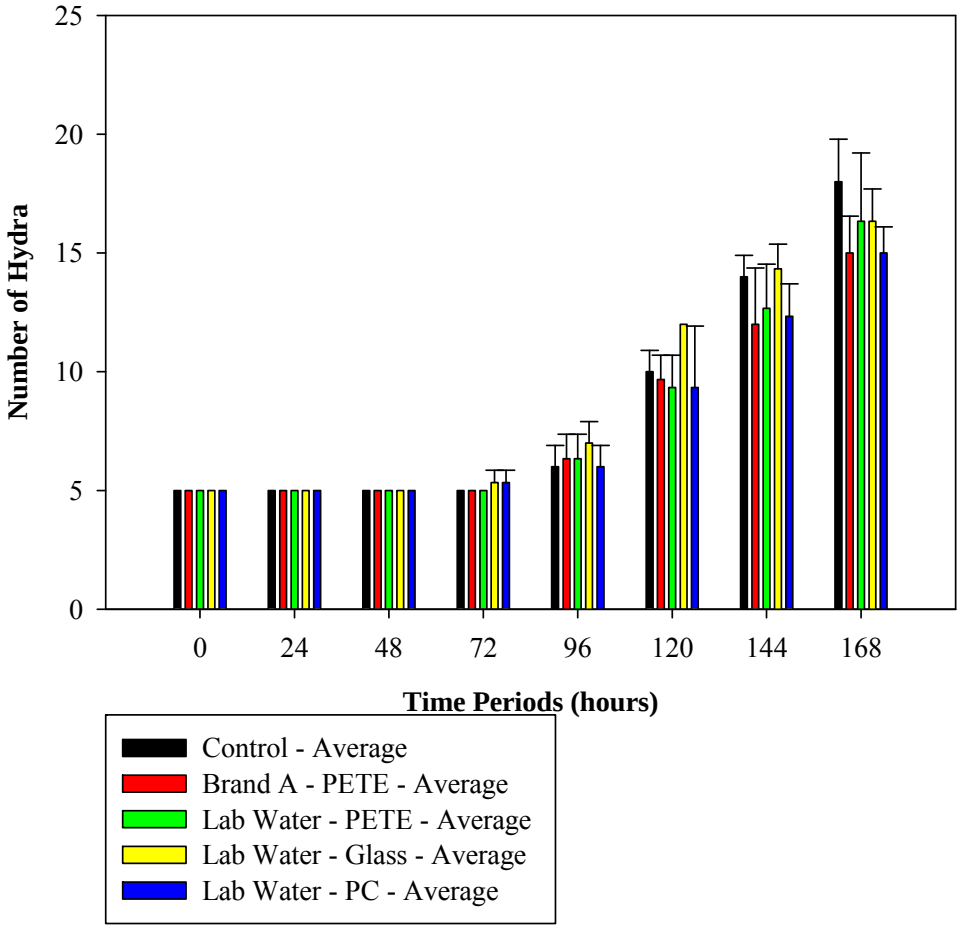


Figure 19. Effect of various treatments on the number of green hydra at week 4 in the darkness environment. Values are displayed as mean number of hydra ± standard deviation. If no superscripts were indicated at specific time periods there were no significant differences.

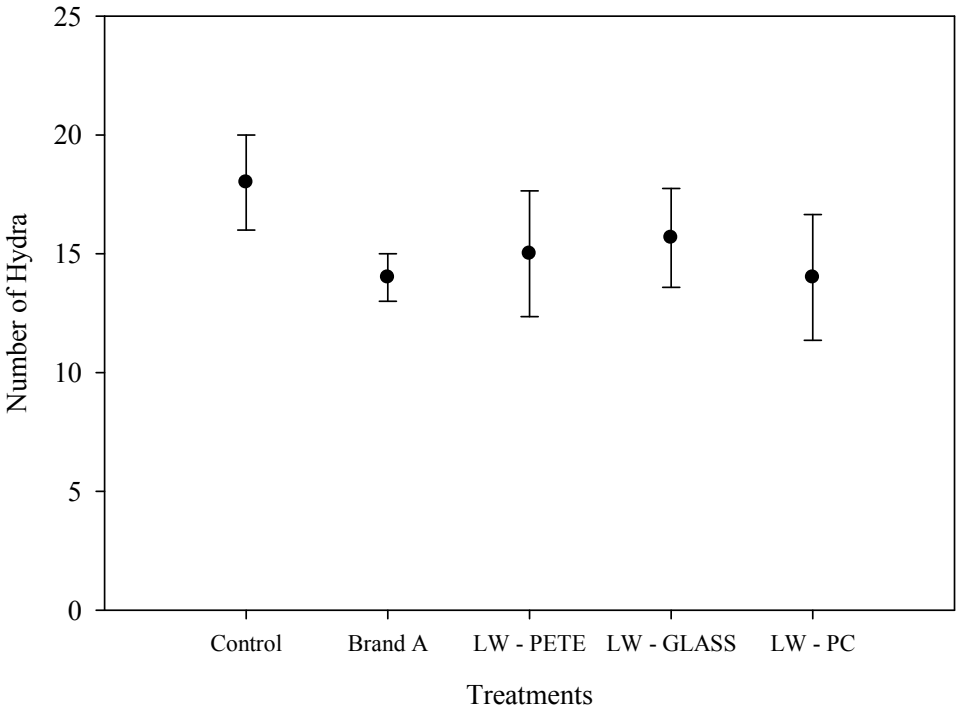


Figure 20. Effect of various treatments on the number of green hydra at week 4 in the sunlight environment on the final day (168 hours). Values are displayed as mean number of hydra ± standard deviation.

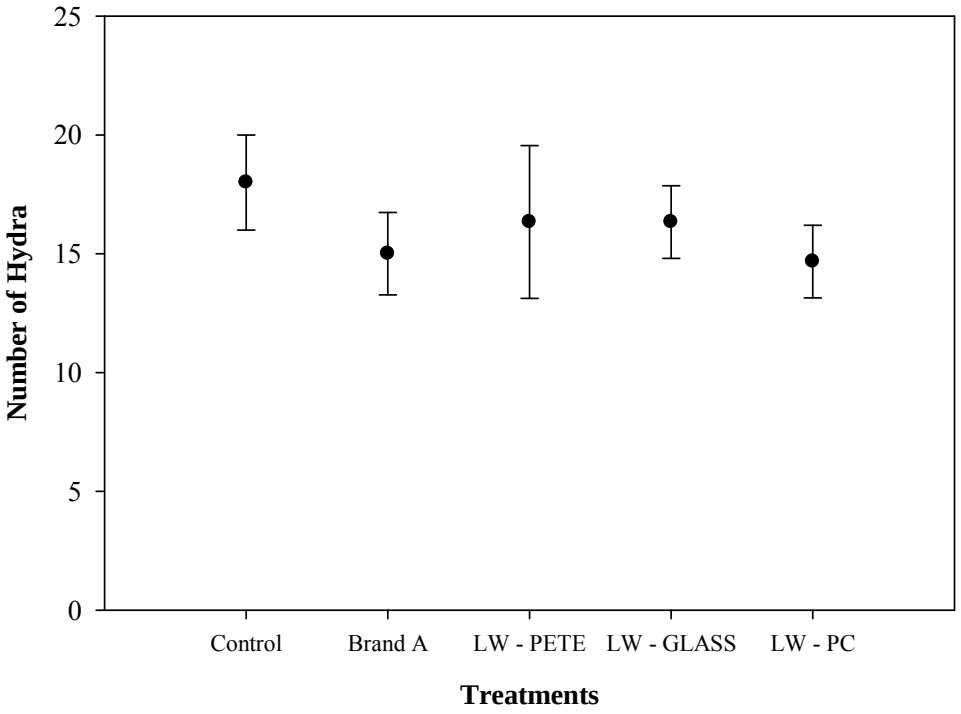


Figure 21. Effect of various treatments on the number of green hydra at week 4 in the darkness environment on the final day (168 hours). Values are displayed as mean number of hydra ± standard deviation.

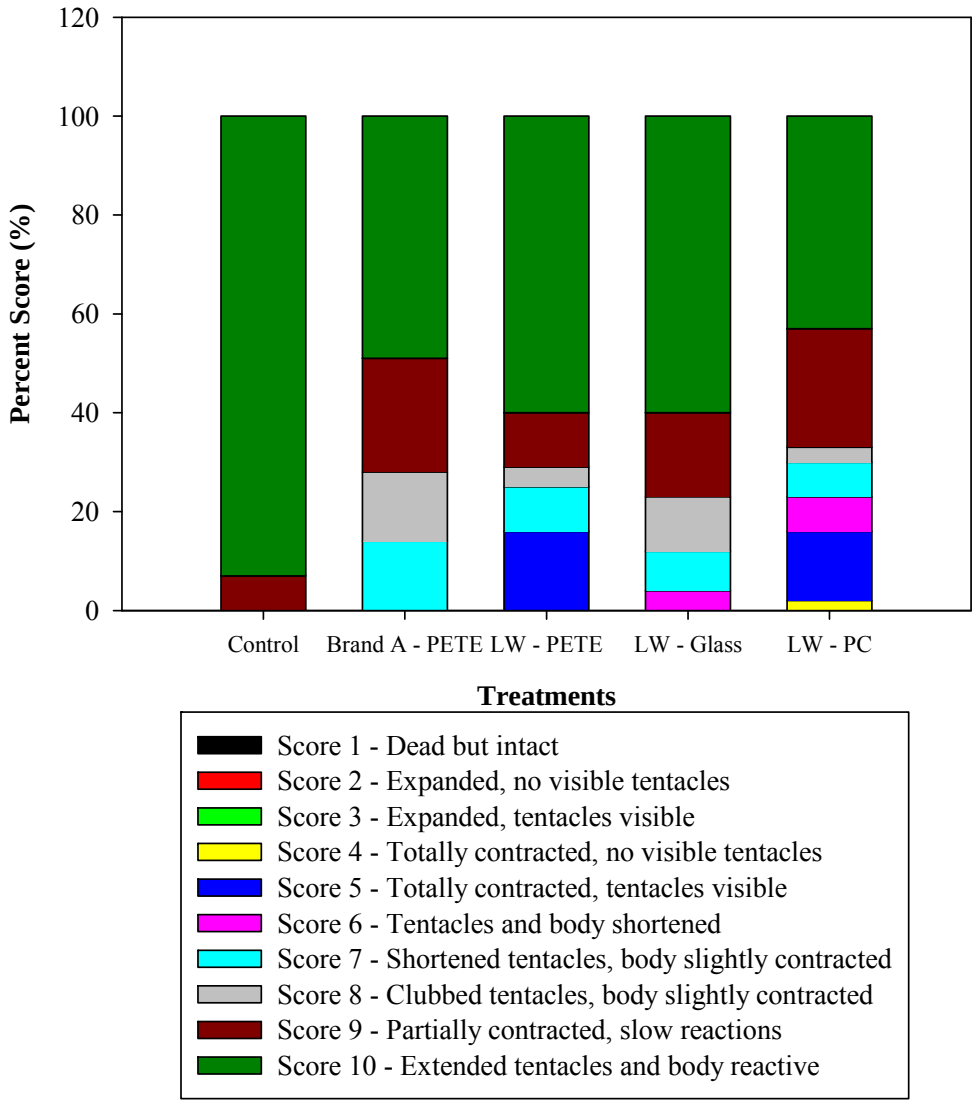


Figure 22. Effect of various treatments on the morphology of green hydra at week 4 at 168 hours in the sunlight environment

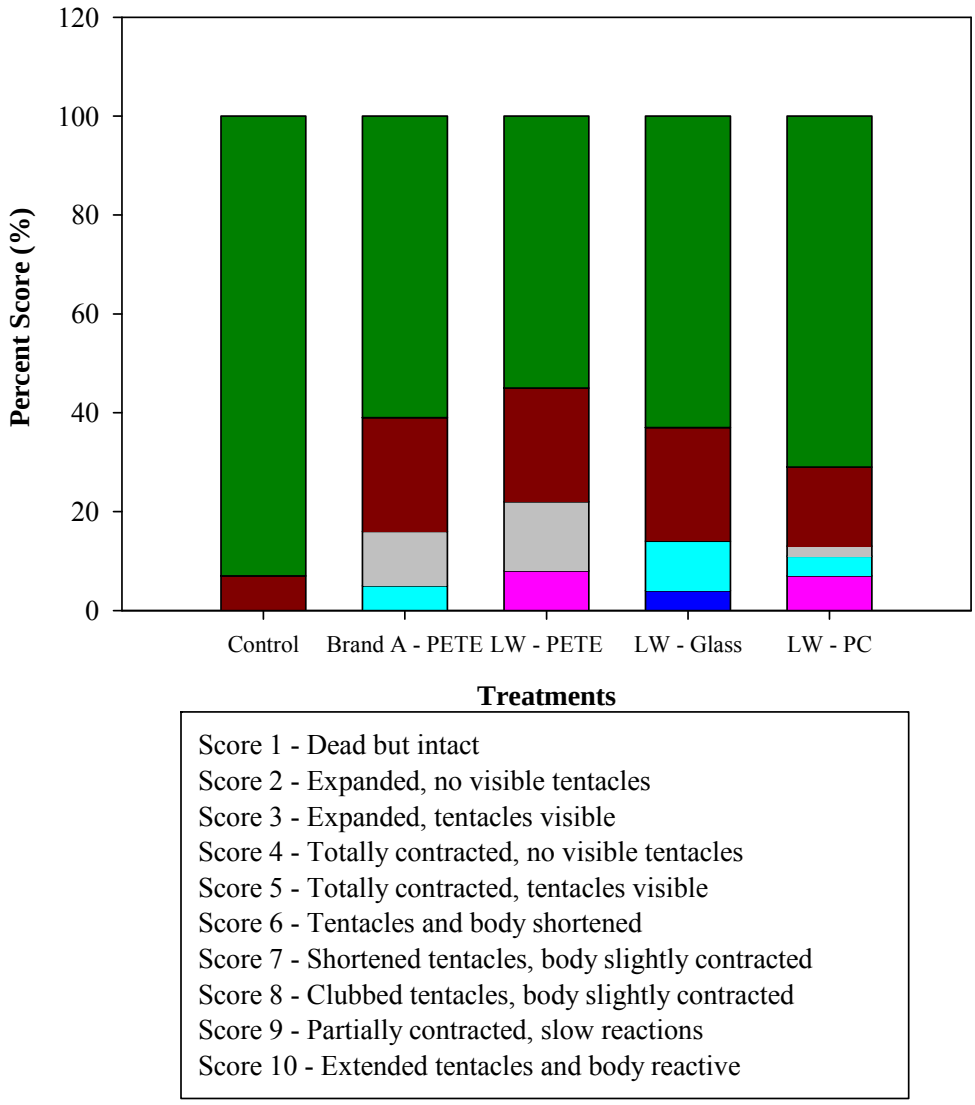


Figure 23. Effect of various treatments on the morphology of green hydra at week 4 at 168 hours in the darkness environment.

sunlight environment was seen at 120 hours (Figure 24 & Appendix 6). The Lab Water-PETE treatment statistically showed significant difference between the Brand A & Polycarbonate treatments (Figure 24 & Appendix 6). At 144 hours the control was significantly different from Brand A and the polycarbonate treatments (Figure 24 & Appendix 6). On the final day of the bioassay the control was significantly different from all treatments and Brand A was significantly different from the glass treatment (Figure 26 & Appendix 6). At the end of the bioassay the number of hydra in the control treatment was much higher than the other treatments (Table 8). On average there were 19 hydra per Petri dish in the control treatment (Table 8). The lowest amount of hydra were found in the Brand A and polycarbonate treatments with a range of 8 to 10 hydra per Petri dish (Table 8). Morphological effects on the hydra included about 5 to 14% of green hydra being at a score of 8 for the treatments Brand A, Lab Water-PETE & Glass (Figure 28). No scores below 8 were observed for the sunlight environment at 168 hours (Figure 28). The polycarbonate treatment had 72% of the hydra at a score of 10 and the remaining percent at a score of 9. The control had on average 96% of the hydra at a score of 10 (Figure 28).

The Week 6 - dark environment results exhibited a similar pattern to the sunlight environment. At 144 hours the control treatment was significant from the glass treatment (Figure 25 & Appendix 6). On the final day of the bioassay at 168 hours the control treatment was significantly different from the Brand A and glass treatment (Figure 25, Figure 27 & Appendix 6). The smallest amount of hydra were found in the Brand A and Glass treatments on average at 168 hours (Table 8). An average of 9 hydra per Petri dish

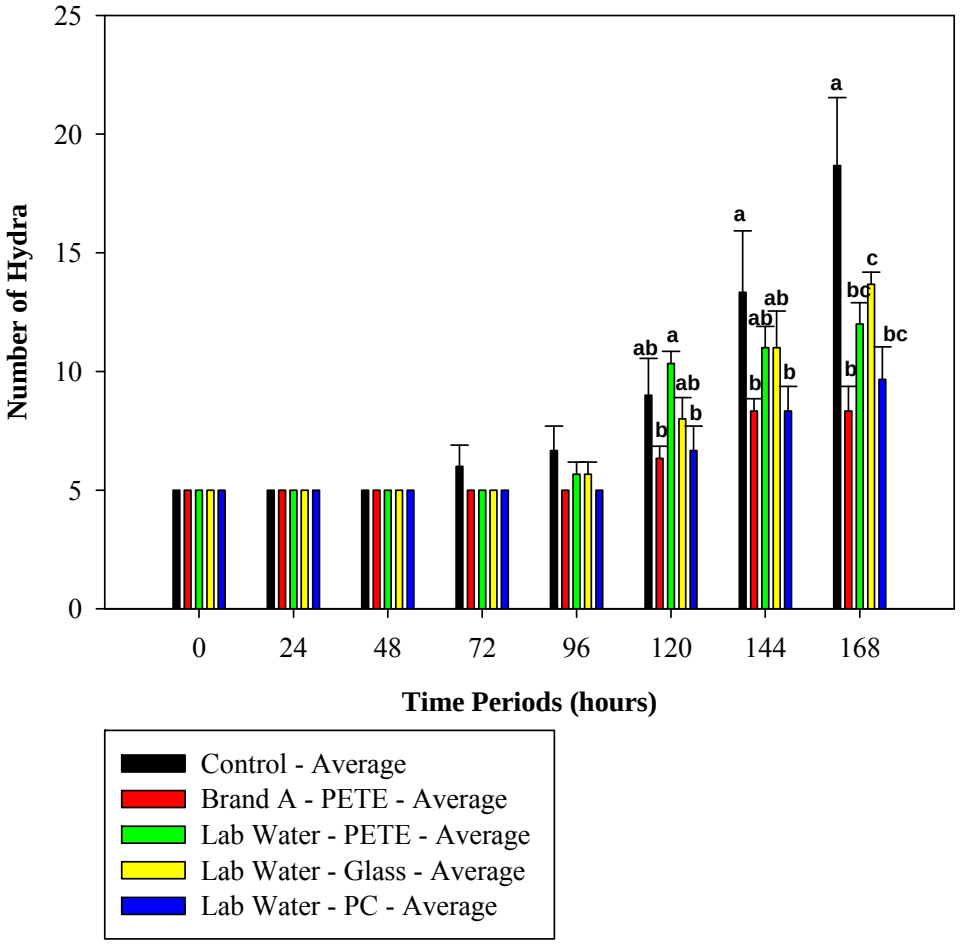


Figure 24. Effect of various treatments on the number of green hydra at week 6 in the sunlight environment. Values are displayed as mean number of hydra ± standard deviation. Number of hydra (within time periods) with alphabetical superscripts in common were not significantly different from each other. If no superscripts were indicated at specific time periods there were no significant differences.

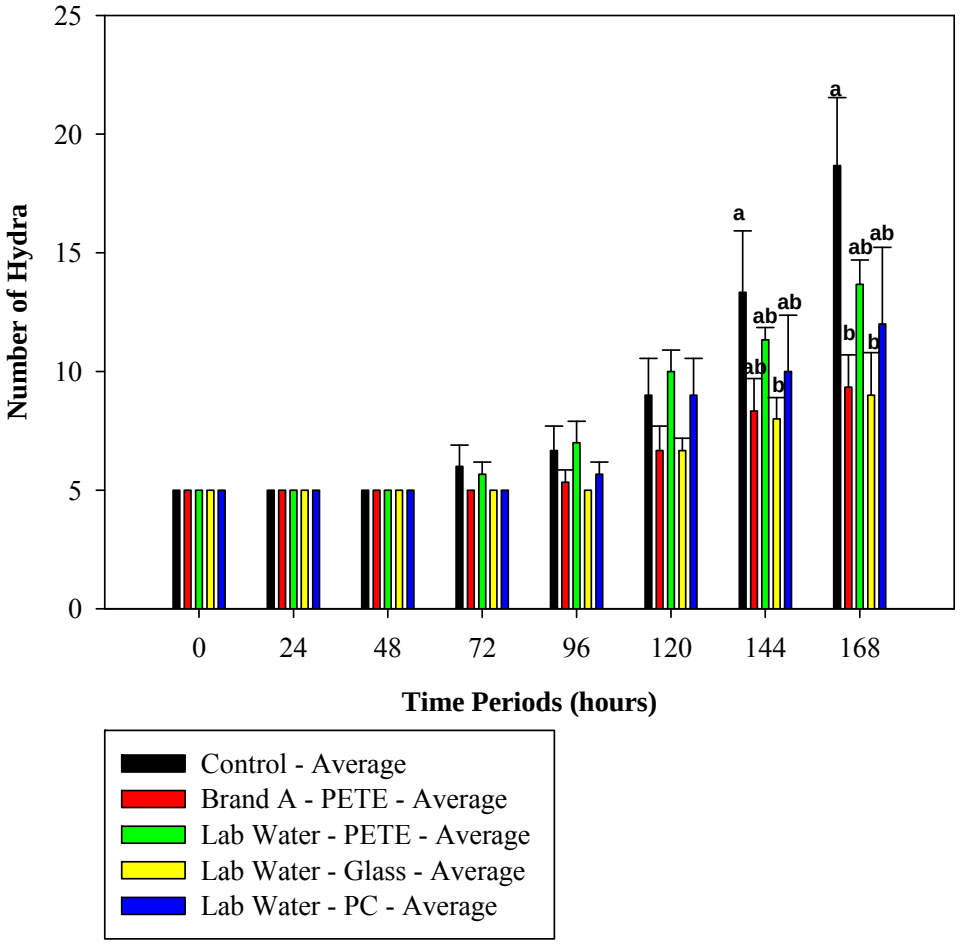


Figure 25. Effect of various treatments on the number of green hydra at week 6 in the darkness environment. Values are displayed as mean number of hydra ± standard deviation. Number of hydra (within time periods) with alphabetical superscripts in common were not significantly different from each other. If no superscripts were indicated at specific time periods there were no significant differences.

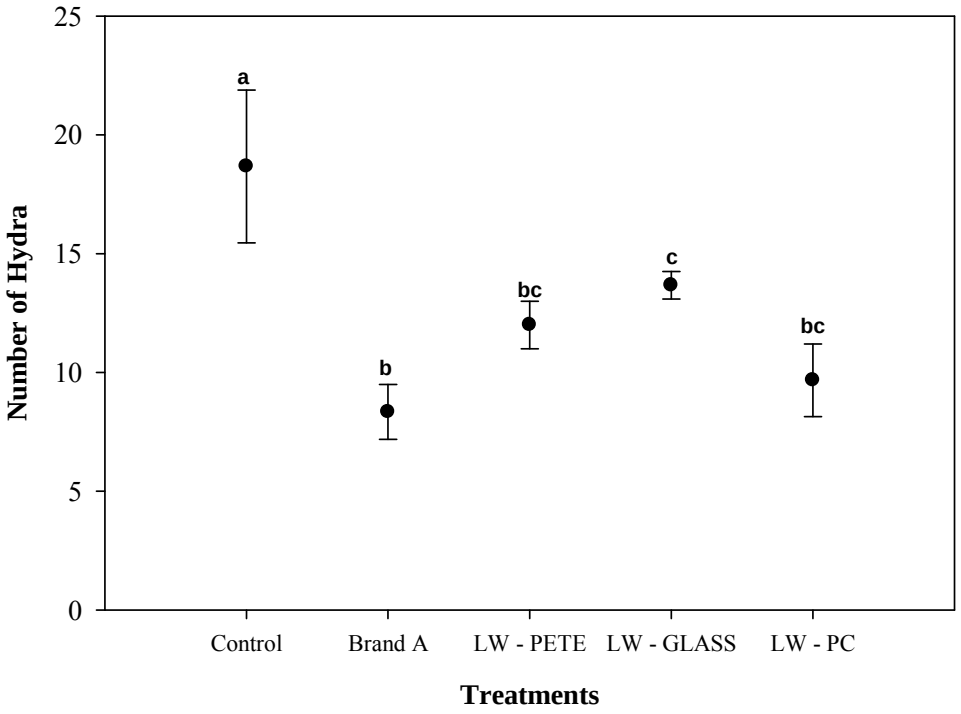


Figure 26. Effect of various treatments on the number of green hydra at week 6 in the sunlight environment on the final day (168 hours). Values are displayed as mean number of hydra ± standard deviation. Number of hydra with alphabetical superscripts in common were not significantly different from each other.

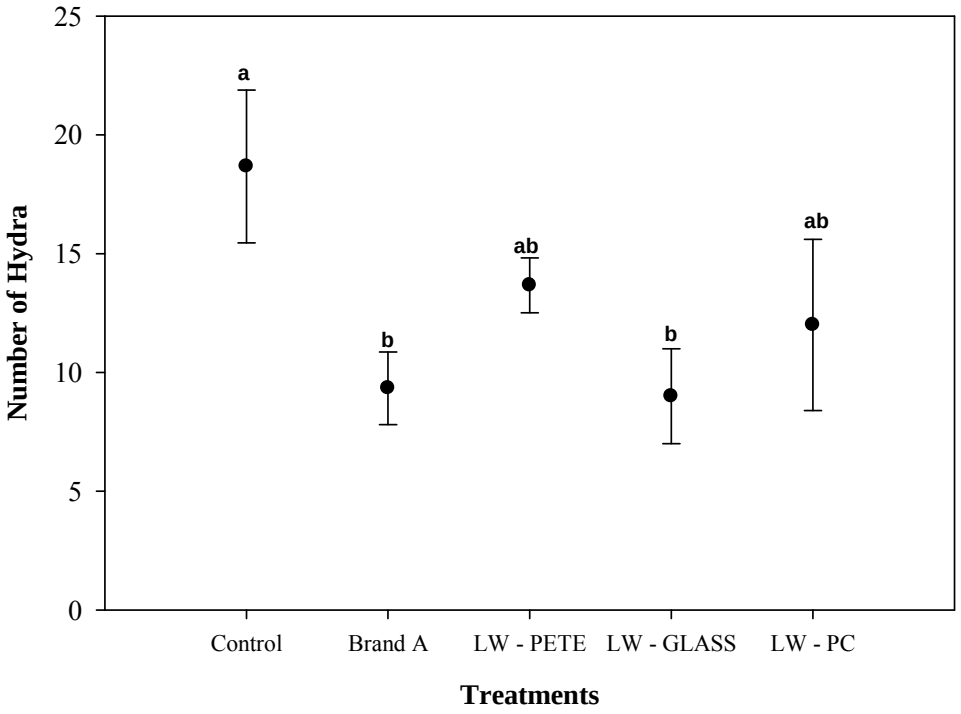


Figure 27. Effect of various treatments on the number of green hydra at week 6 in the darkness environment on the final day (168 hours). Values are displayed as mean number of hydra ± standard deviation. Number of hydra with alphabetical superscripts in common were not significantly different from each other.

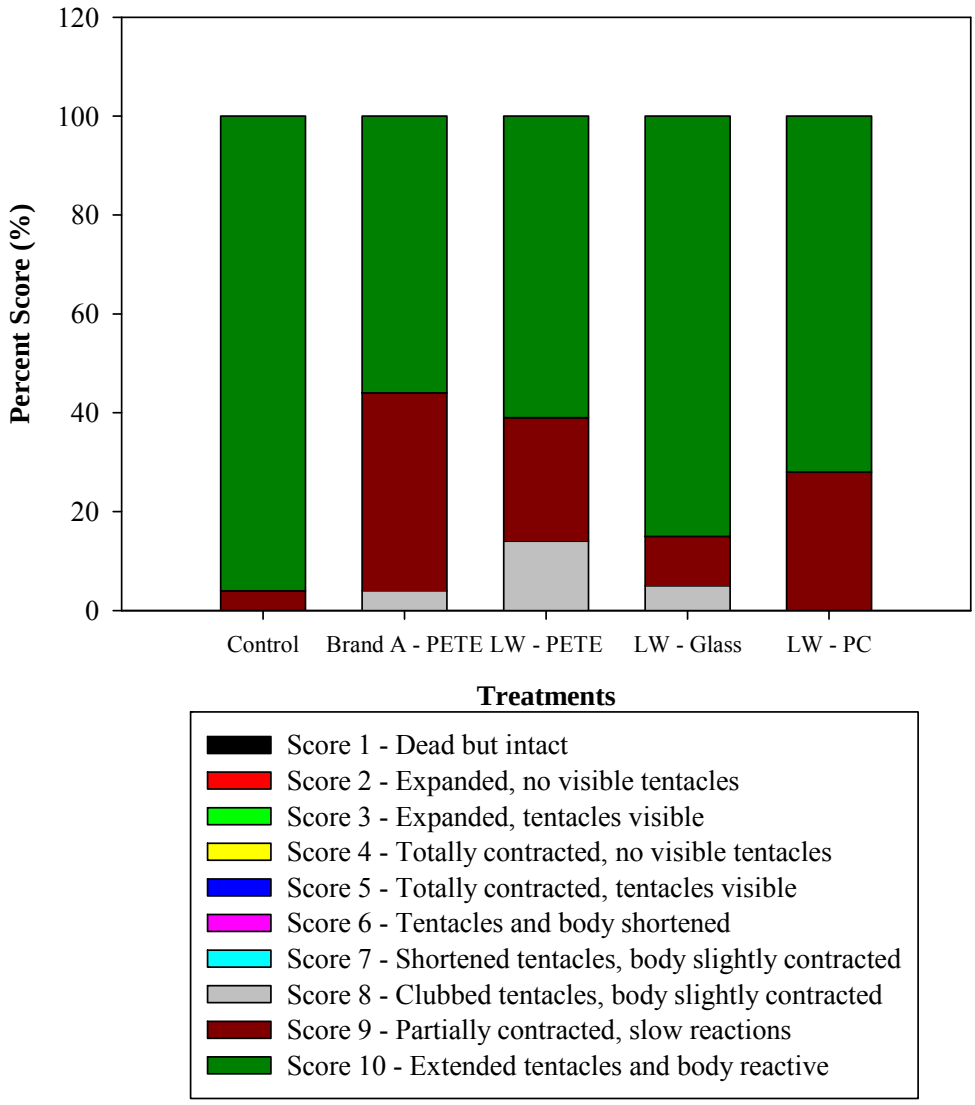


Figure 28. Effect of various treatments on the morphology of green hydra at week 6 at 168 hours in the sunlight environment.

were found in treatments Brand A & Glass, in comparison with 12 to 13 hydra found in the Lab Water-PETE and Glass treatments (Table 8). The control had an average of 19 hydra per Petri dish (Table 8). No major morphological changes were observed on hydra as compared to the previous weeks. The treatments Brand A and Glass had an average of 19 to 39% of the hydra being at a Score of 8 (Figure 29). The control, Lab Water-PETE and Polycarbonate treatments had scores of 9 between the ranges of 4 to 33% of the green hydra population (Figure 29).

The final sampling period of the field experiment was Week 11. The Week 11 analysis showed no difference between replicates and between the two environments (Appendix 7). Significant differences were only found at 168 hours for the sunlight environment (Appendix 7). The control showed significant differences between all treatments which included Lab Water-PETE, Brand A, Glass & Polycarbonate treatments (Figure 30, Figure 32 & Appendix 7). The control's mean number of hydra per Petri dish at 168 hours was 17 hydra which was significantly higher than the other treatments (Table 8 & Appendix 7). The other treatments had a range of 9 to 11 hydra per Petri dish at 168 hours (Table 8). The morphological changes at 168 hours included the Lab Water-PETE, Brand A and Glass treatments of having 4 to 14% of the green hydra at a score of 8 (Figure 34). No scores fell below 8 at 168 hours for any of the treatments. The control had 96% of the hydra at a score of 10 while the polycarbonate had 72% at score 9 (Figure 34).

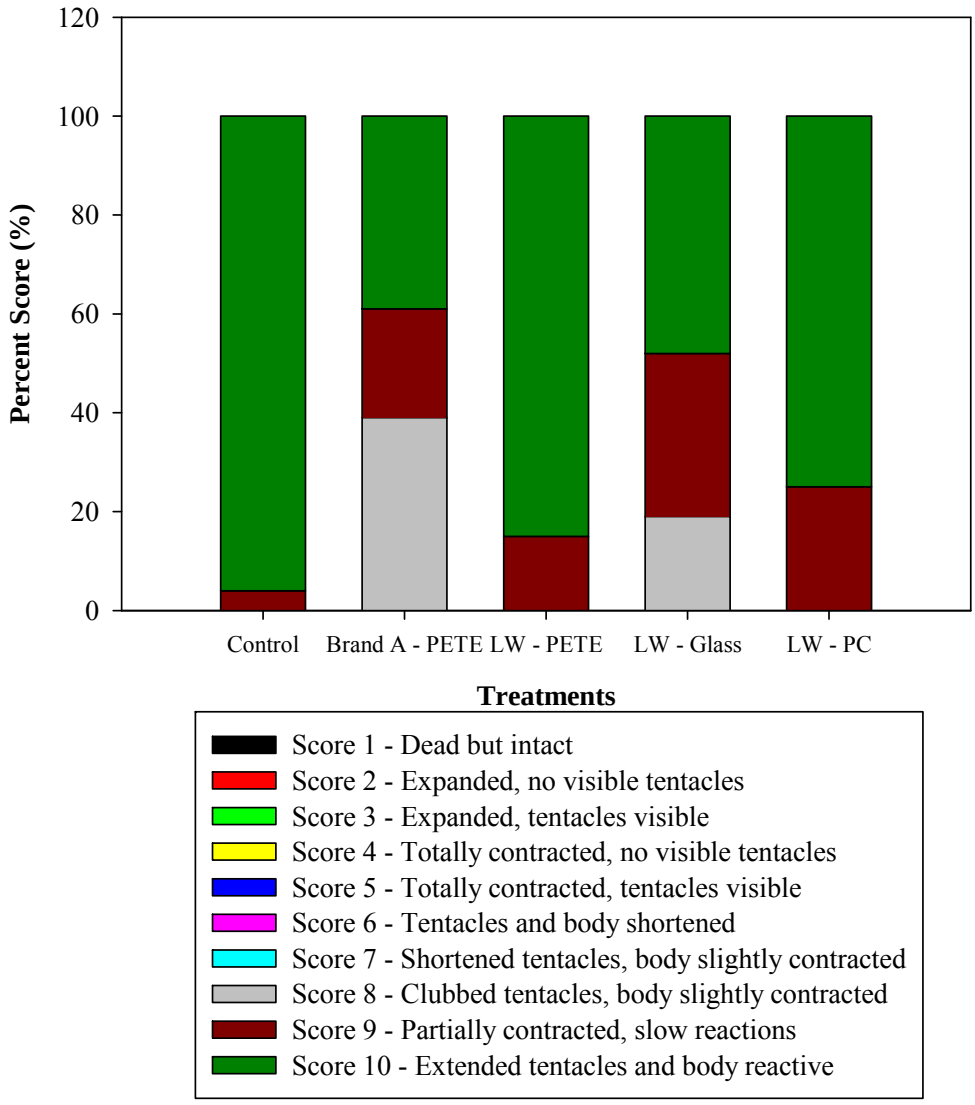


Figure 29. Effect of various treatments on the morphology of green hydra at week 6 at 168 hours in the darkness environment.

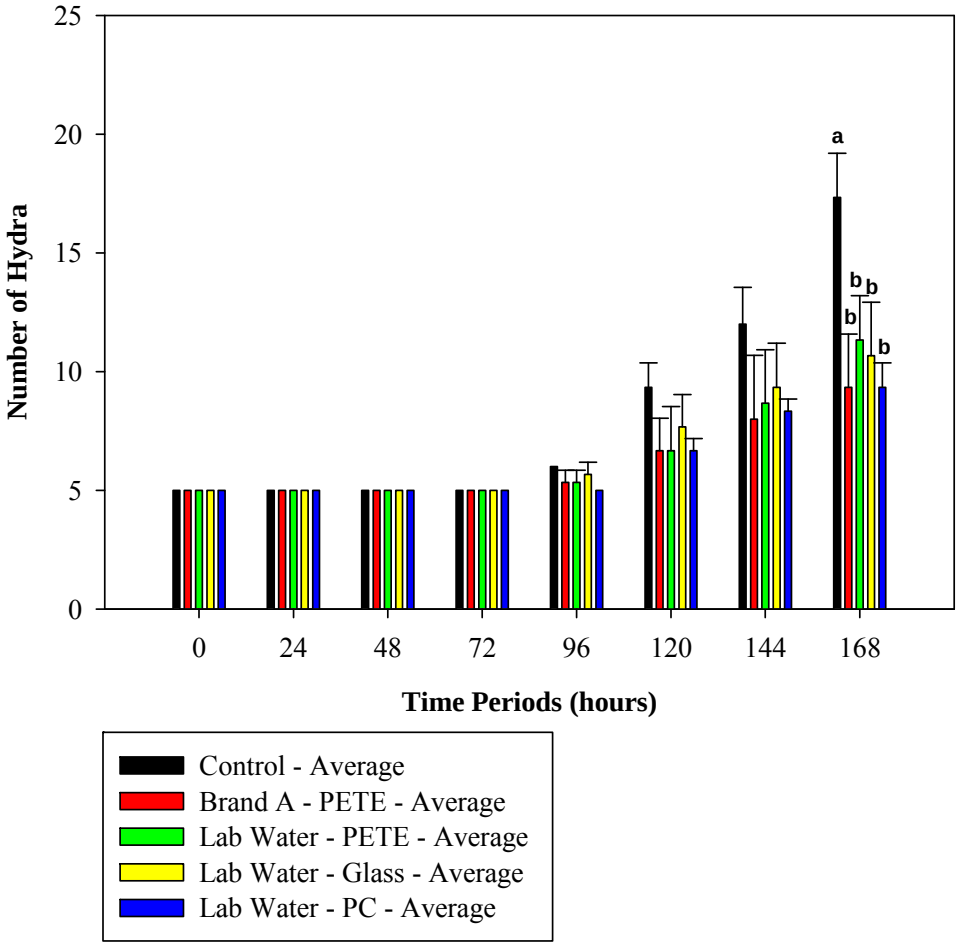


Figure 30. Effect of various treatments on the number of green hydra at week 11 in the sunlight environment. Values are displayed as mean number of hydra ± standard deviation. Number of hydra (within time periods) with alphabetical superscripts in common were not significantly different from each other. If no superscripts were indicated at specific time periods there were no significant differences.

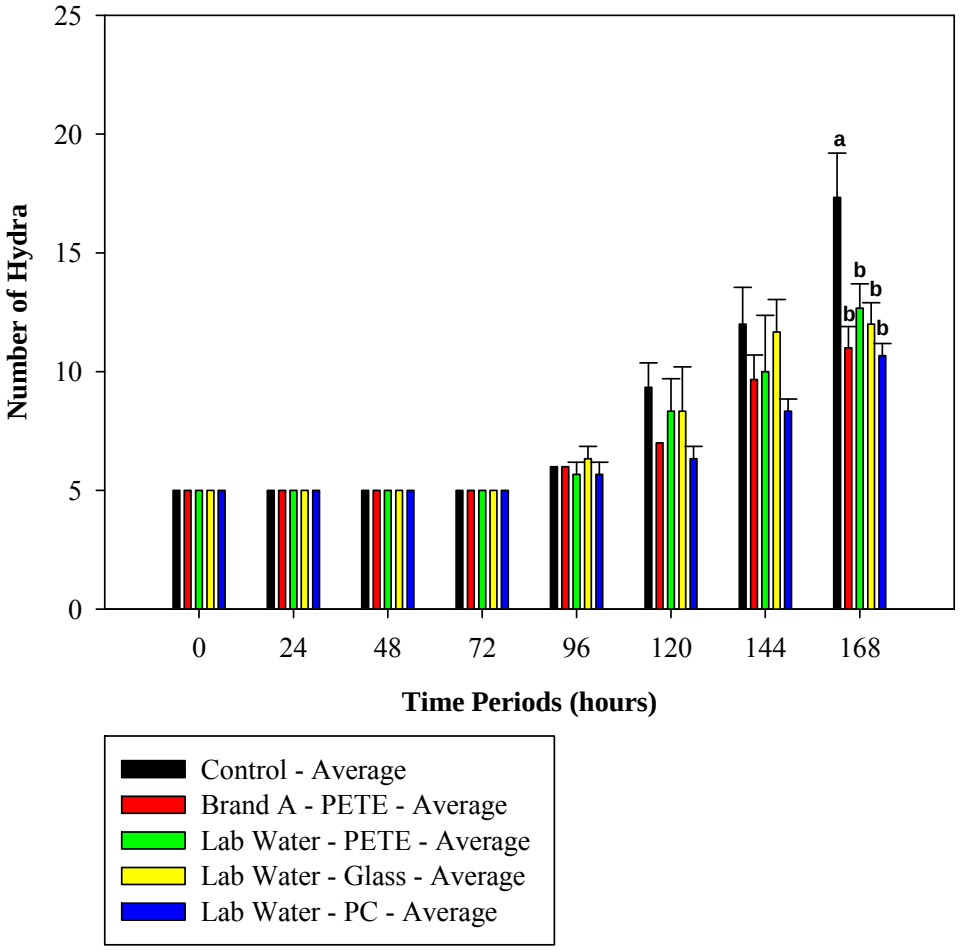


Figure 31. Effect of various treatments on the number of green hydra at week 11 in the darkness environment. Values are displayed as mean number of hydra ± standard deviation. Number of hydra (within time periods) with alphabetical superscripts in common were not significantly different from each other. If no superscripts were indicated at specific time periods there were no significant differences.

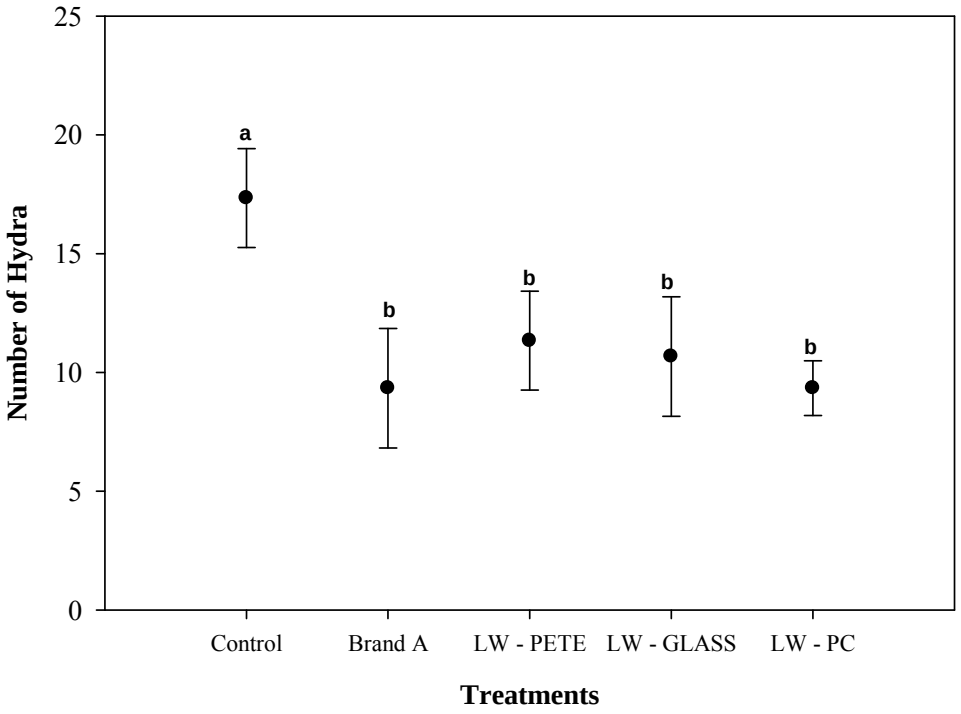


Figure 32. Effect of various treatments on the number of green hydra at week 11 in the sunlight environment on the final day (168 hours). Values are displayed as mean number of hydra ± standard deviation. Number of hydra with alphabetical superscripts in common were not significantly different from each other.

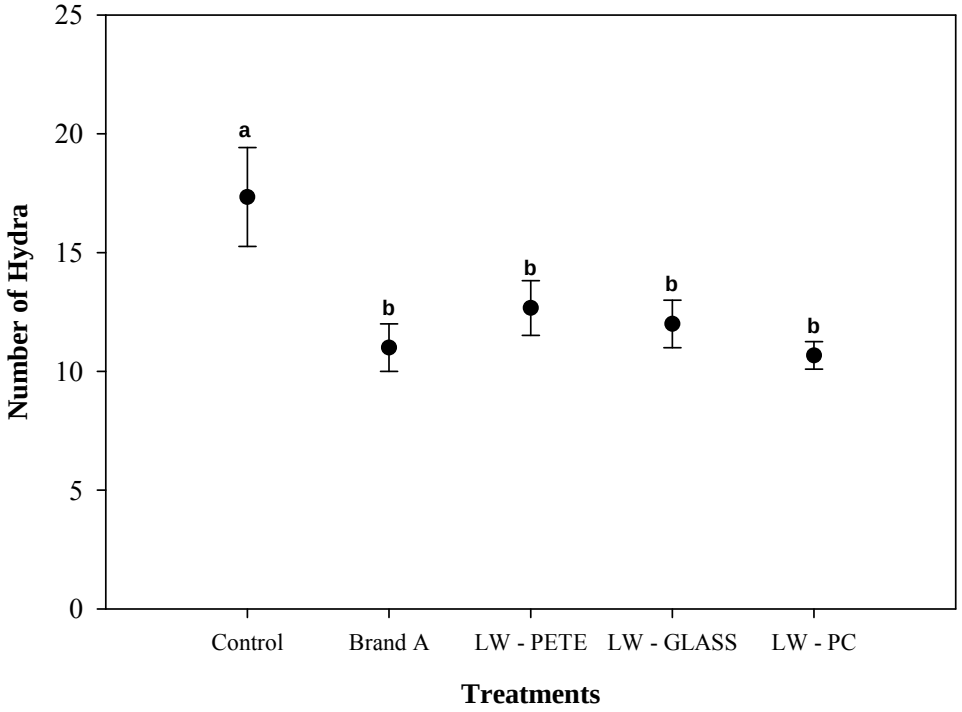


Figure 33. Effect of various treatments on the number of green hydra at week 11 in the darkness environment on the final day (168 hours). Values are displayed as mean number of hydra ± standard deviation. Number of hydra with alphabetical superscripts in common were not significantly different from each other.

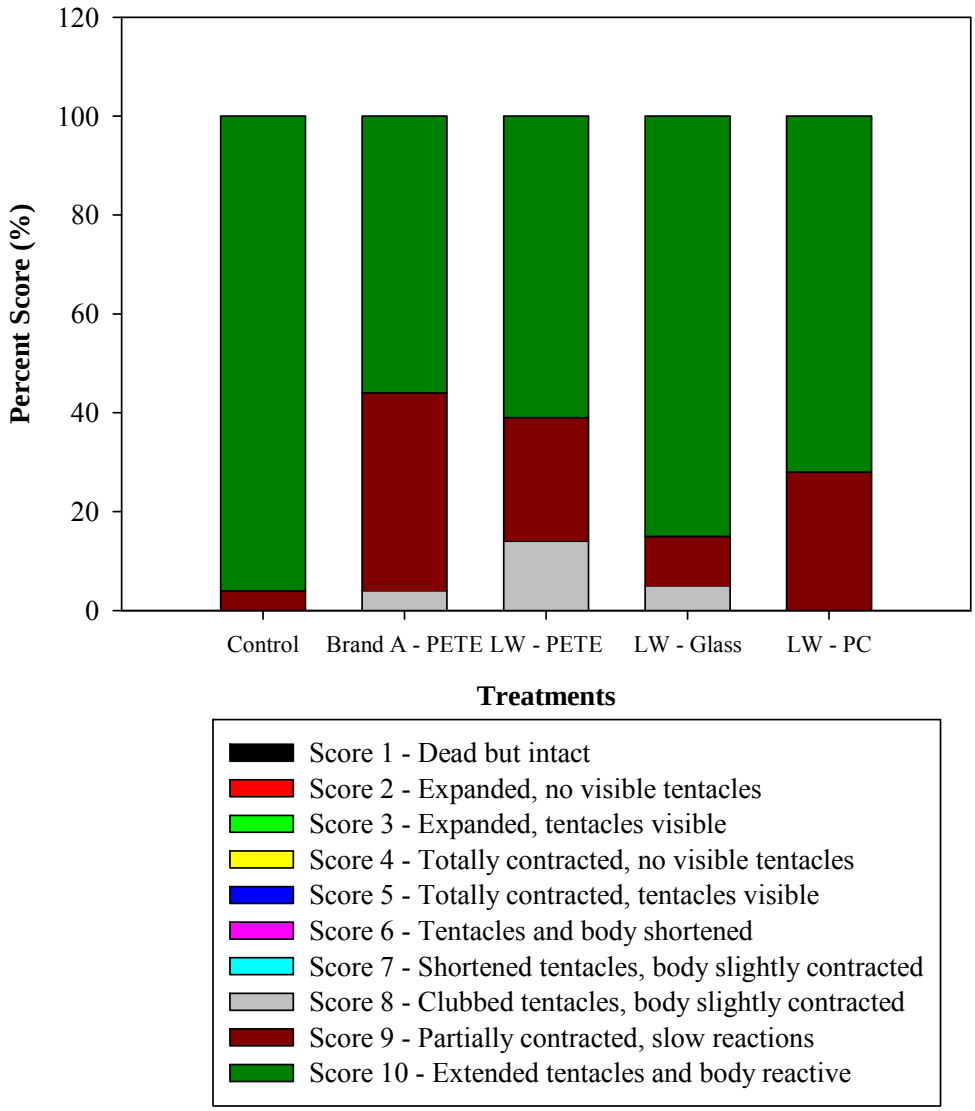


Figure 34. Effect of various treatments on the morphology of green hydra at week 11 at 168 hours in the sunlight environment.

The Week 11 dark environment displayed similar results to the sunlight environment. At 168 hours a significant difference was seen between the control and all treatments (Figure 31, Figure 33 & Appendix 7). Once more the control at the end of the bioassay had a significantly higher number of hydra per Petri dish when in comparison with the other treatments (Table 8, Figure 33 & Appendix 7). The other treatments had a mean number of hydra per Petri dish in a range of 11 to 13 hydra per Petri dish (Table 8 & Figure 33). Morphological results on the green hydra included the Brand A causing 39% of the green hydra to be at a score of 8 while the glass treatment caused 19% of the hydra to be at score of 8 (Figure 35). The remaining treatments including the control, Lab Water-PETE & Polycarbonate treatments had a range of 75 to 96% of the hydra at a score of 10 (Figure 35).

The control from Week 2 to Week 11 of the field experiment averaged 17 to 19 hydra per Petri dish for both environments with the exception of Week 0 where the average was 11 hydra per Petri dish (Table 7 & 8). The Brand A treatment for Week 0 averaged 12 hydra per Petri dish (Table 7). The Brand A treatment started off with 14 hydra per Petri dish for the sunlight environment and 14 to 15 hydra per Petri dish for the dark environment from week 2 and week 4 (Table 8). In week 6 and 11 there was an average of 8 to 9 hydra per Petri dish for the sunlight environment and 9 to 11 hydra per Petri dish for the dark environment (Table 8). The Lab Water-PETE treatment averaged 11 to 15 hydra per Petri dish for the sunlight environment and 13 to 16 hydra per Petri dish for the darkness environment from Week 2 and 4 (Table 8). Between the periods of Week 6 and 11 there was an average of 11 to 12 hydra per Petri dish for the sunlight

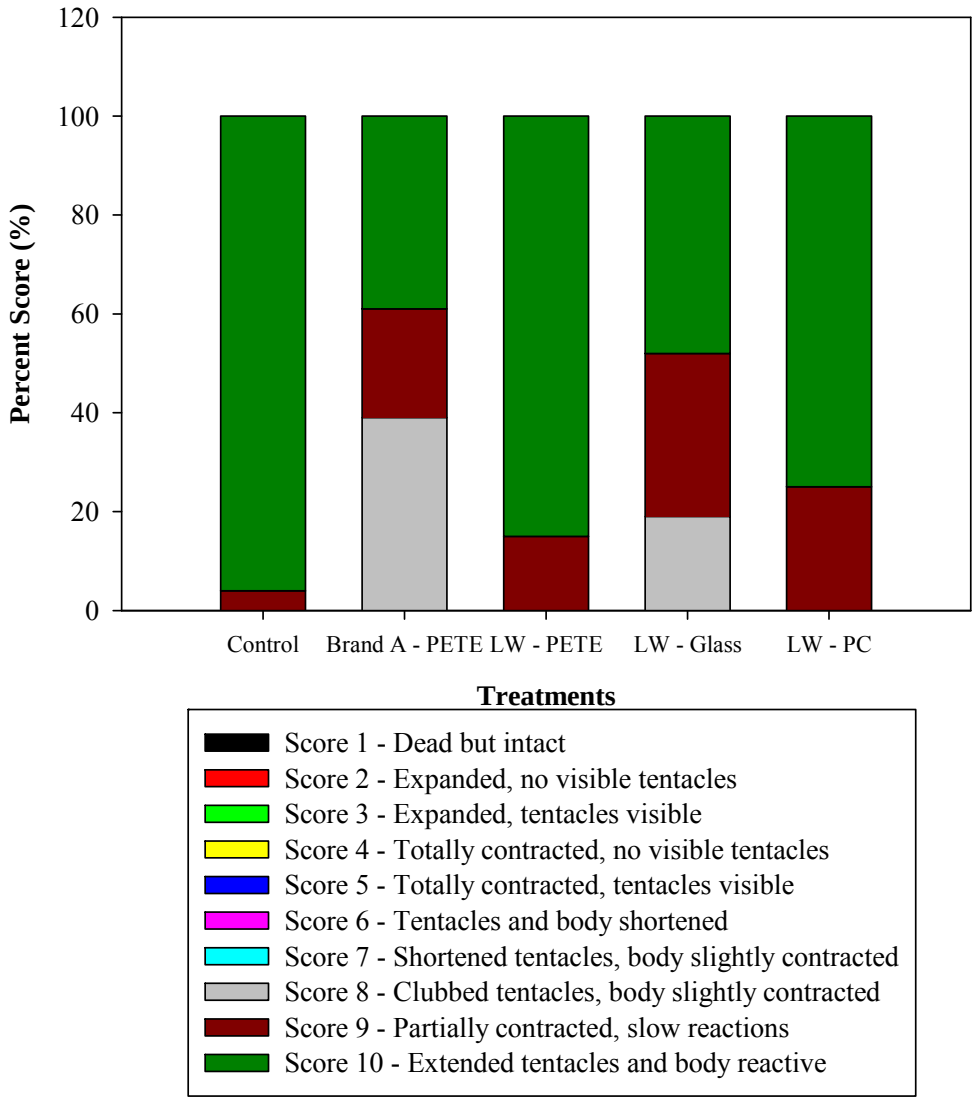


Figure 35. Effect of various treatments on the morphology of green hydra at Week 11 at 168 hours in the darkness environment.

Table 7. Mean ± standard deviation values of green hydra in each treatment at 168 hours (7 days) at week 0.

Treatments: →	Control	Brand A – PETE
Week:	**Average ± SE**	**Average ± SE**
0	10.67 ± 0.52	11.67 ± 0.52

Table 8. Mean ± standard deviation values of green hydra in each treatment at 168 Hours (7 days) in the sunlight and dark environment.

Week:	**Treatments:** →	Control	Brand A – PETE	Lab Water - PETE	Lab Water – Glass	Lab Water - PC
	Environment:	**Average ± SD**	**Average ± SD**	**Average ± SD**	**Average ± SD**	**Average ± SD**
2	Sunlight	17.0 ± 0.89	14.3 ± 3.61	11.0 ± 0.89	10.3 ± 1.03	11.0 ± 1.55
2	Darkness	17.0 ± 0.89	14.0 ± 2.34	13.3 ± 1.37	9.33 ± 1.37	10.3 ± 1.37
4	Sunlight	18.0 ± 1.79	14.0 ± 0.89	15.0 ± 2.37	15.7 ± 1.86	14.0 ± 2.37
4	Darkness	18.0 ± 1.79	15.0 ± 1.55	16.3 ± 2.88	16.3 ± 1.37	15.0 ± 1.10
6	Sunlight	18.7 ± 2.88	8.33 ± 1.03	12.0 ± 0.89	13.7 ± 0.52	9.67 ± 1.37
6	Darkness	18.7 ± 2.88	9.33 ± 1.37	13.7 ± 1.03	9.00 ± 1.79	12.0 ± 3.22
11	Sunlight	17.3 ± 1.86	9.33 ± 2.25	11.3 ± 1.86	10.7 ± 2.25	9.33 ± 1.03
11	Darkness	17.3 ± 1.86	11.0 ± 0.89	12.7 ± 1.03	12.0 ± 0.89	10.7 ± 0.52

environment and an average of 13 hydra per Petri dish for the darkness environment (Table 8). The glass treatment between the periods of Week 2 and 4 averaged 10 to 16 hydra per Petri dish for the sunlight environment and 9 to 16 hydra per Petri dish for the dark environment (Table 8). For the periods of week 6 and 11 the glass treatment averaged 10 to 14 hydra for the sunlight environment and 9 to 12 hydra for the darkness environment (Table 8). The polycarbonate treatment ranged from 11 to 14 hydra per Petri dish for the sunlight environment and 10 to 15 hydra per Petri dish for the darkness environment during the period of Week 2 and 4 (Table 8). During the periods of Week 6 and 11 the hydra averaged 9 to 10 for the sunlight environment and 11 to 12 hydra for the darkness environment (Table 8).

5.3.2 –Chemical Analysis Results (External Lab):

The concentrations of bis (2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP) and bisphenol A (BPA) contained in week 11 water samples in both the sunlight and darkness environment and two baseline samples are listed in Table 9. The limit of detection was 0.08 (Table 9). The controls of lab water and Brand A both had small amounts of DEHP contained in them (Table 9). Brand A-PETE in the sunlight environment had a total of 0.21 µg/L of DEHP contained in it after 11 weeks compared with the darkness environment which had < 0.08 µg/L contained in it. Both glass treatments in both the sunlight and darkness environment contained small amounts of DEHP in them, with 0.21 µg/L in the sunlight glass treatment had 0.23 µg/L in the darkness glass treatment (Table 9). The polycarbonate treatment had

Table 9: Chemical Analysis of Field Experiment Samples

Sample	Compound	Concentration (µg/L)	Concentration (minus blank) (µg/L)
Lab Water – Baseline (Week 0)	DEHP	0.11	-
	BPA	< 0.08	-
	DBP	< 0.08	-
Dasani – Baseline (Week 0)	DEHP	0.10	-
	BPA	< 0.08	-
	DBP	< 0.08	-
Lab Water - PETE Sunlight – Week 11	DEHP	0.10	-
	BPA	< 0.08	-
	DBP	< 0.08	-
Lab Water - PETE Darkness – Week 11	DEHP	0.08	-
	BPA	< 0.08	-
	DBP	< 0.08	-
Brand A - PETE Sunlight – Week 11	DEHP	0.32	0.21
	BPA	< 0.08	-
	DBP	< 0.08	-
Brand A - PETE Darkness – Week 11	DEHP	< 0.08	-
	BPA	< 0.08	-
	DBP	< 0.08	-
Lab Water - Glass Sunlight – Week 11	DEHP	0.32	0.21
	BPA	< 0.08	-
	DBP	< 0.08	-
Lab Water - Glass Darkness – Week 11	DEHP	0.34	0.23
	BPA	< 0.08	-
	DBP	< 0.08	-
Lab Water – PC Sunlight – Week 11	DEHP	0.09	-
	BPA	0.90	0.90
	DBP	< 0.08	-
Lab Water – PC Darkness – Week 11	DEHP	0.61	0.50
	BPA	0.93	0.93
	DBP	< 0.08	-

0.90 µg/L of BPA contained in it for the sunlight environment (Table 9). The polycarbonate treatment for the darkness environment had 0.93 µg/L of BPA in it and 0.50 µg/L of DEHP contained in it (Table 9). All remaining treatments were below 0.08 µg/L (Table 9).

5.4 – In Lab Experiment Results:

5.4.1 – Range Finders:

Range finder tests with each dibutyl phthalate, bis(2-ethylhexyl) phthalate and bisphenol A were conducted with a wide range of concentrations (Appendix 2). The concentrations included 0, 0.1, 1.0, 10 and 100 µg/L for the first set of range finders (Appendix 2). The concentrations were narrowed down and another set of range finders were conducted before a suitable concentration range was found (Appendix 2). The second set of range finders included concentrations of 0, 1.0, 3.2, 10, 32, and 100 µg/L for DEHP and 0, 10, 32, 100, 320 and 1000 µg/L for DBP and BPA.

5.4.2 – Bis (2-ethylhexyl) phthalate (DEHP) Chronic Toxicity Test

The DEHP chronic toxicity test was run for 168 hours (7 days) and no mortality was seen for any of the concentrations. No significant differences were found between replicates (Appendix 8). On the final day of the toxicity test at 168 hours significant differences in the number of hydra were observed between the nominal exposure concentrations 1.0 µg/L and 100 µg/L (Figure 36 & Appendix 8). The control had an

average of 27 hydra per Petri dish on the final day of the bioassay with the highest observed in the 1.0 µg/L concentration with 29 hydra per Petri dish (Table 10). The lowest number of hydra were found in the 100 µg/L concentration with an average of 17 hydra per Petri dish on the final day of the toxicity test (Table 10). The growth rate of the green hydra seemed to rise up to a DEHP concentration of 1.0 µg/L and then a steep decline to the 100 µg/L concentration (Table 10). The highest growth rate was 0.251 for the 1.0 µg/L concentration and the lowest growth rate was seen for the 100 µg/L DEHP concentration with a growth rate of 0.175 (Table 10). There was an almost steady growth rate before the 1.0 µg/L concentration (Table 10).

Morphological changes to the green hydra were seen at the highest concentration of 100 µg/L DEHP with 17% of the green hydra population at 168 hours being at a score of 8 (Figure 37). The 10 µg/L DEHP treatment had about 9% of the hydra at a score of 8 as well (Figure 37). The control and 1.0 µg/L concentrations both had close to 90% of the hydra at a score of 10 at 168 hours (Figure 37). A similar pattern as the growth rate could be seen in the morphological changes of the green hydra. The most impact on the morphology of the green hydra was seen at the 100 µg/L DEHP concentration. No score fell below score 8 for the DEHP chronic toxicity test.

5.4.3 – Dibutyl phthalate (DBP) Chronic Toxicity Test:

Following a seven day (168 hour) exposure to DBP the green hydra numbers increased and reached a peak and then declined (Figure 38). No significant differences

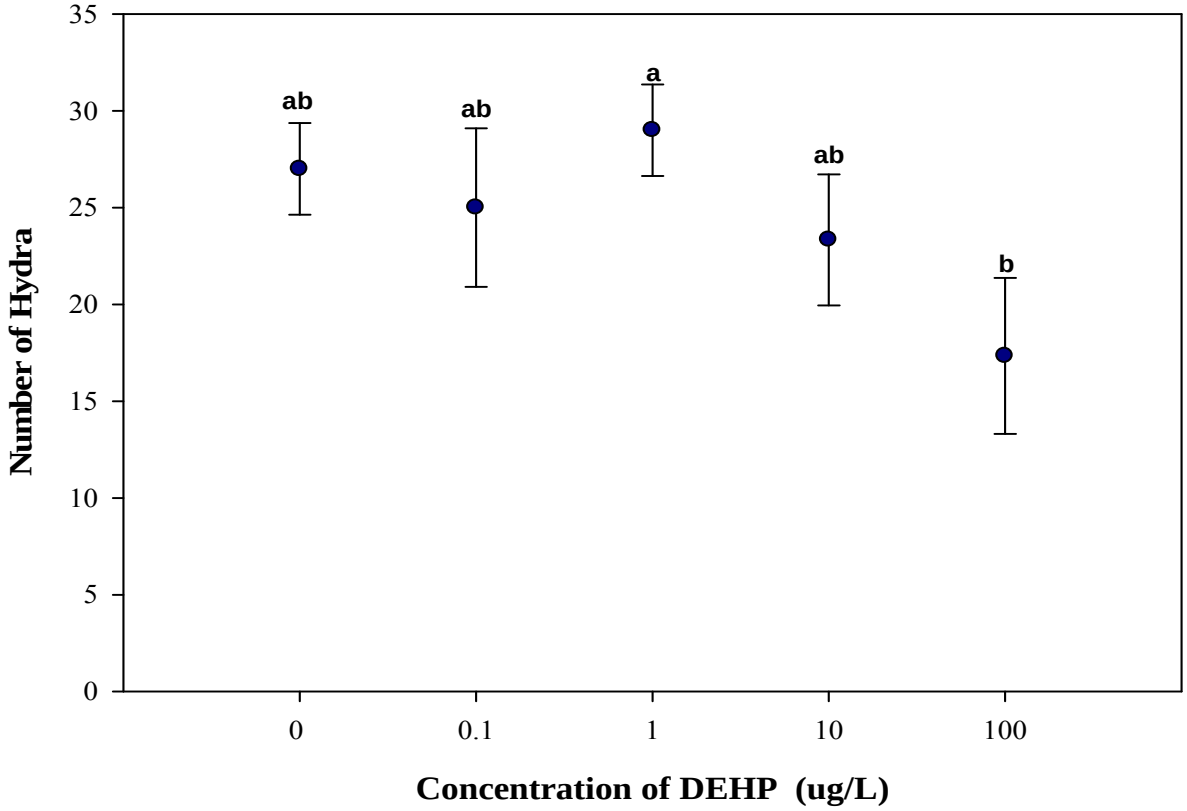


Figure 36. Bis(2-ethylhexyl) phthalate (DEHP) effect on hydra population. Values are displayed as mean number of hydra ± standard deviation. Number of hydra with alphabetical superscripts in common were not significantly different from each other.

Table 10. DEHP chronic toxicity test and mean relative population growth rate (K)

Concentration Group	Offspring Numbers			Average ± SD	K-Values			Average ± SD
-	Rep. 1	Rep. 2	Rep. 3	-	Rep. 1	Rep. 2	Rep. 3	-
Control	29.0	24.0	28.0	27.0 ± 2.65	0.251	0.224	0.246	0.240 ± 0.014
0.1 µg/L	24.0	30.0	21.0	25.0 ± 4.58	0.224	0.256	0.205	0.228 ± 0.026
1.0 µg/L	30.0	31.0	26.0	29.0 ± 2.65	0.256	0.261	0.236	0.251 ± 0.013
10 µg/L	26.0	19.0	25.0	23.3 ± 3.79	0.236	0.191	0.230	0.219 ± 0.024
100 µg/L	22.0	13.0	17.0	17.3 ± 4.51	0.212	0.137	0.175	0.175 ± 0.038

*Offspring numbers are from the final day of the chronic toxicity test (Day 7)

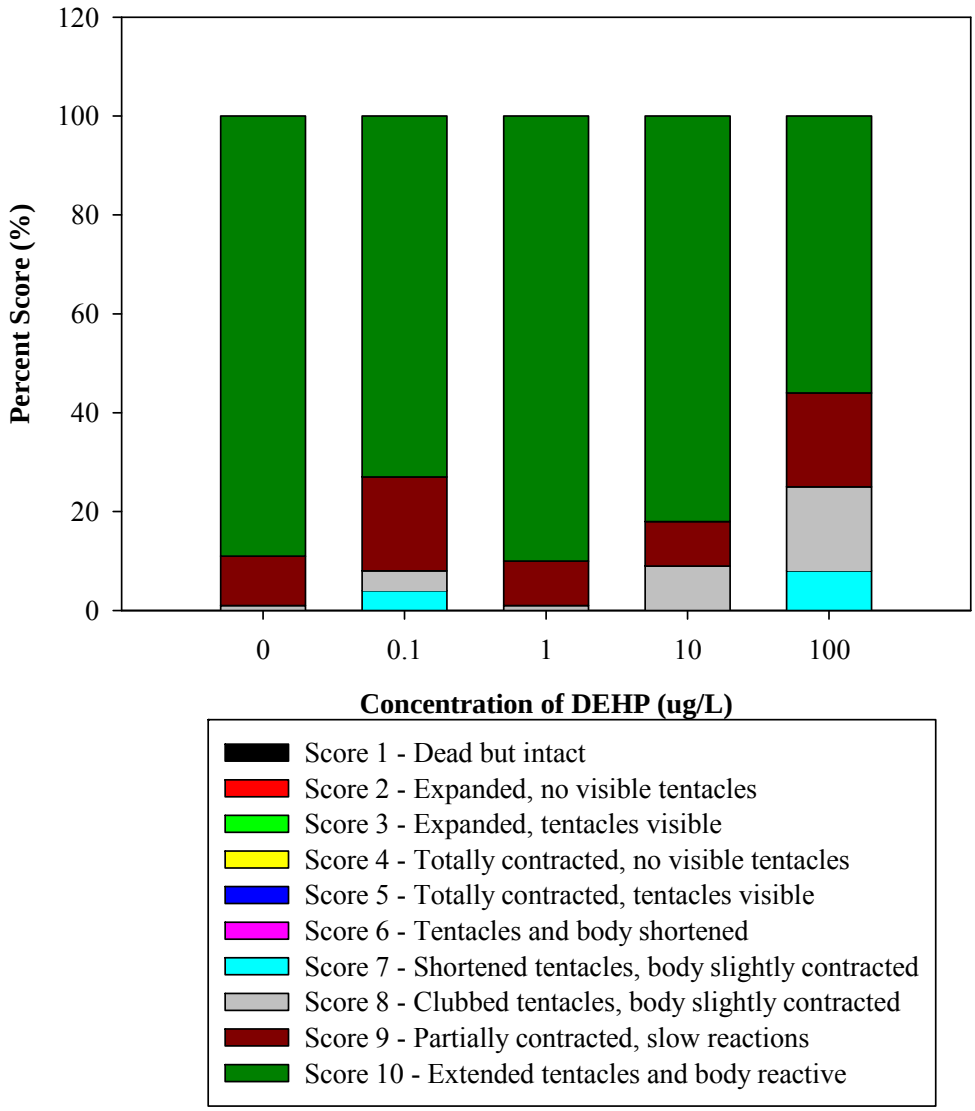


Figure 37. Effect of various concentrations of bis(2-ethylhexyl) phthalate (DEHP) on the morphology of green hydra at 168 hours.

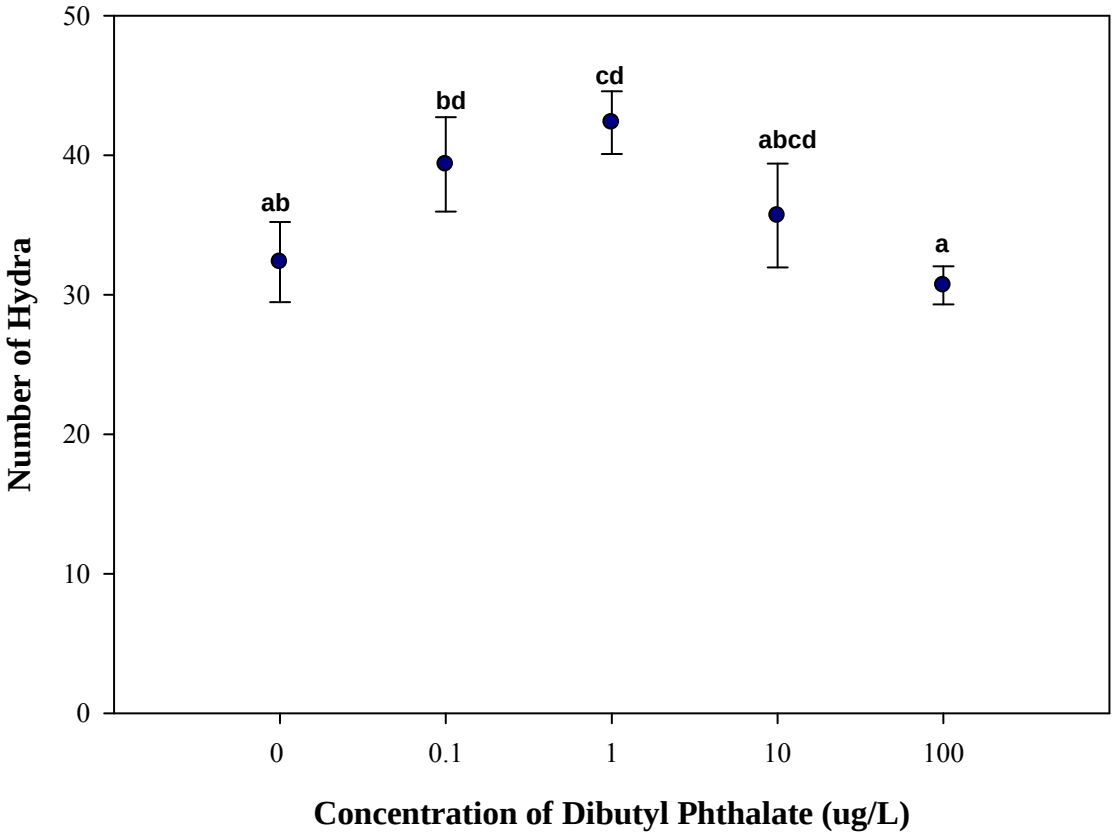


Figure 38. Dibutyl phthalate (DBP) effect on hydra population. Values are displayed as mean number of hydra ± standard deviation. Number of hydra with alphabetical superscripts in common were not significantly different from each other.

were observed between replicates (Appendix 9). Significant differences were seen between many concentrations (Appendix 9). The control hydra numbers were significantly different from the 1.0 µg/L concentration (Figure 38 & Appendix 9). The 0.1 µg/L concentration was significant from the 100 µg/L concentration (Figure 38 & Appendix 9). The 1.0 µg/L concentration was significantly different from the 100 µg/L concentration as well as the control (Figure 38 & Appendix 9). The only concentration that was not significantly different from any of the other concentrations was 10 µg/L (Appendix 9). The pattern that was observed for the DBP chronic toxicity test was constant increase in hydra numbers starting with the control to the 1.0 µg/L concentration and then a steady decline in hydra numbers to the 100 µg/L concentration (Figure 38). The highest amount of hydra were found in the 1.0 µg/L concentration with an average of 42 green hydra per Petri dish at 168 hours (Table 11). The lowest hydra numbers were found in the concentration 100 µg/L with an average close to 31 hydra per Petri dish (Table 11). The control averaged 32 hydra per Petri dish with the 0.1 µg/L, 1.0 µg/L and 10 µg/L averaging higher hydra numbers than the control. The growth rates followed the same pattern with 1.0 µg/L concentration having the highest growth rate of 0.305 and the 100 µg/L concentration having the lowest growth rate of 0.259 (Table 11). The morphology of the green hydra was the lowest at the 100 µg/L DBP concentration with 7% of the hydra being at a score of 6 and 9% at a score of 8 on the final day of the toxicity test (Figure 39). The 10 µg/L DBP concentration had 8% at a score of 8 (Figure 39). The control at 168 hours had 100% of the hydra at a score of 10 (Figure 39). The 0.1 µg/L and 1.0 µg/L DBP treatments both had 92% of the hydra population at a score of 10

Table 11. DBP chronic toxicity test and mean relative population growth rate (K)

Concentration Group	**Offspring Numbers**			**Average ± SD**	**K-Values**			**Average ± SD**
	Rep. 1	**Rep. 2**	**Rep. 3**	**-**	**Rep. 1**	**Rep. 2**	**Rep. 3**	**-**
Control	30.0	36.0	31.0	32.3 ± 3.21	0.256	0.282	0.261	0.266 ± 0.014
0.1 µg/L	35.0	41.0	42.0	39.3 ± 3.79	0.278	0.301	0.304	0.294 ± 0.014
1.0 µg/L	40.0	45.0	42.0	42.3 ± 2.52	0.297	0.314	0.304	0.305 ± 0.009
10 µg/L	31.0	39.0	37.0	35.7 ± 4.16	0.261	0.293	0.286	0.280 ± 0.017
100 µg/L	31.0	32.0	29.0	30.7 ± 1.53	0.261	0.265	0.251	0.259 ± 0.007

*Offspring numbers are from the final day of the chronic toxicity test (Day 7)

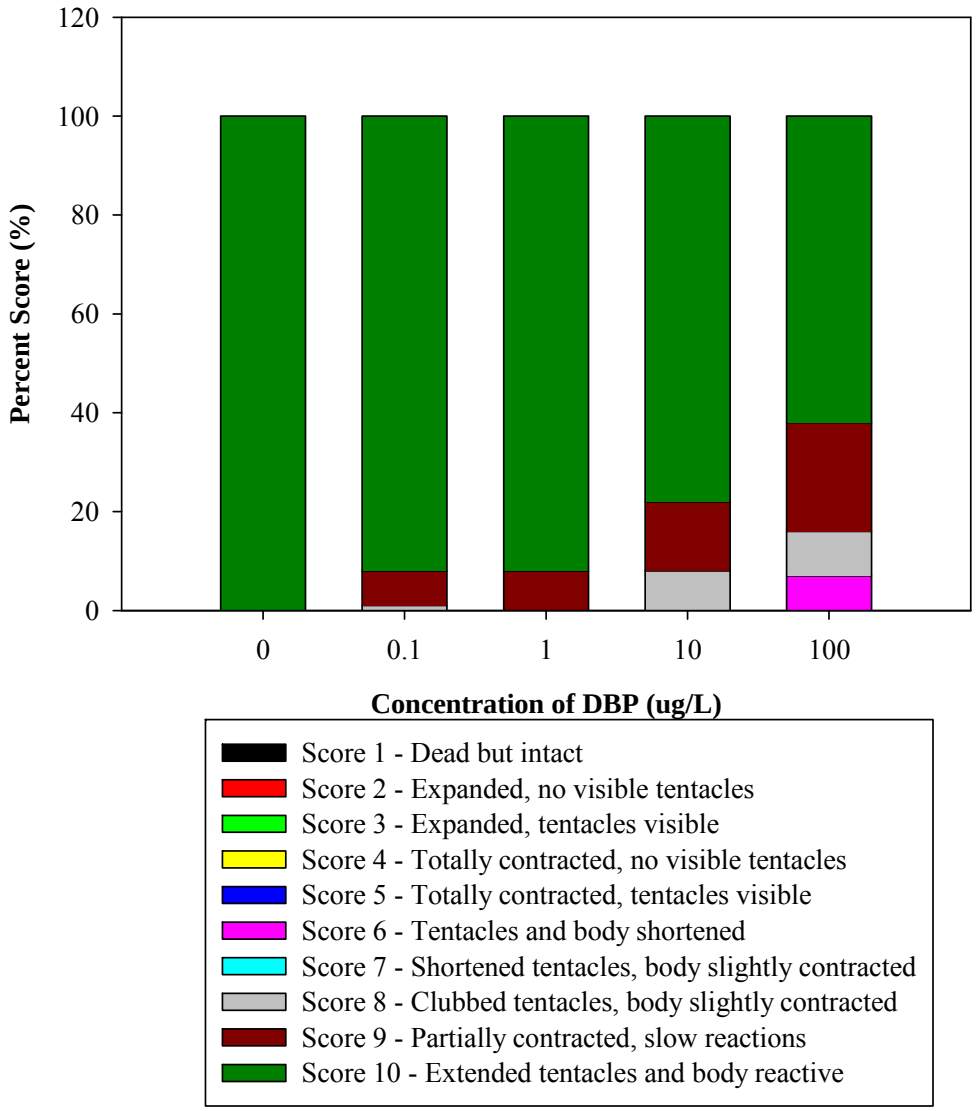


Figure 39. Effect of various concentrations of dibutyl phthalate (DBP) on the morphology of green hydra at 168 hours.

(Figure 39). The DBP chronic toxicity test caused more morphological changes at the higher concentrations which included 10 µg/L and 100 µg/L.

5.4.4 – Bisphenol A (BPA) Chronic Toxicity Test:

For the BPA chronic toxicity test no significant differences were seen between replicates (Appendix 10). Significance was observed between concentrations, with a reduction in hydra numbers observed in the 0.1 µg/L treatment compared to the control, 10 µg/L, 10 µg/L and 100 µg/L (Figure 40 & Appendix 10). The 0.1 µg/L BPA treatment had the least number of hydra at 168 hours, with an average of 22 hydra per Petri dish (Table 12). The remaining treatments including the control ranged from approximately 29 to 31 hydra per Petri dish (Table 12). The population of green hydra remained consistent for the control and other concentrations with the exception of the 0.1 µg/L concentration (Table 12). The population growth rate followed the same pattern with the lowest growth rate (k-value) being 0.212 for the 0.1 µg/L BPA treatment (Table 12). The remaining growth rates ranged from 0.249 to 0.259 (Table 12). The treament that most affected morphology was the highest BPA concentration of 100 µg/L with 2% of the green hydra being at a score of 6 and 19% being at a score of 8 on the final day of the test (Figure 41). The control had approximately 88% of its hydra at a score of 10 (Figure 41). The BPA concentration of 0.1 µg/L had 51% at a score of 10, 37% at 9 and 12% at 8 (Figure 41). All BPA treatments were affected morphologically but the BPA concentrations of 0.1 µg/L and 100 µg/L had a higher percentage of lower scores (Figure 41).

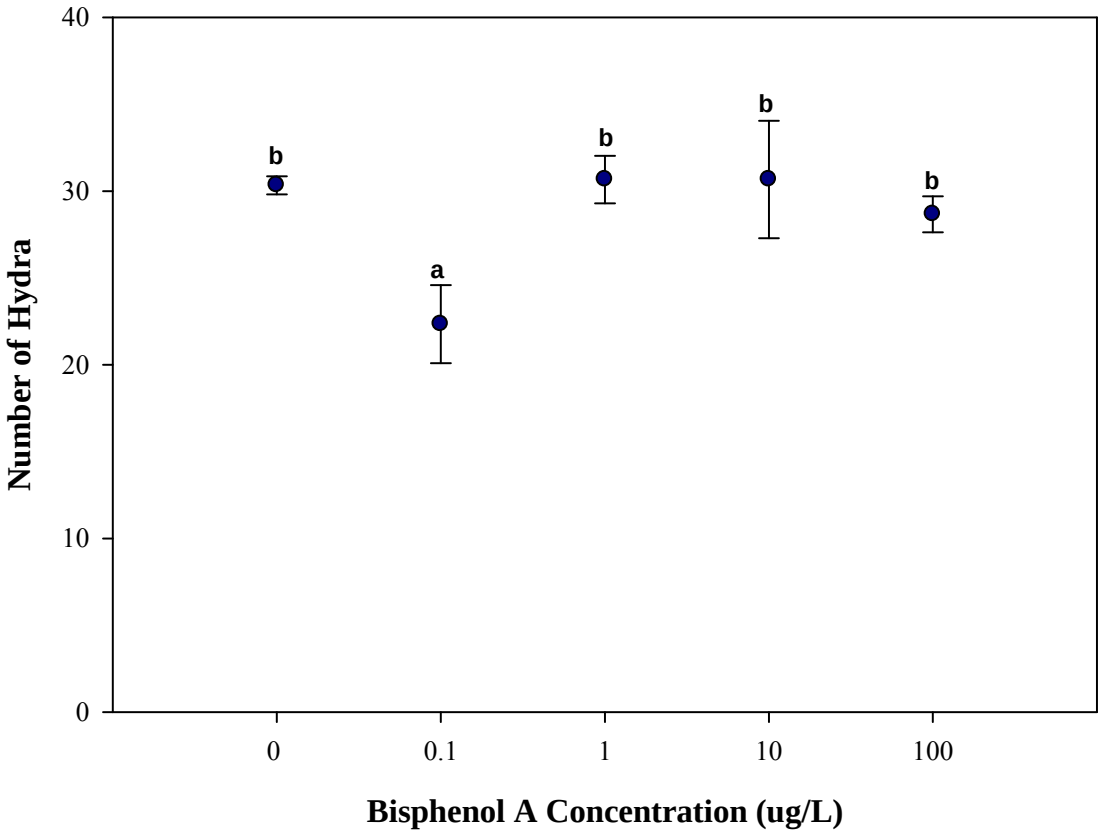


Figure 40. Bisphenol A (BPA) effect on hydra population. Values are displayed as mean number of hydra ± standard deviation. Number of hydra (within time periods) with alphabetical superscripts in common were not significantly different from each other.

Table 12. BPA chronic toxicity test and mean relative population growth rate (K)

Concentration Group	**Offspring Numbers**			**Average ± SD**	**K-Values**			**Average ± SD**
-	**Rep. 1**	**Rep. 2**	**Rep. 3**	**-**	**Rep. 1**	**Rep. 2**	**Rep. 3**	**-**
Control	30.0	30.0	31.0	30.3 ± 0.58	0.256	0.256	0.261	0.258 ± 0.003
0.1 µg/L	22.0	25.0	20.0	22.3 ± 2.52	0.212	0.230	0.198	0.213 ± 0.016
1.0 µg/L	31.0	29.0	32.0	30.7 ± 1.53	0.261	0.251	0.265	0.259 ± 0.007
10 µg/L	28.0	29.0	35.0	30.7 ± 3.79	0.246	0.251	0.278	0.258 ± 0.017
100 µg/L	28.0	30.0	28.0	28.7 ± 1.15	0.246	0.256	0.246	0.249 ± 0.006

*Offspring numbers are from the final day of the chronic toxicity test (Day 7)

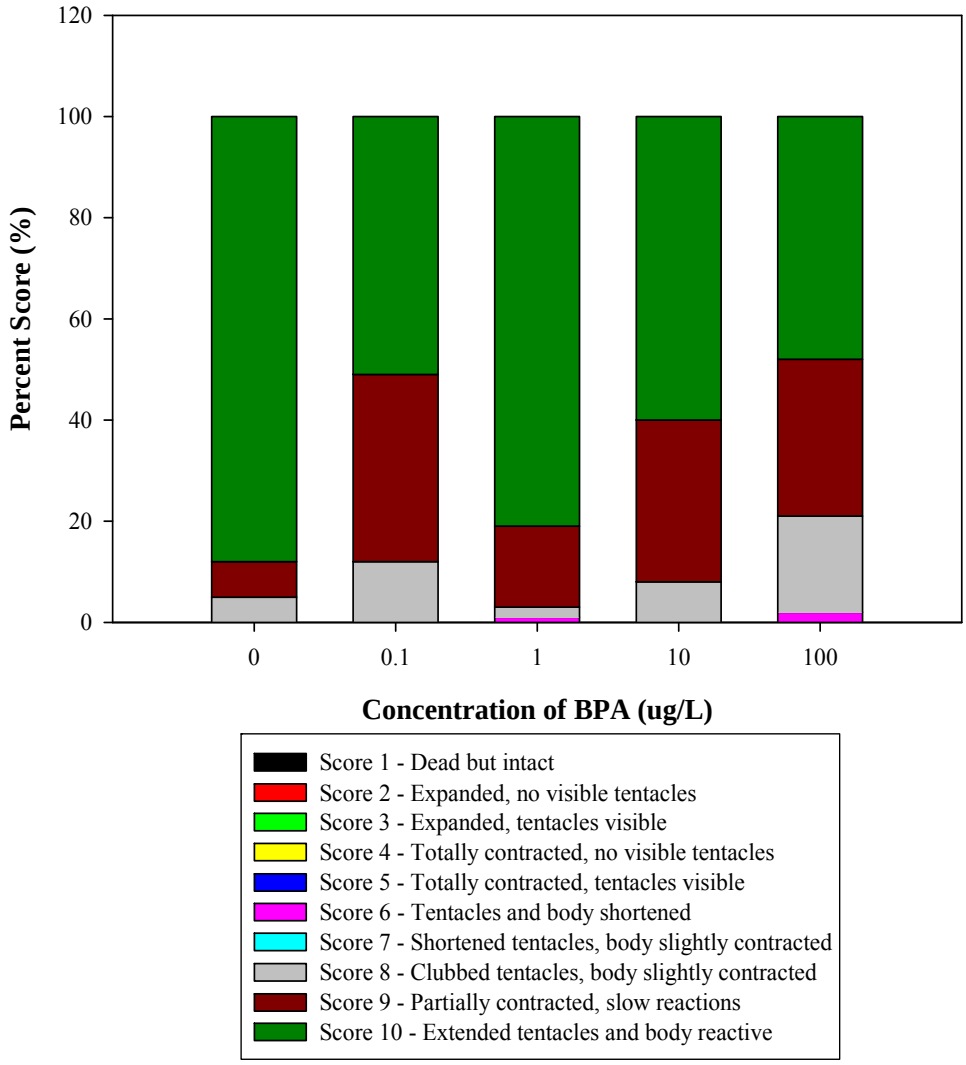


Figure 41. Effect of various concentrations of bisphenol A (BPA) on the morphology of green hydra at 168 Hours.

5.4.5 – 4-chlorophenol Reference Toxicant Test:

Figure 42 shows the response to green hydra to the reference toxicant 4-chlorphoenol (Figure 42). The 96 LC_{50} value for the 1st reference toxicant test was found to be 54.3 mg/L (Figure 42, Table 13). The second reference toxicant test LC_{50} value was calculated to be 52.0 mg/L and the last reference toxicant test produced an LC_{50} value of 50.8 mg/L (Figure 43, Figure 44, Table 13). The mean LC_{50} value for all three reference toxicant tests was 50.8 mg/L (Table 13). There were no significant differences between the LC_{50} value for all three reference toxicant tests.

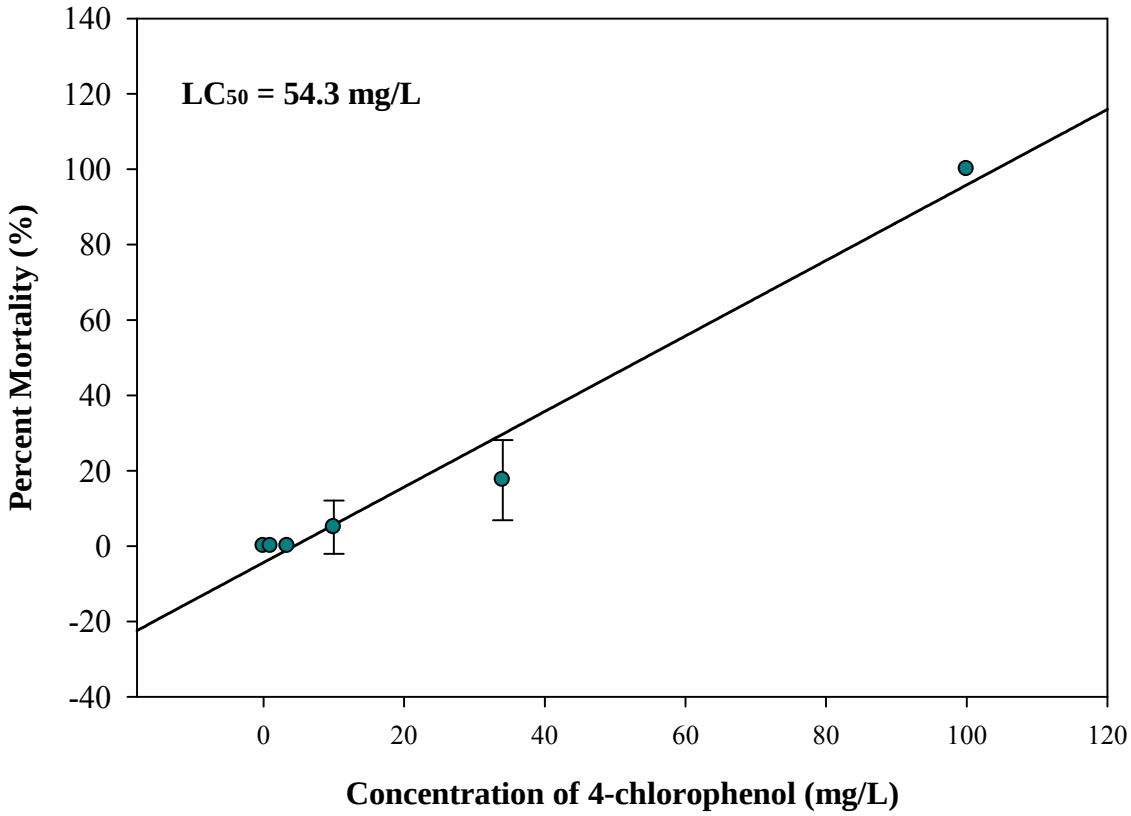


Figure 42. Percentage mortality of green hydra after exposure to 4-chlorophenol for 96 hours (1st reference toxicant test) **.**

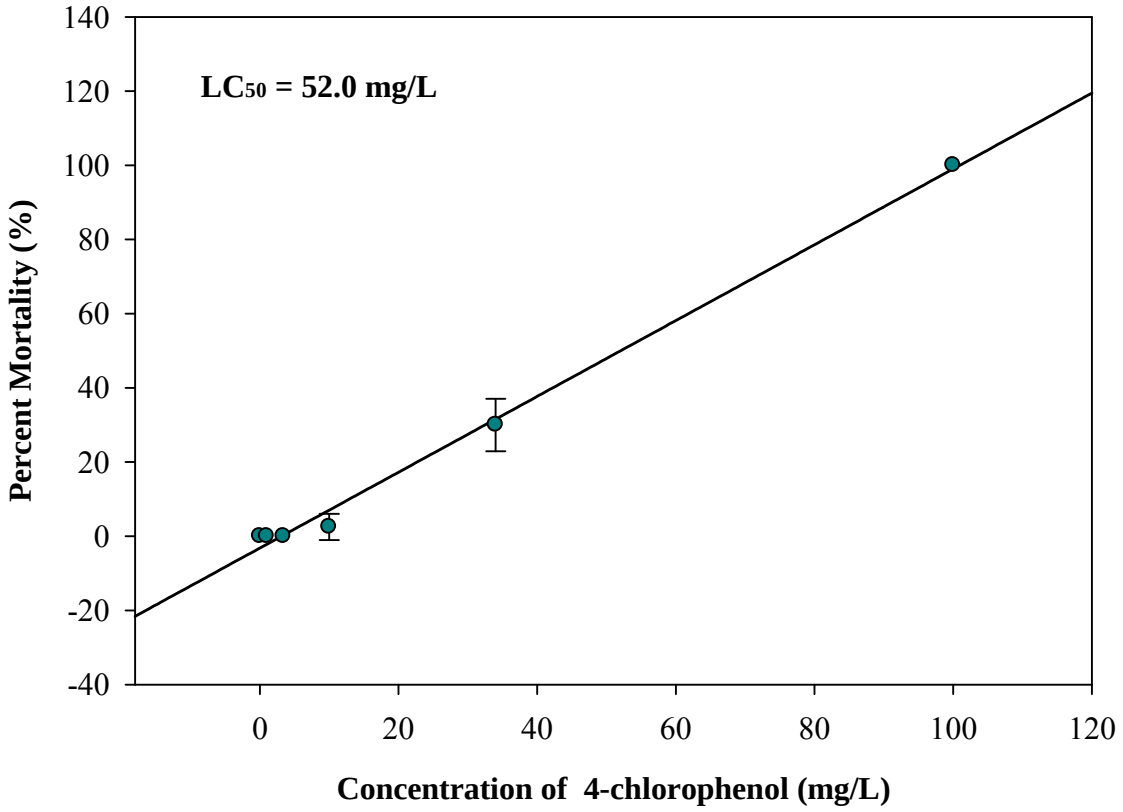


Figure 43. Percentage mortality of green hydra after exposure to 4-chlorophenol for 96 hours (2nd reference toxicant test) **.**

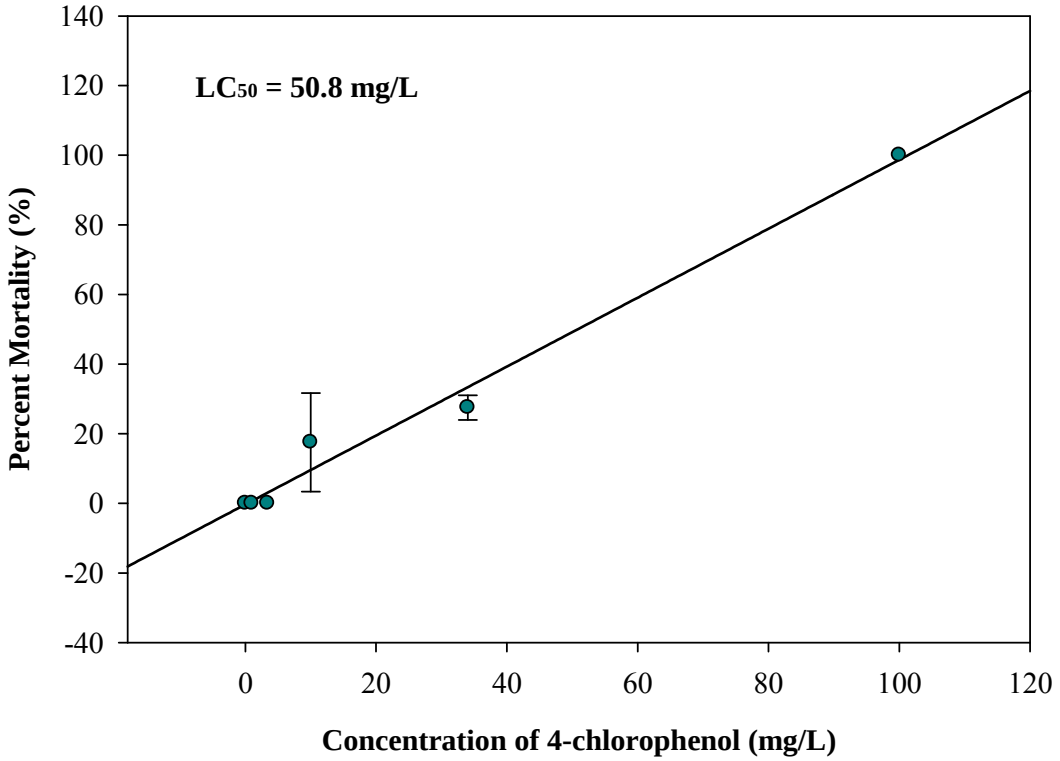


Figure 44. Percentage mortality of green hydra after exposure to 4-chlorophenol for 96 hours (3rd reference toxicant test)**.**

Table 13. LC_{50} values for the reference toxicant tests using 4-chlorophenol.

Reference Toxicant	**LC_{50} Value - 1 (mg/L)**	**LC_{50} Value - 2 (mg/L)**	**LC_{50} Value - 3 (mg/L)**	**Mean LC_{50} (mg/L)**
4-chlorophenol	54.3	52.0	50.8	52.4

6.0 – Discussion & Conclusion:

6.1 – Response of Hydra to Bottled Water:

6.1.1 – Bottled Water Bioassays

Significant differences in hydra population growth between the two storage environments (sunlight and darkness) were not observed in this study for any of the time periods (Week 0, 2, 4, 6 and 11). Each week some but not significant differences were seen between treatments but they varied each time period. Unexpectedly the glass and polycarbonate treatments caused the greatest changes in hydra population over the weeks (Table 8). They also caused the greatest morphological change in hydra when compared with other treatments. The chemical analysis of all week 11 samples including the two controls revealed why these morphological changes may be occurring in the polycarbonate and glass treatments (Table 9). The polycarbonate and glass treatments both had low levels of DEHP contained in them (Table 9). The polycarbonate treatments also had a significant amount of BPA contained in them, in both the sunlight and darkness environment. Overall all treatments with the exception of the control caused some type of morphological or reproductive inhibition.

Many studies have not exposed various PETE bottled water samples to organisms but they have looked at whether storage time and conditions affects migration of phthalates or BPA (Biscardi *et al.,* 2003; Brede *et al.,* 2003; Casjuana & Lacorte, 2003; Montuori *et al.,* 2008). Casjuana & Lacorte (2003) exposed PETE, polyethylene (PE) and glass bottles were analyzed before and after 10 weeks in temperatures up to 30°C

(Casjuana & Lacorte, 2003). An increase in concentration of various phthalates and BPA was seen (Casjuana & Lacorte, 2003). Intial concentrations of the various phthalates and BPA ranged from below detection limit to 0.059 µg/L. The more significant changes that were seen in PETE bottles after 10 weeks of storage included increased DEHP concentration to 0.135 µg/L after 10 weeks storage and increased DEP and BPA concentrations to 0.214 µg/L and 0.007 µg/L respectively (Casjuana & Lacorte, 2003). In this study the PETE bottle containing Brand-A in the sunlight environment specifically had 0.21 µg/L of DEHP at the end of the week 11 sampling period (Table 9).

Significant changes of hydra populations seen in Brand A-PETE and Lab Water-PETE treatments in this study may have occurred due to migration of phthalates from bottles. The Lab Water-PETE had no significant concentrations of DEHP, DBP or BPA for both the sunlight and darkness environments and all were below detection limit (Table 9). On the other hand Brand A-PETE in the sunlight environment had much higher concentration of DEHP than the dark environment with a concentration of 0.21 µg/L (Table 9). Montuori *et al.,* (2008), looked at PETE, PE and glass bottles also (Montuori *et al.,* 2008). Samples were analyzed right after purchasing using solid-phase microextraction and electron-impact gas chromatography-mass spectrometry (Montuori *et al.,* 2008). The study discovered that the use of PETE bottles was correlated with the concentration of phthalates in bottled water and was close to 20 times higher in PETE bottles than glass bottles (Montuori *et al.,* 2008). For example it was found that the DEHP concentration in PETE bottles was 0.17 µg/L while in glass bottles the concentration was found to be a maximum of 0.02 µg/L for PETE bottles (Montuori *et al.,* 2008). Significant differences between Lab Water-PETE and Brand A-PETE hydra

population growth with the control in this study may thus be due to potential leaching occurring from the plastic, as can be seen in the chemical analysis of Brand A-PETE in the sunlight environment with 0.21 µg/L of DEHP (Table 9).

The effect of PETE bottles on *Hydra vulgaris* species was examined by Arkhipchuk *et al.,* (2006), where they looked at the chronic toxicity of waters that humans consumed (Arkhipchuk *et al.,* 2006). A total of 30 brands of bottled drinking waters were looked at and all bottles were packaged in PETE bottles and were separated into two experiments first analyzing 12 brands of water and then the second part analyzing 18 brands of bottled water each (Arkhipchuk *et al.,* 2006). Hydra were exposed to bottled water samples for 21 days and the number of hydra and sublethal and lethal effects were recorded (Arkhipchuk *et al.,* 2006). The percent of lethality was calculated as the ratio of number of animals at tulip and disintegration stages and sublethality was calculated as the number of hydra with clubbed tentacles and shortened tentacles at a specific time period (Arkhipchuk *et al.,* 2006). The results of the first set of experiments looking at 12 brands of water they found that there were no lethal or sublethal effects for hydra following 96 hours but after 8 days of exposure, some samples of bottled water decreased the reproductive rate of hydra (Arkhipchuk *et al.,* 2006). On the last day of the exposure period (21 days) seven samples of the bottled water had caused sublethal or lethal chronic effects on hydra and another five brands had completely inhibited the hydra reproductive rate (Arkhipchuk *et al.,* 2006). In this study a seven day chronic toxicity test was conducted and hydra were affected morphologically during the bioassay.

The second set of experiments looking at 18 brands found 10 samples were toxic for hydra based on sublethality and lethality endpoints (Arkhipchuk *et al.,* 2006). A

chemical analysis was also done, focusing on inorganic substances and all bottled waters were deemed safe because they complied with national and international standards (Arkhipchuk *et al.,* 2006). The study concluded that the hydra toxicity to some bottled water brands may be due to bad quality of water sources, insufficient water treatment technology, microbial contamination and most likely leaching of organic compounds from the PETE plastic material into the water (Arkhipchuk *et al.,* 2006).

Toxicity that was observed in weeks 0, 2, 4, 6, and 11 bioassays that were conducted could be due to low levels of organic toxicants leaching from the plastic. The most significant concentration of PETE bottles leaching phthalates was found in the Brand A-PETE sunlight treatment with 0.21 µg/L of DEHP being found in the week 11 samples (Table 9). Hydra are sensitive organisms and morphological effects occurred in this study in the PETE samples (Figures 22, 23, 28, 29, 34, 35). The total number of hydra in the PETE samples when compared with the control and other samples were also affected at some sampling periods (Figures 12, 13, 24, 25, 30, 31, Table 8). Both Lab Water-PETE and Brand A – PETE bottles caused morphological changes and reproductive effects at most sampling periods.

The plastic polycarbonate has many advantages which include its transparency, strong and flexible and can be sterilized in boiling water (Brede *et al.,* 2003). Polycarbonate bottles exhibited the most changes in hydra population and morphologically. At 168 hours the polycarbonate treatment was different from the control for week 2 sunlight and darkness, week 6 sunlight and darkness and week 11 sunlight and darkness (Figures 12, 13, 24, 25, 30, 31). The polycarbonate treatment had a low number of hydra per Petri dish when compared with the controls. On average the controls at 168

hours had 17 to 19 hydra per Petri dish while the polycarbonate treatment had 9 to 15 hydra per Petri dish (Table 8). There seemed to be an effect on hydra population in the polycarbonate treatments when looking at hydra population. The polycarbonate treatment also affected hydra morphologically in both the sunlight and darkness environments in the initial sampling periods of week 2 and 4 (Figures 16, 17, 22, 23). The week 4 sampling period in the sunlight environment affected the hydra the most with the lowest score being score 4 (Figure 22). The primary chemical that could be leaching from polycarbonate bottles is BPA but small amounts of phthalates could potentially be leaching too. This was confirmed in the chemical analysis with DEHP concentrations of 0.50 µg/L potentially leaching out of the polycarbonate bottles into the lab water (Table 9). BPA concentrations for both the sunlight and darkness environment were also significant for the polycarbonate treatment with a concentration of 0.90 µg/L BPA for the sunlight environment and 0.93 µg/L of BPA for the darkness environment (Table 9). The polycarbonate treatment showed that leaching was occurring even at room temperature in complete darkness (Table 9). The presence of BPA and DEHP may have been the main cause of why hydra had lower numbers and morphological effects in the bioassays.

Polycarbonate bottles are known to contain small amounts of BPA which can leach from the PC bottles into liquid (Brede *et al.,* 2003). Brede *et al.,* (2003) discovered that leaching was occurring from polycarbonate baby bottles (Brede *et al.,* 2003). They discovered if baby bottles were subjected to dishwashing, boiling and brushing there would be a significant increase in leaching of BPA (Brede *et al.,* 2003). Biles *et al.,* (1997) looked at the concentration of BPA stored in polycarbonate products for 39 weeks and they found that the concentration of BPA was 4.7 ng/L (Biles *et al.,* 1997). This

concentration was what leached under room temperature and at a neutral pH (Biles *et al.*, 1997). Even though the concentration is small it may pose a threat to organisms. The hydra population may have been affected by low levels of BPA leaching from the polycarbonate bottles that were seen during the bioassay.

Hoa *et al.*, 2008 looked at the effects of room temperature and 100°C water in polycarbonate bottles (Hoa *et al.*, 2008). They also compared new and used polycarbonate bottles with each other with the various effects (Hoa *et al.*, 2008). Under room temperature on day 7 the new bottles released 0.73 to 1.33 BPA ng/mL while in used bottles the concentration was 0.34 to 0.93 ng/mL (Hoa *et al.*, 2008). The addition of 100°C water into the polycarbonate greatly increased the migration of BPA with concentrations of 3.84 to 7.67 ng/mL in new bottles and 1.92 ng/mL in old bottles (Hoa *et al.*, 2008). They concluded that BPA leached into the water in both new and used polycarbonate bottles, under room temperature and at a higher rate in higher temperatures (Hoa *et al.*, 2008).

This study showed that even at room temperature BPA leaches from polycarbonate bottles and this could be a reason why we saw effects in water stored in polycarbonate bottles in the darkness treatment, both morphologically and in reproduction. The chemical analysis confirmed these results because BPA was present in both darkness and sunlight environment samples with concentrations of 0.90 µg/L for the sunlight and 0.93 µg/L for the darkness environment (Table 9). The darkness environment for the polycarbonate treatment even resulted in a DEHP concentration of 0.50 µg/L (Table 9). BPA was potentially leaching from the plastic even under complete darkness.

Glass bottles seemed to cause the greatest change in hydra population throughout all weeks and changes morphologically. In weeks 2, 6 and 11 the glass treatment was significantly different from the control treatment and always had a lower hydra population when compared with the control (Figures 12, 13, 24, 25, 30, 31). Not only were there changes in the hydra population in the glass treatments but morphological changes were seen for all sampling periods including week 2, 4, 6, and 11 (Figures 16, 17, 22, 23, 28, 29, 34 ,35). Some of the lowest scores were seen in the glass treatment. The slow reproductive rate and change in morphology may be due to binding out of essential nutrients that the hydra requires to remain healthy. Hydra require essential ions/nutrients such as calcium or magnesium and these ions may have been binding out.

Such effects were likely due to a lack of essential nutrients in the water contained in the glass bottles as a consequence of binding very low levels of nutrients. The lab water contains essential metals and during storage in glass, they likely were bound out onto the glass bottles. The glass treatment was meant to be used as another type of control for comparison with plastic but it proved to cause significant effects on hydra population growth. Another reason why the glass treatment in both the sunlight and darkness treatments may have been causing morphological and reproductive effects is because of the significantly higher DEHP concentrations in both glass treatments (Table 9). The glass treatments had concentrations of 0.21 µg/L of DEHP in the sunlight environment and 0.23 µg/L in the darkness environment. The source of phthalates is unknown; contamination may have occurred from the lining of the lids of the glass bottles or from the cleaning of the bottles.

Hydra require essential metals such as zinc or copper and magnesium. for their survival. Karntanut & Pascoe (2002), looked at the acute toxicity of copper, cadmium and zinc to four different hydra species which included *Hydra vulgaris (Zurich), Hydra vulgaris, Hydra oligactis,* and *Hydra viridissima* (Karntanut & Pascoe, 2002). Zinc was the least toxic and seemed to stimulate the hydra's growth in all four species (Karntanut & Pascoe, 2002). Stebbing & Pomroy (1978), looked at copper and its effect on *Hydra littoralis* (Stebbing & Pomroy, 1978). They found that at a concentration of 2.5 μg/L the growth of the hydra was stimulated while at a concentration of 5.0 μg/L the growth of the hydra was inhibited (Stebbing & Pomroy, 1978). Various metals have shown to be essential for healthy hydra growth.

Pollino & Holdway, (2000) looked at cadmium and zinc and their toxicity to two types of species of hydra (Pollino & Holdway, 2000). They found that green hydra were more sensitive to both cadmium and zinc (Pollino & Holdway, 2000). Green hydra may be more sensitive to metals because of their symbiotic zoochlorellae, which may play a part in the sensitivity of green hydra to other essential metals too (Pollino & Holdway, 2000).

Muscatine & Lenhoff (1965), showed that under a controlled environment in the lab with daily feeding green hydra grew rapidly in a solution that contained calcium, sodium, magnesium, and potassium chloride (Muscatine & Lenhoff, 1965). They found that calcium and sodium ions were necessary for the growth of the hydra and that magnesium and potassium only improved the growth rates of the green hydra (Muscatine & Lenhoff, 1965). Some of these essential ions and metals may be binding out onto the glass bottle and affecting hydra morphologically and reproductively.

The glass effects seen in this study could be due to the very low water hardness of the laboratory water which resulted in the loss of essential ions binding out into the glass bottles. Various studies have proven that some types of metals and ions are required for the hydra's survival and growth (Muscatine & Lenhoff, 1965; Stebbing & Pomroy, 1978; Pollino & Holdway, 2000; Karntanut & Pascoe, 2002).

6.2 - Response of Hydra to Toxicants:

6.2.1 - Bis(2-ethylhexyl) phthalate (DEHP) & Dibutyl Phthalate (DBP):

DEHP is a compound that could potentially be migrating from the plastic into the bottled water. The chronic toxicity test of DEHP yielded some interesting results. The concentration of 1.0 µg/L had the highest number of hydra at 168 hours with an average of 29.0 hydra per Petri dish compared with the control with an average of 27 hydra per Petri dish (Table 10). There seemed to be a stimulation of budding in the 0.1 µg/L and 1.0 µg/L concentrations and then a drop in hydra population, with the lowest hydra being seen in the 0.1 µg/mL concentration (Figure 36 & Table 10).

Hormesis is a biphasic dose-response occurrence that shows a pattern of low dose-stimulation and high dose inhibition (Calabrese, 2008). Hormetic effects in response to DEHP have been seen in other organisms. For example a study conducted by the National Toxicology Program (NTP) measured what effect DEHP would have on mice growth (Hunt & Bowman, 2004). Mice were exposed during pregnancy to five various dose levels of DEHP including the control and were measured for body weight, liver weight, uterine weight, dead fetuses and live fetuses (Hunt & Bowman, 2004). The

experiment resulted in a U-shape dose response curve at low doses of DEHP (Hunt & Bowman).

Another study done by Anderson *et al.,* (2006) also exposed DEHP to male and female rats to low and high doses (Anderson *et al.,* 2006). The low doses used were 0.045, 0.135, 0.405, 1.215 mg/ DEHP/kg body weight (bw)/day and the high doses were 5, 15, 45, 135 and 405 mg DEHP/kg bw/day and they were exposure was daily from day 6 to lactation day 21 (Anderson *et al.,* 2006). They discovered on postnatal day 1 in males that aromatase activity was inhibited at low doses and increased at high doses resulting in a J-shaped curve (Anderson *et al.,* 2006). Inhibition was significant at concentrations 0.135 and 0.405 mg DEHP/kg/day and increased activity was seen at 15, 45 and 405 mg/kg/day (Anderson *et al.,* 2006). The results of the study indicated that the response of the dose response curve was non-monotonic and J-shaped with low dose inhibition and high dose stimulation (Anderson *et al.,* 2006). The DEHP chronic toxicity test produced a hormetic response which was an inverted U shaped result (Figure 36). Anderson *et al.,* (2006) indicated that this biphasic response would have been ignored if only the high dose range was tested which is an error that many studies do (Anderson *et al.,* 2006).

The DEHP chronic toxicity test in this study caused stimulation at the 1.0 µg/L concentration but did not stimulate the hydra population at a concentration below 0.1 µg/L (Figure 36 & Table 10). The only significant difference was observed for this test was that the 1.0 µg/L DEHP treatment hydra numbers were significantly greater than the 100 µg/L DEHP treatment numbers (Figure 36). The 1.0 µg/L DEHP treatment had on average 29 hydra per Petri dish while the 100 µg/L DEHP treatment had 17 hydra per

Petri dish (Table 10). The chronic toxicity test showed some evidence of a hormetic response. Morphologically the 100 µg/L concentration had the highest morphological changes with 8% having a score of 7 (Figure 37). DEHP is known to be one of the most potent phthalate esters. It has caused many morphological effects in various organisms, for example liver abscess and testicle abscesses, peritonitis and increase in organ weight in mice and growth reduction in Japanese medaka (Calley *et al.,* 1966; Defoe *et al.,* 1990).

Dibutyl phthalate (DBP) is one of the most widely used phthalate esters and is used largely in polyvinyl chloride products and in cosmetics and various personal care products (Wang *et al.,* 2006). DBP's urinary metabolites have shown that humans are exposed to higher amounts of DBP than other phthalate esters (Williams & Blanchfield, 1975; Wang *et al.,* 2006). The chronic toxicity test of DBP revealed a similar pattern that was seen in the DEHP chronic toxicity test (Figure 38). The DBP concentration of 1.0 µg/L had the most hydra with an average of 42 hydra per Petri dish compared with the controls which had an average of 32 hydra per Petri dish (Figure 38 & Table 11). The 1.0 µg/L DBP treatment was significantly different from the control (0 µg/L) and 100 µg/L concentration (Figure 38 & Table 11). The hydra population increased from the control to the 1.0 µg/L DBP treatment and then dropped up to the final DBP concentration of 100 µg/L (Figure 38). Thus observed chronic effects on hydra population growth appeared to be a hormetic response to DBP.

Wang *et al.,* (2006) looked at the effects of low levels of DBP and its metabolite MBP and focused on low doses (Wang *et al.,* 2006). The study looked at the effects of low concentrations of DBP on steroidogenesis in mouse Leydig tumour cells (Wang *et*

al., 2006). The cells were exposed to concentrations of MBP from 1 to 1000 nmol/L and showed that MBP caused a stimulatory effect on steroidogenesis at 100 and 1000 nmol/L of MBP (Wang *et al.,* 2006). They concluded that MBP was a primary metabolite of DBP and induced a low dose stimulation giving a non-monotonic dose response (Wang *et al.,* 2006). It is important to focus on low doses, since humans and animals are being exposed to low doses of various phthalate esters and they could be producing toxic effects to organisms.

The same pattern that was observed in the Wang *et al.,* (2006) study was observed in this study in the DBP chronic toxicity test results (Figure 38 & Table 11). The growth rate also followed the same pattern with the lowest growth rate being seen in the 100 µg/L concentration followed by the control (Table 11). The highest growth rate was found in the 1.0 µg/L concentration with a growth rate of 0.305 (Table 11). Similar results were seen in *Daphnia magna* conducted by Huang (1999). They exposed *Daphnia* to various concentrations of DBP which included 0, 0.5, 1, 2, 4 and 8 mg/L (Huang, 1999). It was discovered that the growth of the *Daphnia* was not affected significantly at the concentration of 0.5 mg/L but the reproduction was affected significantly at that specific concentration (Huang, 1999). At a concentration of 0.5 mg/L DBP stimulated the reproduction of *Daphnia* and caused the number of *Daphnia* at this concentration to be higher than control numbers (Huang, 1999). At high concentrations such as 1, 2 and 4 mg/L DBP inhibited the reproduction of *Daphnia* and the inhibition increased as the concentration of DBP increased (Huang, 1999).

Morphological effects were seen in green hydra when exposed to DBP in this study (Figure 39). The highest DBP concentration of 100 µg/L had the most

morphological effect, with 7% of the hydra population at a score of 7, 9% at a score 8, 22% at a score of 9 and 62% at a score of 10 in the 100 µg/L treatment (Figure 39). The 10 µg/L DBP concentration had some morphological effects too which included 8% being at a score of 8, 14% at a 9 and 78% at a score of 10 (Figure 39). The remaining DBP treatments and controls did not cause any severe morphological changes as the other concentrations (Figure 39).

Morphological effects from DBP have been seen in many organisms and they include causing 50% reproductive impairment in *Daphnia magna* at concentrations of 1.64, 0.15 and 0.43 mg/L (DeFoe *et al.,* 1990). DBP has been found to be toxic to fathead minnows with LC_{50} values 0.90 and 0.61 mg/L (Defoe *et al.,* 1990). In frogs DBP caused reproductive tract malformations, decrease of anogential distance, germ cell loss and loss of prostate gland and seminal vesicles at concentrations of 0.1 to 10 µm (Ohtani *et al.,* 2000).

An inverted U shape curve was observed in this study for the DBP & DEHP chronic toxicity test (Figure 36, Figure 38). Endocrine disrupting chemicals usually result in an inverted U shaped dose response curve when looking at low dose stimulation (Calabrese, 2008). Calabrese (2008) indicated that the hormetic dose responses may occur because of overcompensation to a disturbance in homeostasis or as a direct stimulatory effect (Calabrese, 2008). It is thought that these hormetic responses occur in many biological systems (Calabrese, 2008). Endocrine disruptors each have their own specific mechanisms of toxicity but all generally follow the same pattern of an inverted U shaped curve (Calabrese, 2008).

6.2.2 - Bisphenol A (BPA):

Bisphenol A is a high production chemical that humans are exposed to on a daily basis through many different types of products. BPA is a known endocrine disrupting chemical which can hinder mammalian growth by mimicking hormones (Howdeshell *et al.,* 1999). Numerous studies have shown low dose effects causing toxic effects to various organisms (Vom Saal *et al.,* 1998, Lyons, 2000, Vom Saal & Hughes, 2005). A low dose effect was seen in the BPA chronic toxicity test, where BPA reduced the hydra population at the lowest concentration of 0.1 µg/L (Figure 40). The BPA treatment of 100 µg/L had the lowest number of hydra with an average of 22 hydra per Petri dish at 168 hours, while the other BPA treatments including the controls averaged 29 to 31 hydra per Petri dish (Table 12). The 0.1 µg/L BPA concentration was significantly different from the other concentrations (Appendix 10).

Fukuhori *et al.,* (2005) looked at the effect of BPA on the species *Hydra oligactis* and concentrations that were used included 0, 0.5, 1, 2, 3 and 4 mg/L (Fukuhori *et al.,* 2005). Asexual and sexual reproduction of the hydra were only hindered at concentrations 0.5 to 4 mg/L (Fukuhori *et al.,* 2005). It was also seen at a concentration of 1 mg/L asexual reproduction was stimulated (Fukuhori *et al.,* 2005). Pascoe *et al.,* (2002) also looked at the effect of BPA on the *Hydra vulgaris* species and concentrations that were used for the acute toxicity test to BPA included 0.22, 0.46, 1.0, 2.2, 4.6, 10.0, 15.0 mg/L (Pascoe *et al.,* 2002). The LC_{50} value for BPA was determined to be 6.9 mg/L after the acute toxicity test was run (Pascoe *et al.,* 2002). The study confirmed that BPA would not pose a threat to the development of hydra species at low concentrations found in natural waters (Pascoe *et al.,* 2002). Both Fukuhori *et al.,* (2005) and Pascoe *et al.,*

(2002) looked at a wide range of concentrations of BPA exposed to hydra but they did not look at concentrations below 0.22 mg/L. The BPA chronic toxicity test that was run looked at no concentration above 100 µg/L with the lowest concentration being 0.1 µg/L (Figure 40).

Vom Saal *et al.,* (1998), showed that exposing female mouse fetuses to BPA caused toxic effects (Vom Saal *et al.,* 1998). Pregnant mice were fed either oil or BPA dissolved in oil to a dose levels that are usually found in the environment (2.4 mg/kg) on days 11 to 17 of gestation (Vom Saal *et al.,* 1998). It was observed that exposing female mice to BPA at an environmentally realistic dose caused altered reproductive function and an altered postnatal growth rate (Vom Saal *et al.,* 1998). Rubin *et al.,* 2001 also focused on low dose effects of BPA on female rats (Rubin *et al.,* 2001). Rats were exposed through their drinking water to close to 0.1 mg BPA/kg body weight/day (low dose) or 1.2 mg BPA/kg bw/day (high dose) following day 6 of the pregnancy through the stage of lactation (Rubin *et al.,* 2001). Offspring that were exposed to BPA displayed an increased weight gain that was observed following birth and lasted into later life (Rubin *et al.,* 2001). Rats also had lower levels of luteinizing hormone into adulthood (Rubin *et al.,* 2001). It was shown that low levels of BPA caused affects especially during the perinatal stage (Rubin *et al.,* 2001).

Even though BPA had the least number of hydra in the lowest test concentration of 0.1 µg/L (Figure 40), morphological effects were seen in hydra exposed to higher BPA concentrations (Figure 41). The treatment that was most affected morphologically was 100 µg/L BPA, with 2% of the green hydra being at a score of 6 and 19% being at a score of 8 (Figure 41). The lowest BPA concentration of 0.1 µg/L was also affected

morphologically with 12% at a score of 8, but no score fell below 8 for the remaining hydra at 168 hours (Figure 41). Morphologically hydra were most affected at the 100 µg/L concentration but this BPA treatment had a higher average of hydra per Petri dish than the 0.1 µg/L concentration (Table 12).

Fukuhori *et al.,* (2005) found that at a concentration of 0.5 mg/L BPA had no effect on asexual reproduction but at a concentration of 1 mg/L budding was stimulated (Fukuhori *et al.,* 2005). It was determined that concentrations of 2 and 3 mg/L BPA caused an inhibition of budding and the rate of asexual reproduction was reduced (Fukuhori *et al.,* 2005). These stimulatory effects are usually seen at low doses of endocrine disrupting chemicals (Fukuhori *et al.,* 2005). Low dose effects such as what was seen in the BPA chronic toxicity test has been found in other various organisms. Effects of endocrine disrupting chemicals have been seen in humans and are consistent with effects that have been seen in other animals (Muncke, 2009). Effects in humans such as an increase in genital abnormality in boys and advanced sexual maturation in girls have been seen as to what has been observed in animals (Vom Saal *et al.,* 1998).

6.2.3 - 4-chlorophenol:

The sensitivity of the green hydra stock cultures were periodically measured by a reference toxicant known as 4-chlorophenol. The chemical 4-chlorophenol was used as a reference toxicant to confirm that the green hydra species sensitivity to an organic toxicant was consistent throughout each reference toxicant test. It was used as a standard

for toxicity test to identify differences in the sensitivity of green hydra over time (Environment Canada, 2005).

The LC_{50} values for the three reference toxicant tests that were conducted remained consistent throughout each toxicant test (Figures 42, 43, 44, Table 13). The LC_{50} values for the three reference toxicant tests ranged from 50.8 mg/L to 54.3 mg/L (Table 12). When hydra were exposed to 4-chlorphenol for 96 hours in Mitchell & Holdway (2000) study, the LC_{50} value was 35 mg/L (Mitchell & Holdway, 2000). In Pollino & Holdway (1999), they exposed 4 chlorophenol to pink and green hydra (Pollino & Holdway, 1999). The pink hydra had a lower LC_{50} value than the green hydra with a value of 32.0 mg/L and 45.0 mg/L for the green hydra (Pollino & Holdway, 2000). Therefore the senstivitiy of the green hydra in this study was comparable to the literature value of 45.0 mg/L with the LC_{50} value in this study being an average of 52.4 mg/L (Pollino & Holdway, 2000; Table 13).

6.3 - Future Work:

Research on the migration of potential organic compounds from plastic bottled water has increased for the polycarbonate plastic field but is still lacking for other types of plastic such as polyethylene terephthalate bottles. The reason why studies are lacking for other types of plastic material may be because the levels of phthalates, BPA and other organic compounds are only found at trace levels. It is thought that these low levels of organic compounds pose no threat to humans or animals but this is being proved wrong by many low dose studies. Many instruments have difficulty detecting these compounds

at trace levels. New techniques need to be developed to detect these compounds at low concentrations. Optimized chemical analysis using appropriate instruments should be developed to detect low doses of phthalates and BPA because of the concern of effects from low doses have on organisms. The effect of long storage time and various storage conditions such as high temperatures and darkness on bottled water needs to be further investigated. The effects that these leached chemicals have on small organisms and fish also needs to be explored and is hardly mentioned in literature. More focus needs to be put on all types of phthalates and other plasticizers that may be posing a risk to organisms.

6.4 - Conclusion:

The issue of migration of potentially toxic chemicals such as phthalates and BPA needs to be given more attention. Research on bottled water and other types of plastic material and the potential migration of these plasticizers need to be evaluated thoroughly. Hydra provided sensitive results on the chemicals present in bottled water. Some significant differences were seen in treatments and in most sampling periods. The chronic toxicity tests showed that BPA caused effects on hydra morphology and population at low doses and DBP and DEHP both showed signs of hormesis. A general trend was observed with concentrations of DEHP, DBP and BPA and toxic reproductive and morphological effects to the green hydra were observed. Many studies look at the microbiological contamination of bottled water but there seems to be a lack of studies on the potential organic content found in bottled water. This may be due to the fact that

phthalate levels in bottled water are found at trace levels. More research needs to be conducted on the fate of organic chemicals from plastic materials under various conditions and the effect of these organic chemicals on organisms at low doses. The concentrations found in bottled waters may be at low concentrations but could have long-term effects on organisms. Consumers need to consider all the factors when purchasing bottled water.

7.0 – Literature Cited

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8.0 – Appendices

Appendix 1:

Rearing of Brine Shrimp Larvae (*Artemia salina*):

Brine shrimp larvae were reared at 25°C in conical shaped Nalgene® funnels. Cultures of brine were aerated. A 7 litre culture solution was made by dissolving 1.5 cups of salt in approximately 7 litres of water. The salt was left to dissolve in the water. Then about 7 teaspoons of commercial brine shrimp cysts (Premium Grade Brine Shrimp Eggs from Brine Shrimp Direct) was added. The eggs were aerated for 48 hours.

To harvest the brine, the aerator was turned off and the hatched brine was allowed to settle at the bottom of the cone. The brine was allowed to flow through a tube and collected into a filter. The brine shrimp was rinsed with laboratory water and washed into a container. All precautions were taken to avoid getting unhatched cysts into the filtered brine.

Appendix 2:

Concentrations Used for Bioassays:

Table 1: Range Finder Concentrations of DBP, DEHP, & BPA Exposed to Green Hydra

Chemicals	Range Finder	Nominal Concentrations (µg/L)
Bis(2-ethylhexyl) phthalate	1	0, 0.1, 1.0, 10, 100
Dibutyl phthalate	1	0, 0.1, 1.0, 10, 100
Bisphenol A	1	0, 0.1, 1.0, 10, 100
Bis(2-ethylhexyl) phthalate	2	0, 1.0, 3.2, 10, 32, 100
Dibutyl phthalate	2	0, 10, 32, 100, 320, 1000
Bisphenol A	2	0, 10, 32, 100, 320, 1000

Table 2: Chronic Toxicity Concentrations of DBP, DEHP, & BPA Exposed to Green Hydra

Chemicals	Range Finder	Nominal Concentrations (µg/L)
Bis(2-ethylhexyl) phthalate	1	0, 0.1, 1.0, 10, 100
Dibutyl phthalate	2	0, 0.1, 1.0, 10, 100
Bisphenol A	3	0, 0.1, 1.0, 10, 100

Table 3: Concentrations of 4-chlorophenol (reference toxicant) Exposed to Green Hydra:

Chemicals	Range Finder	Nominal Concentrations (mg/L)
4-chlorophenol	1	0, 1.0, 3.4, 10, 34, 100
4-chlorophenol	2	0, 1.0, 3.4, 10, 34, 100
4-chlorophenol	3	0, 1.0, 3.4, 10, 34, 100

Appendix 3:

Field Experiment Statistics:

Week 0 – Replicates versus Number of Hydra – One Way ANOVA

Effect	Univariate Tests of Significance for Number of Hydra (Week 0 Worksheet.sta) Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2	NewVar 3
Intercept	2282.521	1	2282.521	414.4805	0.000000			
Replicates	2.667	2	1.333	0.2421	0.785979			
Error	247.813	45	5.507					

Week 0 – Treatment versus Number of Hydra – One Way ANOVA

Effect	Univariate Tests of Significance for Number of Hydra (Week 0 Worksheet.sta) Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2	NewVar 3
Intercept	2282.521	1	2282.521	420.0538	0.000000			
Treatment	0.521	1	0.521	0.0958	0.758267			
Error	249.958	46	5.434					

Appendix 4:

Week 2 – Replicates versus Number of Hydra – One Way ANOVA

Effect	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2	NewVar 3
	Univariate Tests of Significance for Number of Hydra (Week 2 Spreadsheet.sta) Sigma-restricted parameterization Effective hypothesis decomposition							
Intercept	10542.04	1	10542.04	1219.408	0.000000			
Replicates	19.53	2	9.76	1.129	0.325156			
Error	1841.43	213	8.65					

Week 2 – Environment versus Number of Hydra – One Way ANOVA

Effect	SS	Degr. of Freedom	MS	F	p	NewVar	NewVar 1	NewVar 2	NewVar 3
	Univariate Tests of Significance for Number of Hydra (Week 2 Spreadsheet.sta) Sigma-restricted parameterization Effective hypothesis decomposition								
Intercept	8897.130	1	8897.130	1231.222	0.000000				
Environment	0.880	1	0.880	0.122	0.727469				
Error	1372.990	190	7.226						

Week 2 – Treatment versus Number of Hydra at 120 Hours – Sunlight Environment - One Way ANOVA (Significance – $p < 0.05$)

Effect	SS	Degr. of Freedom	MS	F	p	NewVar	NewVar 1
	Univariate Tests of Significance for Number of Hydra (Spreadsheet6) Sigma-restricted parameterization Effective hypothesis decomposition						
Intercept	912.6000	1	912.6000	402.6176	0.000000		
Treatment	37.7333	4	9.4333	4.1618	0.030693		
Error	22.6667	10	2.2667				

Week 2 – Treatment versus Number of Hydra at 120 Hours – Sunlight Environment – Post hoc – Tukey Test

Cell No.	Tukey HSD test; variable Number of Hydra (Spreadsheet6) Homogenous Groups, alpha = .05000 Error: Between MS = 2.2667, df = 10.000 Treatment	Number of Hydra Mean	1	2	NewVar	NewVar 1
5	LAB WATER - PC	6.33333	****			
3	LAB WATER - PETE	6.66667	****	****		
4	LAB WATER - GLASS	7.00000	****	****		
2	BRAND A - PETE	8.33333	****	****		
1	Control	10.66667		****		

Week 2 – Treatment versus Number of Hydra at 168 Hours – Sunlight Environment - One Way ANOVA (Significance – $p < 0.05$)

Effect	Univariate Tests of Significance for Number of Hydra (Spreadsheet6) Sigma-restricted parameterization Effective hypothesis decomposition SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2
Intercept	2432.067	1	2432.067	536.4853	0.000000		
Treatment	97.600	4	24.400	5.3824	0.014167		
Error	45.333	10	4.533				

Week 2 – Treatment versus Number of Hydra at 168 Hours – Sunlight Environment – Post hoc – Tukey Test (Significance)

Cell No.	Tukey HSD test; variable Number of Hydra (Spreadsheet6) Homogenous Groups, alpha = .05000 Error: Between MS = 4.5333, df = 10.000 Treatment	Number of Hydra Mean	1	2	NewVar
4	LAB WATER - GLASS	10.33333	****		
3	LAB WATER - PETE	11.00000	****		
5	LAB WATER - PC	11.00000	****		
2	BRAND A - PETE	14.33333	****	****	
1	Control	17.00000		****	

Week 2 – Treatment versus Number of Hydra at 120 Hours – Darkness Environment – One Way ANOVA (Significance – p < 0.05)

Effect	Univariate Tests of Significance for Number of Hydra (Spreadsheet6) Sigma-restricted parameterization Effective hypothesis decomposition						
	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2
Intercept	992.2667	1	992.2667	676.5455	0.000000		
Treatment	27.0667	4	6.7667	4.6136	0.022745		
Error	14.6667	10	1.4667				

Week 2 – Treatment versus Number of Hydra at 120 Hours – Darkness Environment – Post hoc – Tukey Test

Cell No.	Tukey HSD test; variable Number of Hydra (Spreadsheet6) Homogenous Groups, alpha = .05000 Error: Between MS = 1.4667, df = 10.000					
	Treatment	Number of Hydra Mean	1	2	NewVar 1	NewVar 2
4	LAB WATER - GLASS	7.00000	****			
5	LAB WATER - PC	7.00000	****			
3	LAB WATER - PETE	8.00000	****	****		
2	BRAND A - PETE	8.00000	****	****		
1	Control	10.66667		****		

Week 2 – Treatment versus Number of Hydra at 144 Hours – Darkness Environment – One Way ANOVA (Significance – p < 0.05)

Effect	Univariate Tests of Significance for Number of Hydra (Spreadsheet6) Sigma-restricted parameterization Effective hypothesis decomposition						
	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2
Intercept	1540.267	1	1540.267	1359.059	0.000000		
Treatment	32.400	4	8.100	7.147	0.005498		
Error	11.333	10	1.133				

Week 2 – Treatment versus Number of Hydra at 144 Hours – Darkness Environment – Post hoc – Tukey Test

Cell No.	Treatment	Number of Hydra Mean	1	2	NewVar 1
	Tukey HSD test; variable Number of Hydra (Spreadsheet6) Homogenous Groups, alpha = .05000 Error: Between MS = 1.1333, df = 10.000				
4	LAB WATER - GLASS	8.33333	****		
5	LAB WATER - PC	8.66667	****		
3	LAB WATER - PETE	10.66667	****	****	
2	BRAND A - PETE	10.66667	****	****	
1	Control	12.33333		****	

Week 2 – Treatment versus Number of Hydra at 168 Hours – Darkness Environment – One Way ANOVA (Significance – p < 0.05)

Effect	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2
	Univariate Tests of Significance for Number of Hydra (Spreadsheet6) Sigma-restricted parameterization Effective hypothesis decomposition						
Intercept	2457.600	1	2457.600	819.2000	0.000000		
Treatment	112.400	4	28.100	9.3667	0.002053		
Error	30.000	10	3.000				

Week 2 – Treatment versus Number of Hydra at 168 Hours – Darkness Environment – Post hoc – Tukey Test

Cell No.	Treatment	Number of Hydra Mean	1	2	3	NewVar 1
	Tukey HSD test; variable Number of Hydra (Spreadsheet6) Homogenous Groups, alpha = .05000 Error: Between MS = 3.0000, df = 10.000					
4	LAB WATER - GLASS	9.33333	****			
5	LAB WATER - PC	10.33333	****	****		
3	LAB WATER - PETE	13.33333	****	****	****	
2	BRAND A - PETE	14.00000		****	****	
1	Control	17.00000			****	

Appendix 5:

Week 4 – Replicates versus Number of Hydra – One Way ANOVA

Effect	Univariate Tests of Significance for Number of Hydra (Week 4 Spreadsheet.sta) Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2	**NewVar**
Intercept	14016.67	1	14016.67	846.4243	0.000000			
Replicates	0.08	2	0.04	0.0025	0.997487			
Error	3527.25	213	16.56					

Week 4 – Environment versus Number of Hydra – One Way ANOVA

Effect	Univariate Tests of Significance for Number of Hydra (Week 4 Spreadsheet.sta) Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2	NewVar 3
Intercept	12288.00	1	12288.00	786.2830	0.000000			
Environment	4.69	1	4.69	0.2999	0.584561			
Error	2969.31	190	15.63					

Appendix 6:

Week 6 – Replicates versus Number of Hydra – One Way ANOVA

Effect	Univariate Tests of Significance for Number of Hydra (Week 6 Spreadsheet.sta) Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. of Freedom	MS	F	p	NewVar	NewVar 1	NewVar 2
Intercept	10472.30	1	10472.30	1164.196	0.000000			
Replicates	3.70	2	1.85	0.206	0.814102			
Error	1916.00	213	9.00					

Week 6 – Environment versus Number of Hydra – One Way ANOVA

Effect	Univariate Tests of Significance for Number of Hydra (Week 6 Spreadsheet.sta) Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2	NewVar 3
Intercept	8775.021	1	8775.021	1301.885	0.000000			
Environment	0.333	1	0.333	0.049	0.824255			
Error	1280.646	190	6.740					

Week 6 – Treatment versus Number of Hydra at 120 Hours – Sunlight Environment - One Way ANOVA (Significance – $p < 0.05$)

Effect	Univariate Tests of Significance for Number of Hydra (Template File for Time Data.sta Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. of Freedom	MS	F	p	NewVar	NewVar 1	NewVar 2
Intercept	976.0667	1	976.0667	813.3889	0.000000			
Treatment	32.9333	4	8.2333	6.8611	0.006337			
Error	12.0000	10	1.2000					

Week 6 – Treatment versus Number of Hydra at 120 Hours – Sunlight Environment – Post hoc – Tukey Test

Cell No.	Tukey HSD test; variable Number of Hydra (Template File for Time Data.sta) Homogenous Groups, alpha = .05000 Error: Between MS = 1.2000, df = 10.000 Treatment	Number of Hydra Mean	1	2	NewVar 1	NewVar 2	NewVar 3
2	BRAND A - PETE	6.33333	****				
5	LAB WATER - PC	6.66667	****				
4	LAB WATER - GLASS	8.00000	****	****			
1	Control	9.00000	****	****			
3	LAB WATER - PETE	10.33333		****			

Week 6 – Treatment versus Number of Hydra at 144 Hours – Sunlight Environment - One Way ANOVA (Significance – $p < 0.05$)

Effect	Univariate Tests of Significance for Number of Hydra (Template File for Time Data.sta Sigma-restricted parameterization Effective hypothesis decomposition SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2	NewVar 3
Intercept	1622.400	1	1622.400	579.4286	0.000000			
Treatment	53.600	4	13.400	4.7857	0.020381			
Error	28.000	10	2.800					

Week 6 – Treatment versus Number of Hydra at 144 Hours – Sunlight Environment – Post hoc – Tukey Test

Cell No.	Tukey HSD test; variable Number of Hydra (Template File for Time Data.sta) Homogenous Groups, alpha = .05000 Error: Between MS = 2.8000, df = 10.000 Treatment	Number of Hydra Mean	1	2	NewVar 1	NewVar 2	NewVar 3
2	BRAND A - PETE	8.33333	****				
5	LAB WATER - PC	8.33333	****				
4	LAB WATER - GLASS	11.00000	****	****			
3	LAB WATER - PETE	11.00000	****	****			
1	Control	13.33333		****			

Week 6 – Treatment versus Number of Hydra at 168 Hours – Sunlight Environment - One Way ANOVA (Significance – $p < 0.05$)

Effect	Univariate Tests of Significance for Number of Hydra (Template File for Time Data.sta) Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2	NewVar 3
Intercept	2331.267	1	2331.267	760.1957	0.000000			
Treatment	195.067	4	48.767	15.9022	0.000246			
Error	30.667	10	3.067					

Week 6 – Treatment versus Number of Hydra at 168 Hours – Sunlight Environment – Post hoc – Tukey Test

Cell No.	Tukey HSD test; variable Number of Hydra (Template File for Time Data.sta) Homogenous Groups, alpha = .05000 Error: Between MS = 3.0667, df = 10.000						
	Treatment	Number of Hydra Mean	1	2	3	NewVar 1	NewVar 2
2	BRAND A - PETE	8.33333	****				
5	LAB WATER - PC	9.66667	****	****			
3	LAB WATER - PETE	12.00000	****	****			
4	LAB WATER - GLASS	13.66667		****			
1	Control	18.66667			****		

Week 6 – Treatment versus Number of Hydra at 144 Hours – Darkness Environment - One Way ANOVA (Significance – $p < 0.05$)

Effect	Univariate Tests of Significance for Number of Hydra (Template File for Time Data.sta) Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2	NewVar 3
Intercept	1560.600	1	1560.600	410.6842	0.000000			
Treatment	58.400	4	14.600	3.8421	0.038347			
Error	38.000	10	3.800					

Week 6 – Treatment versus Number of Hydra at 144 Hours – Darkness Environment – Post hoc – Tukey Test

Tukey HSD test; variable Number of Hydra (Template File for Time Data.sta
Homogenous Groups, alpha = .05000
Error: Between MS = 3.8000, df = 10.000

Cell No.	Treatment	Number of Hydra Mean	1	2	NewVar	NewVar 1
4	LAB WATER - GLASS	8.00000	****			
2	BRAND A - PETE	8.33333	****	****		
5	LAB WATER - PC	10.00000	****	****		
3	LAB WATER - PETE	11.33333	****	****		
1	Control	13.33333		****		

Week 6 – Treatment versus Number of Hydra at 168 Hours – Darkness Environment - One Way ANOVA (Significance – p < 0.05)

Univariate Tests of Significance for Number of Hydra (Template File for Time Data.sta
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2	NewVar 3
Intercept	2356.267	1	2356.267	380.0430	0.000000			
Treatment	185.733	4	46.433	7.4892	0.004662			
Error	62.000	10	6.200					

Week 6 – Treatment versus Number of Hydra at 168 Hours – Darkness Environment – Post hoc – Tukey Test

Tukey HSD test; variable Number of Hydra (Template File for Time Data.sta)
Homogenous Groups, alpha = .05000
Error: Between MS = 6.2000, df = 10.000

Cell No.	Treatment	Number of Hydra Mean	1	2	NewVar 1	NewVar 2	NewVar 3
4	LAB WATER - GLASS	9.00000	****				
2	BRAND A - PETE	9.33333	****				
5	LAB WATER - PC	12.00000	****	****			
3	LAB WATER - PETE	13.66667	****	****			
1	Control	18.66667		****			

Appendix 7:

Week 11 – Replicates versus Number of Hydra – One Way ANOVA

Effect	Univariate Tests of Significance for Number of Hydra (Week 11 Spreadsheet.sta) Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2	NewVar 3
Intercept	9936.227	1	9936.227	1327.057	0.000000			
Replicates	5.954	2	2.977	0.398	0.672441			
Error	1594.819	213	7.487					

Week 11 – Environment versus Number of Hydra – One Way ANOVA

Effect	Univariate Tests of Significance for Number of Hydra (Week 11 Spreadsheet.sta) Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2	NewVar 3
Intercept	8413.755	1	8413.755	1464.281	0.000000			
Environment	11.505	1	11.505	2.002	0.158698			
Error	1091.740	190	5.746					

Week 11 – Treatment versus Number of Hydra at 168 Hours – Sunlight Environment - One Way ANOVA (Significance – $p < 0.05$)

Effect	Univariate Tests of Significance for Number of Hydra (Template File for Tim Sigma-restricted parameterization Effective hypothesis decomposition						
	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2
Intercept	2018.400	1	2018.400	445.2353	0.000000		
Treatment	132.267	4	33.067	7.2941	0.005119		
Error	45.333	10	4.533				

Week 11 – Treatment versus Number of Hydra at 168 Hours – Sunlight Environment – Post hoc – Tukey Test

Tukey HSD test; variable Number of Hydra (Template File for Time Data.sta
Homogenous Groups, alpha = .05000
Error: Between MS = 4.5333, df = 10.000

Cell No.	Treatment	Number of Hydra Mean	1	2	NewVar 1	NewVar 2
2	BRAND A - PETE	9.33333	****			
5	LAB WATER - PC	9.33333	****			
4	LAB WATER - GLASS	10.66667	****			
3	LAB WATER - PETE	11.33333	****			
1	Control	17.33333		****		

Week 11 – Treatment versus Number of Hydra at 168 Hours – Darkness Environment - One Way ANOVA (Significance – p < 0.05)

Univariate Tests of Significance for Number of Hydra (Template File for Time Data.sta)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p	**NewVar 1**	NewVar 2	NewVar 3
Intercept	2432.067	1	2432.067	1520.042	0.000000			
Treatment	86.933	4	21.733	13.583	0.000474			
Error	16.000	10	1.600					

Week 11 – Treatment versus Number of Hydra at 168 Hours – Darkness Environment – Post hoc – Tukey Test

Tukey HSD test; variable Number of Hydra (Template File for Time Data.sta
Homogenous Groups, alpha = .05000
Error: Between MS = 1.6000, df = 10.000

Cell No.	Treatment	Number of Hydra Mean	1	2	NewVar 1	NewVar 2
5	LAB WATER - PC	10.66667	****			
2	BRAND A - PETE	11.00000	****			
4	LAB WATER - GLASS	12.00000	****			
3	LAB WATER - PETE	12.66667	****			
1	Control	17.33333		****		

Appendix 8:

In-Lab Experiment Statistics:

DEHP Chronic Toxicity Test:

DEHP – Replicates versus Number of Hydra – One Way ANOVA

Effect	Univariate Tests of Significance for Number of Hydra (DEHP.sta) Sigma-restricted parameterization Effective hypothesis decomposition						
	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2
Intercept	19814.70	1	19814.70	515.3368	0.000000		
Replicates	48.65	2	24.32	0.6326	0.532995		
Error	4498.65	117	38.45				

DEHP – Concentration versus Number of Hydra at 168 Hours - One Way ANOVA (Significance – $p < 0.05$)

Effect	Univariate Tests of Significance for Number of Hydra (Template - 168 Hours.sta) Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2	NewVar 3
Intercept	8881.667	1	8881.667	637.4402	0.000000			
Concentration	238.000	4	59.500	4.2703	0.028516			
Error	139.333	10	13.933					

DEHP – Concentration versus Number of Hydra at 168 Hours – Darkness Environment – Post hoc – Tukey Test

Cell No.	Tukey HSD test; variable Number of Hydra (Template - 168 Hours.sta) Homogenous Groups, alpha = .05000 Error: Between MS = 13.933, df = 10.000					
	Concentration	Number of Hydra Mean	1	2	NewVar 1	NewVar 2
5	0.1 ug/mL DEHP	17.33333	****			
4	0.01 ug/mL DEHP	23.33333	****	****		
2	0.0001 ug/mL DEHP	25.00000	****	****		
1	0 ug/mL (Control)	27.00000	****	****		
3	0.001 ug/mL DEHP	29.00000		****		

Appendix 9:

DBP Chronic Toxicity Test:

DBP – Replicates versus Number of Hydra – One Way ANOVA

Effect	Univariate Tests of Significance for Number of Hydra (DBP Spreadsheet.sta) Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. of Freedom	MS	F	p	NewVar	NewVar 1	NewVar 2
Intercept	36750.00	1	36750.00	357.1799	0.000000			
Replicates	49.95	2	24.97	0.2427	0.784872			
Error	12038.05	117	102.89					

DBP – Concentration versus Number of Hydra at 168 Hours - One Way ANOVA (Significance – $p < 0.05$)

Effect	Univariate Tests of Significance for Number of Hydra (DBP Spreadsheet.sta) Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. of Freedom	MS	F	p	NewVar 1	**NewVar 2**	NewVar 1
Intercept	19512.07	1	19512.07	1925.533	0.000000			
Concentration	279.60	4	69.90	6.898	0.006221			
Error	101.33	10	10.13					

DBP – Concentration versus Number of Hydra at 168 Hours – Darkness Environment – Post hoc – Tukey Test

Cell No.	Tukey HSD test; variable Number of Hydra (DBP Spreadsheet.sta) Homogenous Groups, alpha = .05000 Error: Between MS = 10.133, df = 10.000						
	Concentration	Number of Hydra Mean	1	2	3	NewVar 1	NewVar 2
5	0.1 ug/mL DBP	30.66667	****				
1	0 ug/mL (Control)	32.33333	****	****			
4	0.01 ug/mL DBP	35.66667	****	****	****		
2	0.0001 ug/mL DBP	39.33333		****	****		
3	0.001 ug/mL DBP	42.33333			****		

Appendix 10:

BPA Chronic Toxicity Test:

BPA – Replicates versus Number of Hydra – One Way ANOVA

Effect	Univariate Tests of Significance for Number of Hydra (BPA Spreadsheet.sta) Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2	NewVar 3
Intercept	23998.41	1	23998.41	433.4467	0.000000			
Replicates	12.72	2	6.36	0.1148	0.891608			
Error	6477.88	117	55.37					

BPA – Concentration versus Number of Hydra at 168 Hours - One Way ANOVA (Significance – $p < 0.05$)

Effect	Univariate Tests of Significance for Number of Hydra (BPA Spreadsheet.sta) Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. of Freedom	MS	F	p	NewVar 1	NewVar 2	NewVar 1
Intercept	12212.27	1	12212.27	2475.459	0.000000			
Concentration	152.40	4	38.10	7.723	0.004178			
Error	49.33	10	4.93					

BPA – Concentration versus Number of Hydra at 168 Hours – Darkness Environment – Post hoc – Tukey Test

Cell No.	Tukey HSD test; variable Number of Hydra (BPA Spreadsheet.sta) Homogenous Groups, alpha = .05000 Error: Between MS = 4.9333, df = 10.000					
	Concentration	Number of Hydra Mean	1	2	NewVar 1	NewVar 2
2	0.0001 ug/mL BPA	22.33333		****		
5	0.1 ug/mL BPA	28.66667	****			
1	0 ug/mL (Control)	30.33333	****			
4	0.01 ug/mL BPA	30.66667	****			
3	0.001 ug/mL BPA	30.66667	****			

Appendix 11:

Temperature Data from Weather Station (Oshawa, Ontario):

Date:	Temp. (°C):	Precip. (mm):	Date:	Temp. (°C):	Precip. (mm):
Aug. 28	19.05	2.20	Sept. 17	21.60	0.00
Aug. 29	21.05	13.60	Sept. 18	16.32	0.00
Aug. 30	21.25	0.00	Sept. 19	17.27	0.00
Aug. 31	20.10	0.00	Sept. 20	21.15	0.00
Sept. 1	20.30	0.00	Sept. 21	14.86	0.00
Sept. 2	25.36	0.00	Sept. 22	16.73	0.00
Sept. 3	25.43	0.20	Sept. 23	18.87	0.00
Sept. 4	24.07	0.00	Sept. 24	21.75	0.00
Sept. 5	23.98	0.40	Sept. 25	23.67	0.00
Sept. 6	18.41	13.20	Sept. 26	19.98	0.00
Sept. 7	14.31	8.70	Sept. 27	17.89	3.20
Sept. 8	19.91	7.80	Sept. 28	17.92	0.40
Sept. 9	17.08	13.20	Sept. 29	13.69	0.00
Sept. 10	15.39	0.00	Sept. 30	16.13	16.00
Sept. 11	18.91	0.00	Oct. 1	12.63	0.00
Sept. 12	21.34	0.40	Oct. 2	11.61	2.00
Sept. 13	19.98	26.00	Oct. 3	19.20	0.00
Sept. 14	25.28	16.80	Oct. 4	13.30	0.00
Sept. 15	14.67	0.40	Oct. 5	12.60	0.00
Sept. 16	15.63	0.00	Oct. 6	12.80	0.00

Temperature Data from Weather Station (Oshawa, Ontario) continued...

Date:	Temp. (°C):	Precip. (mm):
Oct. 7	15.20	0.00
Oct. 8	16.30	12.50
Oct. 9	20.50	0.00
Oct. 10	17.90	0.00
Oct. 11	20.40	0.00
Oct. 12	22.50	0.00
Oct. 13	26.30	0.00
Oct. 14	9.65	0.00
Oct. 15	9.32	3.60
Oct. 16	10.56	1.80
Oct. 17	5.30	0.00
Oct. 18	4.53	0.00
Oct. 19	6.56	0.00
Oct. 20	5.44	8.60
Oct. 21	3.42	1.20

*** August 7th to August 27th was unavailable due to malfunctioning of weather station**

Appendix 12:

In-Lab Temperature Data:

Date:	Temp. (°C):	Date:	Temp. (°C):	Date:	Temp. (°C):
Aug. 7	21.5	Aug. 27	21.4	Sept. 16	21.5
Aug. 8	21.5	Aug. 28	21.5	Sept. 17	21.5
Aug. 9	21.5	Aug. 29	21.5	Sept. 18	21.2
Aug. 10	21.2	Aug. 30	21.4	Sept. 19	21.4
Aug. 11	21.4	Aug. 31	21.5	Sept. 20	21.5
Aug. 12	21.5	Sept. 1	21.5	Sept. 21	21.5
Aug. 13	21.5	Sept. 2	21.5	Sept. 22	21.5
Aug. 14	21.5	Sept. 3	21.5	Sept. 23	21.5
Aug. 15	21.4	Sept. 4	21.4	Sept. 24	21.5
Aug. 16	21.4	Sept. 5	21.5	Sept. 25	21.3
Aug. 17	21.5	Sept. 6	21.5	Sept. 26	21.2
Aug. 18	21.5	Sept. 7	21.5	Sept. 27	21.4
Aug. 19	21.5	Sept. 8	21.4	Sept. 28	21.5
Aug. 20	21.5	Sept. 9	21.5	Sept. 29	21.5
Aug. 21	21.5	Sept. 10	21.4	Sept. 30	21.5
Aug. 22	21.4	Sept. 11	21.4	Oct. 1	21.4
Aug. 23	21.5	Sept. 12	21.5	Oct. 2	21.5
Aug. 24	21.5	Sept. 13	21.5	Oct. 3	21.5
Aug. 25	21.5	Sept. 14	21.5	Oct. 4	21.4
Aug. 26	21.5	Sept. 15	21.4	Oct. 5	21.3

In-Lab Temperature Data continued...

Date:	Temp. (°C):
Oct. 6	21.3
Oct. 7	21.3
Oct. 8	21.3
Oct. 9	21.3
Oct. 10	21.3
Oct. 11	21.3
Oct. 12	21.3
Oct. 13	21.3
Oct. 14	21.3
Oct. 15	21.3
Oct. 16	21.4
Oct. 17	21.3
Oct. 18	21.3
Oct. 19	21.3
Oct. 20	21.3
Oct. 21	21.3

Appendix 13:

YORK-DURHAM
Regional Environmental Laboratory

901 McKay Road Pickering, Ontario L1W 3A3 Telephone: (905) 686-0041Fax: (905) 686-0664

ORGANICS ANALYSIS REPORT

Work Order : 2105823 **Submission :** 146384

Comments:

Empty cell = Analysis not requested
< = Less than
^ = Result exceeds limit
MDL = Method detection limit
E = Sample exhausted
I = Possible interference interpret with caution

	Analyte	MDL/Units	Limit	Sample I.D.: 455330 Waterworks No.: N/A Sample Location: Bottled Water Sub Location: Leaching Study Sample Type: Pete - Sun - LW - Date Sampled: Week 11 - MG Client/Field I.D.: Cartridge 2008-10-31 4
BphnAphthalates	Bis(2-ethylhexyl)phthalate	0.08 ug/L		0.10
	Bisphenol-A	0.08 ug/L		<0.08
	Di-n-butyl phthalate	0.08 ug/L		<0.08

Comments:

Empty cell = Analysis not requested
< = Less than
^ = Result exceeds limit
MDL = Method detection limit
E = Sample exhausted
I = Possible interference interpret with caution

	Analyte	MDL/Units	Limit	Sample I.D.: 455331 Waterworks No.: N/A Sample Location: Bottled Water Sub Location: Leaching Study Sample Type: Dasani - Sun - LW - Date Sampled: Week 11 - MG Client/Field I.D.: Cartridge 2008-10-31 5
BphnAphthalates	Bis(2-ethylhexyl)phthalate	0.08 ug/L		0.32
	Bisphenol-A	0.08 ug/L		<0.08
	Di-n-butyl phthalate	0.08 ug/L		<0.08

Comments:

Empty cell = Analysis not requested
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	Analyte	MDL/Units	Limit	Sample I.D.: 455332 Waterworks No.: N/A Sample Location: Bottled Water Sub Location: Leaching Study Sample Type: Dasani - Dark - LW - Date Sampled: Week 11 - MG Client/Field I.D.: Cartridge 2008-10-31 6
BphnAphthalates	Bis(2-ethylhexyl)phthalate	0.08 ug/L		<0.08
	Bisphenol-A	0.08 ug/L		<0.08
	Di-n-butyl phthalate	0.08 ug/L		<0.08

Comments:

Empty cell = Analysis not requested
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E = Sample exhausted
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	Analyte	MDL/Units	Limit	Sample I.D.: 455333 Waterworks No.: N/A Sample Location: Bottled Water Sub Location: Leaching Study Sample Type: Glass - Sun - LW - Date Sampled: Week 11 - MG Client/Field I.D.: Cartridge 2008-10-31 7
BphnAphthalates	Bis(2-ethylhexyl)phthalate	0.08 ug/L		0.32
	Bisphenol-A	0.08 ug/L		<0.08
	Di-n-butyl phthalate	0.08 ug/L		<0.08

YORK-DURHAM
Regional Environmental Laboratory

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ORGANICS ANALYSIS REPORT

Work Order : 2105823 **Submission :** 146384

Comments:

Empty cell = Analysis not requested
< = Less than
^ = Result exceeds limit
MDL = Method detection limit
E = Sample exhausted
I = Possible interference interpret with caution

	Analyte	MDL/Units	Limit	Sample I.D.: 455334 Waterworks No.: N/A Sample Location: Bottled Water Sub Location: Leaching Study Sample Type: Glass - Dark - LW - Week 11 - MG Client/Field I.D.: Cartridge 2008-10-31 8
BphnAphthalates	Bis(2-ethylhexyl)phthalate	0.08 ug/L		0.34
	Bisphenol-A	0.08 ug/L		<0.08
	Di-n-butyl phthalate	0.08 ug/L		<0.08

Comments:

Empty cell = Analysis not requested
< = Less than
^ = Result exceeds limit
MDL = Method detection limit
E = Sample exhausted
I = Possible interference interpret with caution

	Analyte	MDL/Units	Limit	Sample I.D.: 455335 Waterworks No.: N/A Sample Location: Bottled Water Sub Location: Leaching Study Sample Type: PC - Sun - LW - Week 11 - MG Client/Field I.D.: Cartridge 2008-10-31 9
BphnAphthalates	Bis(2-ethylhexyl)phthalate	0.08 ug/L		0.09
	Bisphenol-A	0.08 ug/L		0.90
	Di-n-butyl phthalate	0.08 ug/L		<0.08

Comments:

Empty cell = Analysis not requested
< = Less than
^ = Result exceeds limit
MDL = Method detection limit
E = Sample exhausted
I = Possible interference interpret with caution

	Analyte	MDL/Units	Limit	Sample I.D.: 455336 Waterworks No.: N/A Sample Location: Bottled Water Sub Location: Leaching Study Sample Type: PC - Dark - LW - Week 11 - MG Client/Field I.D.: Cartridge 2008-10-31 10
BphnAphthalates	Bis(2-ethylhexyl)phthalate	0.08 ug/L		0.61
	Bisphenol-A	0.08 ug/L		0.93
	Di-n-butyl phthalate	0.08 ug/L		<0.08

Approved By:

Marco Giuliacci, Group Leader (Ext 4321)

J. Mirsch, Superintendent (Ext 4304)

ASSOCIATION OF THE CHEMICAL PROFESSION OF ONTARIO
CHARTERED
Jurgen Mirsch
CHEMIST

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ORGANICS ANALYSIS REPORT

Work Order : 2105823 **Submission :** 146384

ORGANICS ANALYSIS SUMMARY

TEST GROUP	QUANTITY	SAMPLE MATRIX	INSTRUMENT	METHOD
BphnAphthalates	10	Water	GC/MS EXTR	SPE GCMS

NOTE: All supporting analytical information including measurement uncertainty is available upon request.

Report Comments:

LAB SAMPLE_#	COMMENT
Results are not blank corrected.	

York-Durham Regional Environmental Laboratory

901 McKay Road,
Pickering, Ontario
L1W 3A3
Phone: 905-686-0041 Fax: 905-686-0664

Date: June 24, 2009

SPIKE AND SURROGATE RECOVERIES

Blank Surrogate Recovery

Analyte	% Recovery
Di-n-butyl phthalate-D4	84
Bis-2-ethylhexylphthalate-D4	42

Spike 1 Recovery (0.11ug/L) and Surrogate Recovery

Analyte	% Recovery
Di-n-butyl phthalate	60
Bis-2-ethylhexylphthalate	63
Bisphenol A	150

Analyte	% Recovery
Di-n-butyl phthalate - D4	46
Bis-2-ethylhexylphthalate - D4	52

Spike 2 Recovery (0.53ug/L) and Surrogate Recovery

	Spike	Spk Duplicate
Analyte	% Recovery	% Recovery
Di-n-butyl phthalate	48	50
Bis-2-ethylhexylphthalate	49	50
Bisphenol A	59	67

	Spike	Spk Duplicate
Analyte	% Recovery	% Recovery
Di-n-butyl phthalate - D4	46	42
Bis-2-ethylhexylphthalate - D4	26	58