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Toxicity and bioaccumulation of 2,2', 4,4'-tetrabromodiphenyl ether (BDE47) in a laboratory aquatic food chain

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TOXICITY AND BIOACCUMULATION OF 2,2´,4,4´-TETRABROMODIPHENYL ETHER (BDE47) IN A LABORATORY AQUATIC FOOD CHAIN

by

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ABSTRACT

TOXICITY AND BIOACCUMULATION OF 2,2′,4,4′-TETRABROMODIPHENYL ETHER (BDE47) IN A LABORATORY AQUATIC FOOD CHAIN

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Rising levels of polybrominated diphenyl ethers (PBDEs) have been observed in the environment, humans, and animals. Studies have shown that these compounds can elicit toxic effects in animals (e.g. neurotoxicity and thyroid toxicity). This research investigated the effects of BDE47 on the survival and reproduction of *Daphnia magna* over two generations. The impacts of water-borne exposure were compared to dietary exposure using the following treatments: dosed water (DW), dosed algae (DA) and dosed water and algae (DWA). In the first generation, significant impacts on reproduction were observed in daphnids in the DA and DWA treatments. In the second generation, no significant impacts on reproduction were observed indicating a recovery from maternal exposure. When second generation daphnids were exposed to BDE47, there was high mortality in the DWA treatment and reduced reproduction in all dosed treatments. Dietary exposure to BDE47 had a more profound impact on daphnid reproduction than water exposure. In the second generation, dietary exposure affected both survival and reproduction and water exposure reduced reproduction, indicating that maternal exposure was a factor.

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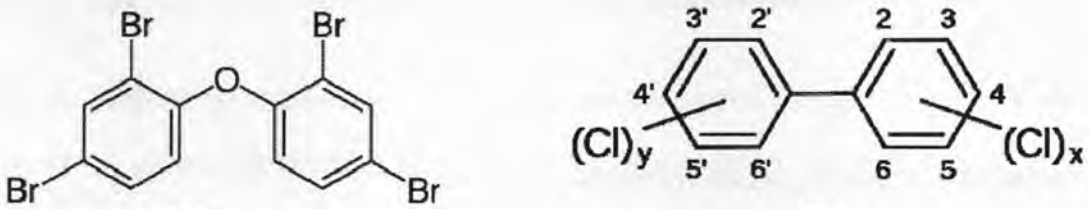
1.0 INTRODUCTION

Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardants that are added to consumer products (e.g. home electronics, furniture, and textiles) to prevent or minimize fires (Alaee *et al.*, 2003). Recently, the importance of these compounds has grown due to the increasing use of flammable materials (i.e. polymers) in consumer products. PBDEs, like other brominated flame retardants, provide people with greater protection from fires and reduce the costs associated with fire damage. These compounds interrupt the free radical chain of combustion; they decompose before the polymer, preventing the formation of flammable gases and decreasing and/or delaying the occurrence of a major fire (Rahman *et al.*, 2001; D'Silva *et al.*, 2004). PBDEs are additive flame retardants, those which are blended with the polymer before, during, or after polymerization along with plasticizers. Because PBDEs are not permanently bound to the polymer, they are therefore more likely to leach out into the environment (D'Silva *et al.*, 2004) and this has resulted in significant increases in levels of these compounds in the terrestrial and aquatic environment over the past few decades (Meironyté & Norén 1999; Norstrom *et al.*, 2002; Zhu & Hites, 2004).

PBDEs have been in use since the 1970s, historically as hydraulic fluid by the German coal mining industry as well as the U.S. offshore oil drilling industry during the early 1990s (Sellström, 1996; Renner, 2000). The structure of PBDEs (Figure 1) is similar to polychlorinated biphenyls (PCBs) with the exception of an ether bond joining the two biphenyl rings.



BDE47 PCB

Figure 1. Chemical structure of BDE47 versus the general PCB structure.

Theoretically, 209 possible congeners can form; however, due to the directing properties of the oxygen and steric hindrance (Alaee *et al.*, 2003) fewer congeners exist. There are three PBDE technical mixtures, pentaBDE, octaBDE and decaBDE. The congener 2,2'-4,4'-tetrabromodiphenyl ether (BDE47) is one of the main components (37%) of the the pentaBDE technical mixture, Bromkal 70-5DE, along with BDE99 (Sjödin *et al.*, 1998). It is principally used in polyurethane foams (Alcock *et al.*, 2003) and until the mid-1990s, in circuit boards (Hale *et al.*, 2003). Table 1 shows the use of PBDE commercial mixtures in various applications. DecaBDE, currently the dominant PBDE mixture in use, has largely replaced the penta and octaBDE mixtures due to concerns over toxicity to humans and wildlife.

Table 1. Applications of the PBDE commercial mixtures (Source: D'Silva *et al.*, 2004).

Resins and polymers	DecaBDE	OctaBDE	PentaBDE
Acrylonitrile–butadiene–styrene		√	
Epoxy-resins	√		
Phenolic resins	√		√
Polyacrylonitrile	√		
Polyamide	√	√	
Polybutadiene terephthalate	√	√	
Polyethylene	√		
Polyethylene terephthalate	√		
Polypropylene	√		
Polystyrene (high impact)	√	√	
Polyvinyl chloride	√		√
Polurethane			√
Polyesters	√		√
Rubber	√		√
Paints/lacquers	√		√
Textiles	√		√

PBDEs are persistent, bioaccumulative, and toxic to some animals (Sellström *et al.*, 1996). Generally, the BDE congeners have low vapour pressures (3.85 to 13.3 Pa at 20-25°C) (WHO, 1994), low water solubilities, (WHO, 1994), and are lipophilic (WHO, 1994; de Wit, 2002). The log K_{ow} values are greater than five (indicating bioaccumulation) and increase with increasing bromination. Due to their chemical and physical properties, PBDEs are persistent and bioaccumulative, and can thus biomagnify up the food chain, resulting in their accumulation in

humans and wildlife. Additionally, various toxic effects, including neurotoxicity, endocrine disruption, reproductive toxicity, and immunotoxicity, have been reported in laboratory animals exposed to PBDEs (Meerts *et al.*, 2000; Eriksson *et al.* 2001; Zhou *et al.* 2001; Hallgren & Darnerud, 2002). This has raised public concern over the widespread usage of these contaminants and possible environmental and human health effects. Select physicochemical and toxicological properties of BDE47 are listed in Tables 2 and 3.

Table 2. Select physicochemical properties of BDE47.

Physicochemical Properties		
BDE47: 2,2',4,4'-tetrabromodiphenyl ether ($C_{12}H_6Br_4O$)		
Molecular Weight	g/mol	485.8
Melting Point (T_m)	°C	83.5-84.5[a]
Water Solubility ($S_{w,25°C}$)	µg/L	15[a], 10.9[b]
Henry's Law Constant ($H_{25°C}$)	Pa·m^3/mol	1.5[a]
Liquid subcooled vapour pressure ($P°_{L,25°C}$)	Pa	1.86×10^{-4} [a], 3.19×10^{-4} [c]
Log K_{OW}		6.39[d], 6.81[e]
Log K_{OA}		10.53[f], 10.44[d]

[a]Tittlemier *et al.*, 2002; [b]EU, 2000; [c]Wong *et al.*, 2001; [d]Wania & Dugani, 2003; [e]Braekevelt *et al.*, 2003; [f]Harner & Shoeib, 2002

Table 3. Toxicity of BDE47 and the PentaBDE technical mixture (which contains a large proportion of BDE47) on aquatic organisms (green algae and *Daphnia*).

Toxicity			
Organism	**Toxicity Endpoint**	**BDE47 (µg/L)**	**PentaBDE (µg/L)**
Algae			
Skeletonema costatum	NOEC	6.6[a]	
S. costatum	LOEC	21[a]	
S. costatum	EC_{50}	70[a]	
Pseudokirchneriella subcapitata	NOEC		3.3-6.5[b]
Daphnia			
Daphnia magna	NOEC	14[a*]	4.9[b], 5.3[b*]
D. magna	LOEC	45[a*]	9.8[b*]
D. magna	L/EC_{50}	92[c]	14[b*]
Ceriodaphnia dubia	NOEC	1.4[d*]	
C. dubia	LOEC	2.0[d*]	
C. dubia	LC_{50}	4.6, 2.95[d]	

[a]Kallqvist *et al.*, 2006; [b]EU, 2000; [c]Masekoameng, 2006; [d]Wisconsin DNR, 2004
*chronic, 21 day

Many jurisdictions have restricted or banned the use and production of some PBDE technical mixtures, mainly the penta and octaBDE mixtures. In 2003, Great Lakes Chemical (the only U.S. producer) agreed to voluntarily discontinue the production of penta and octaBDE mixtures by the end of 2004, due to pressure from the United States Environmental Protection Agency (USEPA) (Talsness, 2008). Products containing pentaBDE and octaBDE have recently been prohibited in 10 states and partial restrictions on the use of decaBDE have also been approved in Maine and Washington (Talsness, 2008). In all jurisdictions, there has been a reluctance to regulate the decaBDE mixture due its widespread use and reported safety. Canada currently has no legislation in place

restricting the use of PBDEs. These compounds are not produced in Canada but are imported (mostly from the U.S.); therefore, it was determined that restrictions were unnecessary (EC, 2004).

Use of the pentaBDE mixture has been restricted within Europe for over 10 years (Alcock *et al.*, 2003) and both the penta and octaBDE mixtures have been banned from all use in the European Union since 2004 (Talsness, 2008). The decaBDE mixture, which was originally included in this ban, was granted an exemption by the European Commission in 2005 which was subsequently annulled in April 2008 due to procedural errors (EBFRIP, 2008; Talsness, 2008).

Presently, the focus of most of the research in the literature is on levels and effects of PBDEs in humans. Humans may be exposed to these compounds through food consumption and occupational exposure. Up until now, toxicity studies have mainly been performed on laboratory animals in order to estimate potential human toxicity. Very little research has focused strictly on the toxic effects of PBDEs on animals, especially in aquatic systems. Some research on PBDE levels and bioaccumulation in aquatic systems is currently available in the literature; however, there is a lack of research focusing on aquatic toxicity and trophic transfer of PBDEs.

1.1 Sources and Fate of PBDEs

PBDEs can contaminate the environment through various sources, including releases from production sites, leaching from landfills, discharge from wastewater treatment plants, emissions from incineration and recycling sites, as well as natural sources. Output from PBDE production facilities may contribute significantly to regional levels but do not have major impacts on national or international levels (Hale *et al.*, 2003). However, several facilities in operation incorporate PBDEs into polymers and other products, which may have larger impacts on the levels in the environment (Hale *et al.*, 2003). Disposal and recycling of PBDEs have also been identified as possible sources. Products containing PBDEs can be either incinerated or disposed of in landfills. Limited leaching and volatilization from landfills is hypothesized (Palm *et al.*, 2002; Alcock *et al.*, 2003); however, since PBDEs are persistent, this may be a significant

continuous diffuse source (WHO, 1994) to the environment. Hale *et al.* (2002) showed that after exposure to outdoor conditions for four weeks, polyurethane foam containing pentaBDE became brittle and began to disintegrate. As polyurethane foams are used in many products which are ultimately disposed of, this may be a major route for PBDEs to enter the environment. PBDE levels in the outdoor environment are expected to increase in well populated areas due to the added product use (Palm *et al.*, 2002). Landfill fires are a possible source of brominated or mixed chlorinated and brominated dioxins and furans to the atmosphere and surrounding environment (WHO, 1994).

Electronic waste ("E-waste") is becoming of greater concern as the demand for newer and faster technology rises and the life span of these electronics decline. Older and out-dated electronics are accumulating in landfills and recycling depots in significant amounts worldwide, the vast majority of which end up in Asia due to the low labour costs and less stringent environmental regulations (Wong *et al.*, 2007). Primitive recycling techniques used in these facilities have resulted in the release of toxic compounds into the air and surrounding environment, ultimately impacting the health of the workers and residents in nearby communities (Wong *et al.*, 2007).

Throughout the life of a product, breakdown can occur, releasing PBDE gases or particulates (dust). This can be very important in indoor environments, where many flame retarded products are in use. Unlike PCBs, for which the usual route of exposure is via food (of animal origin), PBDE exposure in humans is more likely from indoor exposure (Schecter *et al.*, 2005). High levels of PBDEs have been found in indoor air, house dust, and dryer lint (Schecter *et al.*, 2005; Stapleton *et al.*, 2005). Indoor sources become even more important in areas with colder climates where people spend more time indoors. PBDEs in indoor environments can ultimately reach aquatic environments. As dust and particles are washed down the drain (i.e. from washing machines, after house-cleaning), PBDEs make their way to wastewater treatment plants and into lakes and rivers. As described below in Section 1.2.3, PBDE levels in sediment near wastewater treatment plant discharge pipes in Lake Ontario are higher than elsewhere in the lake and levels in sewage sludge are very high, indicating that these compounds are not being removed during the treatment process. There are also some natural sources of PBDEs.

Hydroxylated and methoxylated PBDEs are reportedly produced by marine sponges, algae, and acorn worms (Marsh *et al.*, 2004; Malmvärn *et al.*, 2005; Teuten *et al.*, 2005) making them difficult to distinguish from anthropogenic sources.

Alcock *et al.* (2003) used BDE47 in a mass balance model and determined that soil was the ultimate sink with an overall residence time of 125 days. Palm *et al.* (2002) also modeled PBDE fate and found soil to be the dominant sink, storing 98% of the PBDEs. These compounds are expected to bind strongly to the organic fraction of particulate matter. Loss of BDEs is greatest via reactions in soil, which become more important in the more highly brominated congeners as removal processes in air and water become less important (Palm *et al.*, 2002). Debromination of highly brominated congeners, such as BDE209, may occur in soils by photolysis (WHO, 1994), but is less likely in sediments. Biodegradation reactions (i.e. microbial activity) may also occur in sediments and soils which can result in lower brominated congeners. The bromine industry disputes this suggestion, speculating that these lower brominated congeners are a result of historic emissions (Renner, 2000).

Palm *et al.* (2002) found that all the BDE congeners could undergo moderate long range atmospheric transport. In the Great Lakes, it is believed that BDE loadings are dominated by atmospheric deposition (Dodder *et al.*, 2002), although effluent from wastewater treatment plants is likely also a major contributor. Gouin and Harner (2003) suggest that PBDEs undergo surface-to-air exchange as a result of seasonal variation in temperature which may allow for the "grasshopper effect", a series of deposition and volatilization "hops". They also hypothesized a spring pulse of PBDEs into the atmosphere following winter snow scavenging processes. Additionally, it was speculated that vegetation can have a filtering effect by scavenging the PBDEs from the atmosphere. Palm *et al.* (2002) concluded that tetra and pentaBDE congeners are of most concern due to their bioavailability, whereas decaBDE is of less concern because it binds mostly to soils and sediments. However, in aquatic systems, partitioning to sediments may become a significant source to benthic invertebrates and higher trophic organisms through biomagnification.

1.2 Environmental Levels

Due to their high hydrophobicity, PBDEs are not very soluble in water; however, they still enter the aquatic environment and accumulate in sediment and organisms (Breitholtz & Wollenberger, 2003). PBDEs were first reported in fish collected from the Viskan River in Sweden in 1981 (Andersson & Blomkvist, 1981), and since then have been reported in air (Strandberg *et al.,* 2001), water (Hale *et al.*, 2003), soil (Hale *et al.*, 2003), sediment (Song *et al.*, 2004, 2005a, 2005b), biota (Manchester-Neesvig *et al.*, 2001; Norstrom *et al.*, 2002), and human blood, adipose tissue, and milk (Ryan & Patry, 2000; She *et al.*, 2002; Thomsen *et al.*, 2002). In contrast to other halogenated organic compounds (e.g. PCBs, DDE), levels of PBDEs in the environment have been increasing considerably since manufacture began in the 1970s, reflecting an increase in production and use. The lower brominated congeners, which make up a large portion of the pentaBDE mixture, are most often observed in environmental matrices even though there has been a marked reduction in their use, likely due to past use and debromination of the higher brominated congeners (e.g. BDE209; Lorber, 2008).

1.2.1 Humans

Worldwide concern over human exposure to PBDEs developed when Meironyté & Norén (1999) released data showing exponentially increasing levels of total PBDEs in pooled human milk samples from Swedish mothers. The study showed an increase from 0.07ng/g lipid in 1972 to 4.02ng/g lipid in 1997; a doubling time of five years (Norén & Meironyté, 2000). In all of the milk samples, BDE47 dominated at 60 to 70% of the total, followed by BDE99 and 153. In subsequent research (Figure 2), a decrease in PBDE levels in Swedish mother's milk after 1997 was observed, possibly due to restrictions on the use of the penta- and octa-BDE mixtures (Meironyté Guvenius & Norén, 2001). In Canada, Ryan *et al.* (2002) found total PBDEs ranging from 2.6 to 281.9ng/g lipid (mean=42.8ng/g lipid) in Vancouver and Quebec mothers, an increase by an order of magnitude from a mean of 4.7ng/g lipid in 1992 (Ryan & Patry, 2000). Again, BDE47 dominated, followed by smaller but significant contributions from BDE99, 153, 100, 28, 154, and 183, respectively. In breast milk samples from Texas, U.S.A., total PBDEs ranged from 6.2 to 419ng/g lipid (mean=73.9ng/g lipid), up to 100 times the levels in Swedish mothers, but only 1.5-fold higher than Canadian mothers (Schecter *et al.*, 2003). The congener

pattern was similar in the U.S. samples, with BDE47 dominating at 54% of the total PBDEs. It has been estimated that exposure to indoor dust accounts for 80 to 90% of human exposure, with food ingestion the primary source of the remainder (Lorber, 2008).

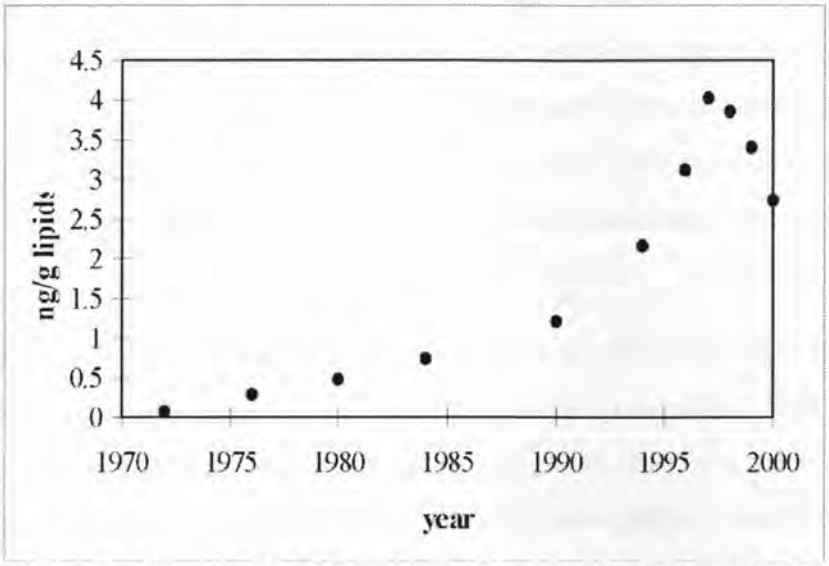


Figure 2. Mean total PBDEs in Swedish mother's milk from 1972 to 2000 (Source: Meironyté & Norén, 2001).

Meironyté Guvenius *et al.* (2003) analyzed maternal blood, breast milk, and cord blood from Swedish mothers and their offspring to check for maternal transfer. Median PBDEs in the maternal blood and breast milk were similar (2.07 and 2.14ng/g lipid, respectively), whereas the cord blood was lower (1.69ng/g lipid). Median PCB levels in all samples were two orders of magnitude higher than PBDEs. BDE47 dominated the samples, followed by BDE153, 99, and 100. The results showed that the lower brominated congeners crossed the placenta to a higher extent than the higher brominated congeners. However, Mazdai *et al.* (2003) determined that all PBDE congeners had a similar potential for placental transfer. They analyzed cord and maternal blood plasma in Indiana, U.S.A. and found no statistical difference between the concentrations of total PBDEs (39 and 37ng/g lipid, respectively). As in breast milk, U.S. levels were substantially higher than those found in Sweden (Meironyté Guvenius *et al.*, 2003). Mazdai and coworkers concluded that lipophilic compounds move into the blood stream of the fetus with maternal lipids and this can have major effects on brain development during the third trimester when maternal fat stores are mobilized.

Levels in human blood have also increased in the past two decades. Thomsen *et al.* (2002) analyzed blood bank samples donated between 1977 and 1999 by Norwegian males. The concentrations of total PBDE increased from 0.44ng/g lipid in 1977 to 3.3ng/g lipid in 1999.

Again, BDE47, 99 and 153 dominated, but BDE100 was also high in the more recent samples. For 1998, samples from various age groups were analyzed to determine changes in levels and exposure. The youngest age group, ages 0 to 4 years, had the highest levels of PBDEs in their blood, possibly due to prenatal exposure as well as exposure from breast milk. She *et al.* (2002) determined levels of PBDEs in breast adipose tissue from women living in the San Francisco Bay area in the late 1990s. They found higher PBDE concentrations than any reported up until then. Total PBDEs ranged from 17.2 to 462ng/g lipid. These extremely high levels were attributed to the strict regulations in California that require all furnishings to pass flammability testing.

1.2.2 Aquatic Organisms

As stated above, PBDEs were first measured in eel and pike muscle and liver samples from the Viskan River in Sweden in 1981 (Andersson & Blomkvist, 1981). Levels were very high, even compared to recent concentrations, with total PBDEs (quantified as the sum of BDE47 and two unidentified penta congeners) in pike ranging from 20,000 to 27,000 ng/g lipid in muscle and 71,000 to 110,000 ng/g lipid in liver samples. In eel, levels were much lower, ranging from 0.001 to 0.017ng/g lipid. Upon analysis of the congener pattern, they determined that BDE47 was the most abundant congener (65 to 85% of the sum PBDEs) and that different species contained different proportions of the congeners. Sellström *et al.* (1993) found even higher levels of PBDEs, a maximum of 36,900ng/g lipid in perch, from the Viskan River in 1987. They determined that the source was the plastic and textile industries located along the river (Sellström *et al.*, 1993). In more recent years (1995), levels of PBDEs have been determined in muscle, eggs, and blood of Atlantic salmon from the Baltic Sea (Asplund *et al.*, 1999). In most samples, levels of PBDEs in the blood were highest, followed by the muscle and then eggs. The mean PBDEs (sum of BDE47, 99, and 100) were 270 to 330 ng/g lipid in the blood, 280 to 300ng/g lipid in muscle, and 98 to 100 ng/g lipid in the eggs. BDE47 was the dominant congener, followed by BDE99 and 100. Interestingly, both hydroxylated and methoxylated PBDEs were found at concentrations similar to the PBDE congeners.

Analysis of Great Lakes fish showed exponentially increasing levels of PBDEs between 1980 and 2000 (Zhu & Hites, 2004). Archived composite samples of lake trout from Lakes Michigan, Superior, Huron, and Ontario and walleye from Lake Erie were collected annually

from the same site, away from urban centres or point sources. As shown in Figure 3, the concentrations of total PBDEs (sum of BDE47, 99, 100, 153, and 154) in fish from the Great Lakes increased significantly between 1980 and 2000.

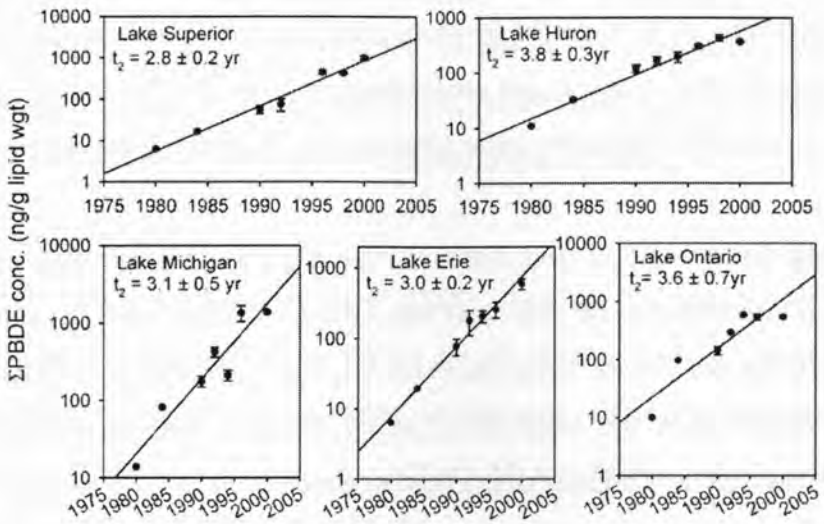


Figure 3. Total PBDE concentrations in fish from the Great Lakes. The doubling times (t_2) were calculated from the slope of the fitted exponential curves (Source: Zhu & Hites, 2004).

Doubling time of the PBDEs was calculated between 2.8 and 3.8 years, with levels in Lake Superior doubling at the fastest rate and levels in Lake Huron at the slowest. In 2000, PBDEs in lake trout from Lakes Michigan and Superior were the highest, 1400 and 990ng/g lipid, respectively, and Lakes Erie, Ontario, and Huron had lower levels, 600, 550, and 370ng/g lipid, respectively. The unexpectedly high levels in Lake Superior are likely due to atmospheric transport as well as the colder water temperatures which can slow down contaminant removal processes. In most other years, the fish from Lakes Michigan and Ontario were the most highly contaminated, likely due to the large populations living on their shores and the concentrated industrial activity. These results show PBDE levels in Great Lakes fish have been increasing exponentially and are five times higher than those in Europe (Hites, 2004). More recently, Batterman *et al.* (2007) found a different trend. Archived (1979-1995) and fresh (1996-2005) lake trout, walleye and rainbow smelt from the Great Lakes were analyzed for total PBDEs (BDE47, 100, 99 and 153) and although levels were found to increase over the 25 year period, it

was not at an exponential rate. The most recent data showed that accumulation rates were slowing and concentrations of the penta- and hexa-congeners started to decrease in lake trout from Lakes Ontario and Michigan and smelt from Lake Ontario in the mid-1990s. The authors did find a similar pattern of concentrations as Zhu & Hites (2004) in the lake trout from 2000 (i.e. highest in Lake Michigan, with similar levels in Lakes Superior and Ontario) but concluded that accumulation rates were slowing and concentrations could be decreasing. Most recent concentrations in smelt were highest in Lakes Michigan and Ontario, followed by Lake Huron, Superior, and Erie.

PBDEs have also been found in river systems around North America. Hale *et al.* (2001a) measured PBDEs in the Roanoke and Dan River watersheds in Virginia, U.S.A, in the vicinity of textile and furniture manufacturers. Total PBDEs (sum of BDE7, 99, 100, 153, 154 and 49) were highest in carp muscle from the Hyco River, at 47,900ng/g lipid, exceeding the former maximum value measured in fish (36,900ng/g lipid in perch from the Viskan River in Sweden; Sellström *et al.*, 1993). Rayne *et al.* (2003) examined levels of PBDEs in mountain whitefish and largescale suckers from the Columbia River system in the northwestern U.S.A. and Canada and found a 12-fold increase in total PBDEs (~6 to 72ng/g wet weight (ww)) between the sampling years at one site. The diet of immature largescale sucker consists of water fleas and copepods, but in adulthood, they shift to bottom feeding of invertebrates. Mountain whitefish are mainly bottom feeders, whose diet consists of aquatic insect larvae, small mollusks, and occasionally, fish. Levels of PBDEs in the largescale sucker were lower than the whitefish reflecting these trophic differences.

As expected, levels of PBDEs are generally lower in mussels than they are in fish, however, very little research has focused on invertebrates. Due to their filter-feeding lifestyle, mussels are directly exposed to particle-bound contaminants in the water column and can be optimal indicators of the contaminant levels that are bioavailable to aquatic organisms. Covaci *et al.* (2005) measured levels of PBDEs in zebra mussels and fish from lakes and canals in Flanders, Belgium in 2002. The mean levels of total PBDEs in the mussels ranged from 0.15 to 1.8ng/g ww. BDE47 and 99 dominated the samples followed by BDE100, closely resembling the congener pattern in the pentaBDE technical mixture. Levels of total PBDE ranged from 2 to

14ng/g ww in eel liver and below detection to 6ng/g ww in carp muscle. In the fish samples, BDE47 and 100 dominated, with BDE99 at very low concentrations or not present at all. Blue mussels from southern Greenland in 2000 were found to have lower levels of PBDEs than the zebra mussels from Belgium (Glasius *et al.*, 2005). The mean total PBDE in the mussels was 0.12ng/g ww and BDE47 and 99 dominated. Levels in fish from southern Greenland ranged from 1.8ng/g ww in sculpin to 12ng/g ww in cod. In this study, PBDEs in both mussels and fish were at lower concentrations than the chlorinated organics measured.

1.2.3 Sediment and Biosolids

Due to the hydrophobic properties of PBDEs, they are normally bound to particles, and sediment can be a major source of PBDEs to an aquatic system. Sediment mixing and breakdown causes resuspension of these compounds. Benthic organisms living in and consuming the sediment are primarily exposed to the PBDEs, which results in increased exposure of organisms at higher trophic levels.

In 2001 and 2002, Song *et al.* (2004, 2005a, 2005b) completed a comprehensive study on sediment levels of PBDEs in the Great Lakes. They measured PBDE levels in various sections of sediment cores to determine recent and historical levels and trends. In all cases, the maximum concentrations were found in the top layers and as depth increased PBDE concentrations decreased. This reflects the increasing production and use of these compounds since the 1970s. Total PBDEs (sum of the nine tri to hexaBDE congeners) in sediments were highest in Lake Ontario, followed by Lakes Michigan, Erie, and both Lakes Huron and Superior, which had similar levels, contrary to the concentration pattern in fish. BDE209 was highest in all lakes and was reported separately, followed by BDE47 and 99. The dominance of the decaBDE congener is expected due to production and use of the decaBDE technical mixture in the Great Lakes as well as the increased affinity this congener has for sediment. BDE209 levels were also highest in Lake Ontario followed by Lakes Erie and Michigan, which had similar levels, then Lake Huron followed by Lake Superior.

The average concentration of total PBDE in Lake Ontario sediment was 5.6ng/g dry weight (dw) whereas the average BDE209 concentration was 226.6ng/g dw. Kolic *et al.* (2004) reported higher concentrations of total PBDEs (including BDE209) in the sediment from various

Lake Ontario tributaries, ranging from 12 to 430ng/g dw. The samples analyzed in this study may have been impacted more by point sources, such as industrial and/or municipal discharges (e.g. wastewater treatment plants), since they were collected in the tributaries and not in the open lake. In Lake Michigan, total PBDEs and BDE209 averaged 2.99 and 63.1ng/g, respectively. Similarly, in Lake Erie, total PBDEs and BDE209 averaged 1.9 and 52.8ng/g, respectively. Lake Huron sediment contained 1.5 and 28.8ng/g of total PBDEs and BDE209, respectively. The lowest levels reported were in Lake Superior, where total PBDEs averaged 1.4ng/g and BDE209 averaged 10.5ng/g. Muir *et al.* (2003) detected low levels of decaBDE in sediment cores collected from the Canadian Arctic.

The biosolids that remain after wastewater is treated is often applied to cropland, as a natural source of fertilizer. This reduces the need for landfill disposal or incineration. Significant levels of PBDEs have been measured in sewage sludge samples in North America. Hale *et al.* (2001b) quantified PBDEs in pre-treated (to reduce odour and pathogen content) biosolid samples from Virginia, Maryland, New York state and California. Total pentaBDE congeners ranged from 1100 to 2290ng/g dw while BDE209 ranged from 84.8 to 4890ng/g dw. These levels are 10 to 100 times greater than levels in sludge from Europe. At these sites, concentrations of BDE47, 99, and 100 were higher than the major PCB congeners present. Another study examined sludge samples from sewage treatment plants adjacent to tributaries flowing into Lake Ontario (Kolic *et al.*, 2004). The levels of total PBDEs in the biosolids ranged from 1700 to 3500ng/g, 28 to 56 times higher than sediment samples collected from the tributaries. BDE209 dominated all samples except at one location, where concentrations of BDE47, 99, and 209 were similar.

1.2.4 Air

Strandberg *et al.* (2001) reported PBDE concentrations in air sampled around the Great Lakes between 1997 and 1999 at one remote site, two rural sites, and an urban site (Chicago, Illinois). In the Chicago area, there are known sources of airborne contaminants, including plastics factories that utilize the decaBDE mixture. Levels of total PBDEs were highest in Chicago, followed by two of the rural sites with averages of 52, 15, and 7.2pg/m^3, respectively. The dominant congeners were BDE47 (50 to 65%) and BDE99 (35 to 40%), which is a function of

the higher vapour pressure of BDE47. BDE209 was only measured at the Chicago site, and only at trace levels. These results are much lower than indoor air levels at an electronics recycling plant in Sweden, where a maximum level of 308,000pg/m^3 was found near a shredder (Sjödin *et al.*, 2001). In the Swedish recycling plant, BDE183 and 209 were dominant whereas BDE47 was dominant in a teaching hall and a circuit-board assembly plant. The congener patterns in the outdoor air of the Great Lakes matched the pentaBDE technical mixture which was unexpected since the decaBDE mixture is manufactured in the area. This may be due to historical sources of the pentaBDE mixture, or the biodegradation of the decaBDE mixture before it is released to the air. Due to the fact that PBDEs were found in all samples, it was concluded that they are widely dispersed in Great Lakes' air.

Indoor air is a significant source (80 to 90%) of PBDE exposure to humans. North Americans spend much of their time indoors, especially during the winter months in colder regions, such as Canada. Most indoor environments, either homes or offices, are filled with electronic devices (e.g. computers, televisions) and textiles (e.g. furniture made of polyurethane foam). In these environments, dust accumulates and does not undergo degradation as it would in the outdoor environment, where it is exposed to the elements. Ingestion of dust, especially by young children who are in close contact to the floor and often put objects into their mouths, is another exposure route. Wilford *et al.* (2005) reported concentrations of PBDEs in dust samples taken out of previously-used vacuums from homes in Ottawa, Ontario in 2003. PBDEs were found in all samples and the maximum value of total PBDEs measured was 170,000ng/g, the highest concentration found in a domestic indoor environment. This study lacked control over the sample collection, however, and this elevated result may have been due to direct vacuuming at a source (e.g. foam couch). The median level of total PBDEs in the Ottawa homes was 1800ng/g, which was mostly made up of BDE209 (42%), followed by BDE99 (29%) and BDE47 (19%). Stapleton *et al.* (2005) measured PBDE levels in dust and dryer lint from homes in the Washington D.C. and Charleston, South Carolina, areas of the U.S.A in 2004. Total PBDEs in dust ranged from 780 to 30,100ng/g. The penta and decaBDE congeners dominated the samples while levels of the octaBDE congeners were very low. As in the Canadian study, BDE99 generally contributed more than BDE47; however, there

was great variation in congener patterns and some instances where BDE47 dominated. This variation could reflect differences in electronic equipment in the homes. PBDEs were lower in the lint samples, ranging from 480 to 3050ng/g. It was unknown if PBDEs were present in the dryer itself. Results from both studies show that levels of PBDEs found in North American dust are generally higher than those found in Europe, with the exception of the United Kingdom, which has reported some high levels, possibly due to stringent fire regulations and past manufacture.

1.3 Uptake and Metabolism in Aquatic Systems

In the literature, research on the movement of PBDEs in aquatic systems has focused on higher trophic levels, such as fish. Several different fish species have shown preferential uptake of specific congeners, e.g. BDE47 and 99, and in specific tissues. Kierkegaard *et al.* (1999) exposed rainbow trout to purified decaBDE (i.e. no polybrominated dibenzodioxins and furans (PBDD/Fs)) via food (7.5 to 10mg/kg body weight (bw)/day) to determine uptake. Low bioavailability of decaBDE is generally assumed due to its large size and high log Kow. After 16 days, the mean muscle concentration of total BDE was 10ng/g (ww) and increased to 38ng/g at 120 days, whereas the mean concentration in the liver rose from 560 to 870ng/g in the same time period. After depuration, muscle levels decreased significantly, by a factor of two. In both liver and muscle samples from the treated fish, BDE47, 99, 100, 153, and 154 were observed and liver concentrations of these congeners increased after depuration. Levels of BDE154 increased by 20-fold in muscle and 22-fold in liver tissue. Levels of decaBDE decreased significantly during depuration while levels of the hexa, hepta and octaBDEs remained steady. It was concluded that selective uptake of octa and heptaBDEs, impurities in the decaBDE mixture, was occurring. It was speculated that the presence of the tetra, penta, and hexaBDEs may indicate metabolism of decaBDE in fish. This hypothesis was confirmed by Stapleton *et al.* (2004a) after exposure of juvenile carp to 950ng decaBDE/day for 60 days. No BDE209 was detected in the fish, but increasing levels of BDE154 and 155 as well as several unidentified penta, hexa, hepta and octaBDE were observed.

In a previous experiment, Stapleton *et al.* (2002) had also determined that BDE183 was debrominated to BDE154 and an unknown hexaBDE in juvenile carp exposed via food. Exposure to a number of different BDE congeners showed that uptake of most of the BDEs occurred within the first five days of exposure. BDE47 had the most rapid uptake rate of 70ng/g lipid/day, whereas uptake of BDE99 and 153 was minimal. BDE47 was also depurated at the fastest rate, 0.32 day^{-1}. They also found differences in congener composition in the muscle and liver. BDE47 was the dominant congener in both muscle and liver tissue, however, the liver showed elevated levels of BDE154 as well.

In subsequent research, Stapleton *et al.* (2004b) exposed carp to BDE99 and 183 via food. In those fish exposed to BDE99, approximately 9.5% was converted to BDE47. BDE99 was detected in intestinal tissue but not in liver or whole body tissues during the experiment. Appreciable levels of BDE47 were detected in whole body tissues along with BDE28, indicating that BDE47 is rapidly debrominated as well. It was concluded that intestinal and/or liver enzymes were responsible for the rapid debromination of BDE99. Between exposure days 44 and 62, concentrations of BDE47 dropped in the liver but continued to rise in the intestines and whole body tissue indicating further debromination of BDE47 within the liver. Approximately 17% of the BDE183 was debrominated into two unknown hexaBDE congeners. The rate of debromination of BDE99 was more rapid than for BDE183.

PBDEs have been shown to accumulate in lower trophic levels. Gustafsson *et al.* (1999) reported rapid uptake of PCBs and PBDEs by blue mussels (*Mytilus edulis*) via cultured green algae (*Scenedesmus obtusiusculus)*. The uptake rates were significantly higher for PBDEs than for the PCBs. Uptake of BDE47 was 6 to 7 fold higher than PCB52 and 77 and uptake of BDE99 was 12 to 30 fold higher than PCB118 and 153. These differences in uptake rates may be due to different partitioning behaviour or debromination within the mussel. Depuration rates for PCBs decreased with increasing hydrophobicity (K_{ow}), however, for PBDEs, depuration rates appeared to be independent of K_{ow}. BDE47 and 99 showed the highest bioaccumulation factors (BAF). The calculated BAFs for BDE47 and 99 were 13 and 14, respectively, five to eight times higher than similar-sized PCBs.

Along with uptake and bioaccumulation, biomagnification of PBDEs up trophic levels has been demonstrated. Andersson *et al.* (1999) showed that PBDEs biomagnify in the zebrafish (*Danio rerio*). They exposed the fish to 10μg/g total PBDEs for 21 days followed by depuration. Levels in fish increased over time and the highest time-specific biomagnification factors (BMF) were observed for BDE28 and 47. As compared to chlorinated diphenyl ethers, BMFs for PBDEs were higher and more dependent on substitution pattern. TetraBDEs had the highest BMFs indicating that molecular size likely affects biomagnification. Of the higher brominated congeners, BDE100, 153 and 154 magnified most. The authors speculated that adjacent bromines were unfavourable for biomagnification or could lead to debromination. Using stable nitrogen isotopes, Burreau *et al.* (1999) was also able to show biomagnification of a number of PBDE congeners between sprat (*Sprattus sprattus*), herring (*Clupea harengus*), and salmon (*Salmo salar*) from the Baltic Sea.

Tomy *et al.* (2004) examined bioaccumulation, biotransformation, and biochemical effects of various BDEs in juvenile lake trout. They exposed the fish to 11 different congeners via food. Depuration occurred by a first-order process, however many of the BDEs showed rapid depuration initially, followed by a slower rate of depuration. The half lives varied between the low and high dose groups and ranged from 38 to 346 days. Assimilation efficiencies ranged from 0.3% for BDE209 to 52.9% for BDE28 and the BMFs all exceeded one except for BDE209. This study reiterated the difficulties in determining bioaccumulation of BDEs in fish due to biotransformation. Debromination likely increases the elimination rates of some congeners, making it difficult to measure assimilation efficiency, depuration rate, and BMF. Bioformation of lower brominated congeners via debromination of higher brominated congeners adds to the difficulty of determining bioaccumulation. Bioformation results in slower depuration rates and longer half lives, evidenced by the fact that the half lives of some BDE congeners are much longer than PCB congeners with similar K_{ow} values. Three congeners not present in the fish food were identified, including one pentaBDE, and two hexaBDEs (one identified as BDE140), further demonstrating debromination.

Hydroxylated and methoxylated versions of PBDEs have been identified in red algae, blue mussels, and salmon from the Baltic Sea (Marsh *et al.*, 2004; Malmvärn *et al.*, 2005).

Hydroxylated congeners have also been identified in fish from the Detroit River (Valters *et al.*, 2005). It is unknown if these products are formed within these organisms or if they are accumulating from natural sources (e.g. marine sponges and tunicates). Regardless, the presence of these compounds is important due to their similarities to thyroid hormones and associated toxicities. Also, hydroxylated forms are more water soluble and can therefore be transported to all cells in the body via the blood stream.

In the literature, limited research has focused on trophic transfer or food chain effects of PBDEs. Wolkers *et al.* (2004) looked at congener-specific accumulation and food chain transfer of PBDEs in Arctic food chains in Norway. They focused on two food chains: polar cod (*Boreogadus saida*) to ringed seal (*Phoca hispida*) to polar bear (*Ursus maritimus*) and polar cod to beluga whale (*Delphinapterus leucas*) and determined species specific differences in PBDE metabolism and accumulation. Biomagnification was estimated by determining the Metabolic Index (MI), the ratio between the relative presence in predator and prey. The calculated MI is compared to the MI for PCB153, a very persistent congener that most animals cannot metabolize. MI values greater than or less than one indicate higher or lower accumulation than PCB153, respectively. BDE47, 99, 100 as well as several methoxylated congeners were identified in the animals, however BDE47 was dominant at all trophic levels. For BDE47 and 99, levels in seals were almost an order of magnitude higher than in cod indicating biomagnification. In polar bears, only BDE47 was detected which confirms reports of very high metabolic activity in these animals. Levels of BDE47 were higher in polar bears than in seals, also indicating biomagnification. In the other food chain, whales had a very complex congener pattern compared to the other animals, including detection of methoxlated tetraBDEs, not detected in cod. The complexity of the congener pattern in the whales confirms reports of low metabolic activity in these animals. The MI values determined that BDE47 and 99 accumulate to the same magnitude as PCB153 in seals and whales. Seals and whales are at similar trophic levels and share the same diet (cod) however seals were able to metabolize BDE100 to a greater extent. At the top of the food chain, polar bears were shown to metabolize all PBDEs and it was concluded that biomagnification is negligible in these animals. Wolker *et al.* also observed that BDE47, 99, 100, and 154 all have at least two ortho and para bromines, but are

being metabolized in these animals. By comparison, this structural pattern in PCBs is stabilizing, resulting in very little metabolism, even in polar bears. It is evident that the properties that result in persistence of PBDEs are not the same as that for PCBs.

1.4 Toxicity

Toxicity studies on brominated flame retardants, especially PBDEs, have been conducted mainly on laboratory animals (mice and rats), and less frequently on aquatic organisms. These studies have demonstrated five main types of toxicity: neurotoxicity, dioxin-like toxicity, endocrine disruption including effects on thyroid and estrogen hormone systems, reproductive toxicity, and immunotoxicity. Acute toxicity of many of the PBDEs has been shown to be very low (WHO, 1994). Currently, there is only evidence of carcinogenicity for BDE209 and the USEPA has determined that there is "suggestive evidence of carcinogenic potential" (Lorber, 2008).

1.4.1 Neurotoxicity

Eriksson *et al.* (2001) studied neurotoxicity in mice orally exposed to BDE47 during the neonatal brain growth spurt, a period of rapid brain growth in mammals spanning the first three to four weeks in rodents. At two and four months, the mice were placed in a new environment and monitored for motor activity. Normally, increased motor activity is observed during the first 20 minutes followed by a decrease over the next 40 minutes, as the animal becomes accustomed to its surroundings. Compared to the controls, those exposed to high doses of BDE47 and 99 and low doses of BDE99 displayed hyperactivity after familiarizing themselves with their surroundings, especially at four months. Also, the mice exposed to high doses of BDE99 showed decreases in relearning and memory in a swim maze test. Eriksson and coworkers concluded that exposure to these two congeners during this specific brain development stage can lead to permanent changes in spontaneous behaviour that worsen with age, as well as decreased learning and memory in the adults. In a follow-up study, Eriksson *et al.* (2002) exposed mice to BDE99 at different times and concentrations and found similar disturbances in spontaneous motor behaviour in a dose-dependent manner. Radio-labeled

BDE99 was also observed in the brain suggesting that it is responsible for the disturbances in neurological behaviour.

In a subsequent study, Eriksson *et al.* (2006) exposed mice to BDE99 and PCB52 individually at low and high doses, and combined at low doses. Mice exposed to the combined dose and the high doses of each had significantly impaired spontaneous motor behaviour and habituation at four and six months. They also found that the neurobehavioural impairments worsened with age for those mice exposed to the combined dose. Exposure to the low doses alone did not illicit any significant impacts, indicating that the impacts of the PCB52 and BDE99 may be more than additive. Protein samples isolated from the brain cortex of mice 24 hours after a 12mg/kg dose of BDE99 resulted in altered expression levels of proteins associated with the cytoskeleton, mitochondria, and oxidative phosphorylation (Alm *et al.*, 2008). These cytoskeletal proteins are important for cell migration, proliferation, and differentiation and may be the underlying mechanisms of the observed impacts on behaviour outlined above (Alm *et al.*, 2008).

1.4.2 Dioxin-like Toxicity

Dioxin-like toxicity is mediated through the binding of compounds to the Aryl hydrocarbon receptor (AhR) (Okey *et al.*, 1994). Cytochrome P450 is an enzyme involved with reactions in the liver that aid in the excretion of hydrophobic compounds and is encoded by the CYP1A1 gene. Measurement of ethoxyresorufin-O-deethylase (EROD), pentoxyresorufin-O-deethylase (PROD), and methoxyresorufin-O-deethylase (MROD) activity in an organism serve as biomarkers of exposure to a contaminant that has dioxin-like toxicity (Whyte *et al.*, 2000). Several studies have shown increased induction of these enzymes after exposure to pure and hydroxylated PBDE congeners as well as mixtures (Tjarnlund *et al.*, 1998 and Tomy *et al.*, 2004 in fish; Fowles *et al.*, 1994; Chen *et al.*, 2001; Hallgren *et al.*, 2001; Zhou *et al.*, 2001; Hallgren & Darnerud, 2002; Stoker *et al.*, 2004; and Staskal *et al.*, 2005 in laboratory animals).

Meerts *et al.* (1998) determined *in vitro* AhR agonistic or antagonistic activity in rat hepatoma cells exposed to PBDEs alone and in combination with tetrachlorodibenzo-*p*-dioxin (TCDD). BDE85 showed the highest induction, while BDE99 and 119 were also found to be partial agonists and antagonists. Overall, the congeners mainly acted as antagonists to AhR

activity. PBDEs were found to have weaker binding affinity for the AhR than TCDD in rat hepatic cells (Chen *et al.*, 2001). BDE85 was also found to have the most binding activity in this study as well, at 2% of TCDD.

More recent research has shown that dioxin-like activity of PBDEs is negligible in comparison to other environmental contaminants. Kuiper *et al.* (2004) showed that PBDE exposure alone to carp hepatocytes does not induce EROD activity. In fact, exposure of the hepatocytes to PBDEs (congeners and technical mixtures) along with TCDD caused an inhibition in the TCDD-induced EROD activity. Sanders *et al.* (2005) looked at gene expression of CYP1A, 2B, and 3A in rats after exposure to BDE congeners (the major components of the penta-mixture) and found them to be poor AhR agonists, contributing nothing or very little to the dioxin-like toxicity. They found that the PBDD/F present in the mixture were likely responsible for any associated dioxin-like toxicity. Chen and Bunce (2003) re-examined the agonistic/antagonistic behavior of several PBDE congeners at sequential stages of the AhR signal transduction pathway leading to CYP1A1. Contrary to their own previous research (Chen *et al.*, 2001, described above), they found that the relative induction potencies of the most active PBDEs towards CYP1A1 are approximately 0.0001 compared with TCDD, and essentially zero for the environmentally-prominent congeners like BDE47. They concluded that PBDE contribution to the total TEQ "dioxin load" is negligible. It would seem from this research that the other toxic pathways (i.e. thyroid and neurotoxicity) may be of greater concern for these compounds.

1.4.3 Thyroid Effects

PBDEs have been shown to disrupt endocrine processes, with the most notable effects on thyroid homeostasis. There are three different levels at which the thyroid hormone system can be disrupted: at the thyroid gland, in thyroid hormone metabolism, or with thyroid hormone transport proteins (Meerts *et al.*, 2000). Stoker *et al.* (2004) observed histological effects on the thyroid after *in vivo* mouse exposure to DE-71 (pentaBDE mixture), including an increase in follicular cell height and a decreased colloid area of the thyroid cells, indicative of a hypothyroid state. Due to the ether linkage, PBDE congeners have structural resemblances to thyroid hormones, such as thyroxine (T_4). T_4 and triiodothyronin (T_3), its more biologically active form

(Figure 4), are involved in energy metabolism, the cardiovascular system, and lipid metabolism (Marsh *et al.*, 1998).

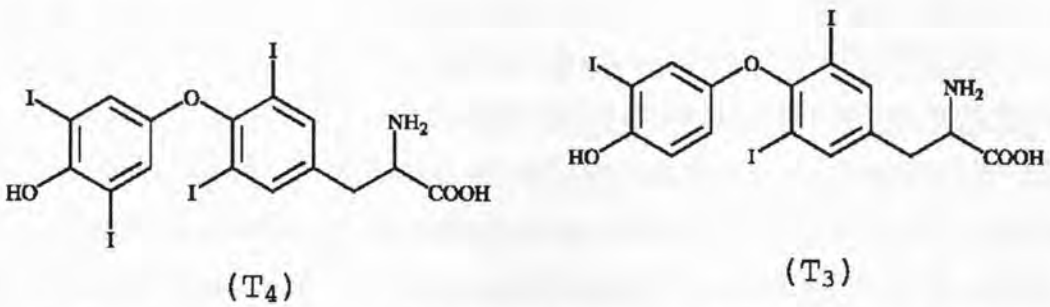


Figure 4. The chemical structures of the thyroid hormones 3,3',5,5'-tetraiodo-L-thyronine (thyroxine or T_4) and its 5'-deiodinated congener 3,3',5-triiodothyronine (T_3). Source: de Wit, 2002.

Circulating levels of T_4 and T_3 spur the hypothalamus to release thyrotropin-releasing hormone (TRH) which stimulates the pituitary gland to release thyroid-stimulating hormone (TSH) causing the thyroid to produce more T_4. The T_4 is then converted to T_3 and binds to transthyretin (TTR), the hormone receptor. This negative feedback system is stabilizing and necessary to maintain thyroid hormone homeostasis (Campbell, 1996). Disruption of this system may occur if PBDEs compete with T_4 for binding sites on transthyretin (Meerts *et al.*, 2000) or bind directly to thyroid hormone receptors (Marsh *et al.*, 1998).

Increased elimination of T_4 has been observed in animals exposed to different PCBs as well as PBDEs (Fowles *et al.*, 1994; Hallgren & Darnerud, 2002; and Stoker *et al.*, 2004 in laboratory animals and Tomy *et al.*, 2004 in lake trout). Zhou *et al.* (2001) exposed rats orally to the technical mixtures DE-71, DE-79 and the decaBDE mixture, DE-83R, and found a significant dose-dependent decrease in serum T_4 levels for the DE-71 and DE-79 mixtures. A lower effect on T_3 and no effect on TSH levels were observed.

Metabolites of PBDEs have also been shown to bind to the thyroid hormone receptor, in some cases more effectively than the thyroid hormones themselves. Meerts *et al.* (2000) showed that the hydroxylated metabolites most similar to thyroid hormones T_3 and T_4 were bound to TTR with more potency than T_4. The pure congeners did not compete with T_4-TTR binding. Meerts *et al.* (2000) concluded that the degree of bromination and the nature of the halogenated substituents play major roles in binding potency. A similar study was conducted

by Marsh *et al.* (1998) where human THR was mixed with radio-labeled T_3 and various hydroxylated BDEs. They found that the T_3-like hydroxylated BDE congener had the most affinity for THR, however, it was not higher than the natural ligands, T_3 and T_4. Binding of hydroxylated PBDEs to TTR could be involved in facilitative transfer of BDEs across the placenta and blood-brain barrier and could result in high levels in the fetus (Meerts *et al.*, 2000).

Thyroid hormones are also responsible for the regulation of amphibian metamorphosis, including the transformation of a tadpole into a froglet and the subsequent regression of the tail. Schriks *et al.* (2006) observed that exposure of *Xenopus laevis* tadpoles to BDE206 was antagonistic to T_3-induced tail tip regression in a dose-dependent manner. Exposure of the tadpoles to BDE206 alone (without the natural hormone T_3) did not have any effect on tail tip regression. The authors found that these impacts occurred at BDE concentrations only 5 to 50 times higher than T_3.

1.4.4 Endocrine Disruption and Reproductive Toxicity

Along with thyroid hormone effects, PBDEs also have been shown to have effects on the estrogen hormone receptor. This is an important toxic effect because disruption of this system may cause cancer of estrogen-sensitive tissues in humans and/or wildlife (Colborn *et al.,* 1993). Meerts *et al.* (2001) tested the possible (anti)estrogenic effects of pure and hydroxylated PBDEs in a human breast cancer cell line transfected with an estrogen-responsive luciferase reporter gene. They found that 11 out of 17 pure PBDE congeners exhibited luciferase induction in a dose-dependent manner (BDE100>BDE75>BDE51>BDE30>BDE119). However, this induction was 250,000-390,000 times less potent than estradiol, the natural ligand. The hydroxylated PBDEs that were closest to T_4 (Figure 4) showed no estrogenic effect, however, the T_2 and T_3 (thyroid hormone)-like congeners showed the highest potencies, which exceeded the natural ligand. The potency of the T_2-like hydroxylated BDE was in line with that of bisphenol A, a known environmental estrogen mimic that has been shown to increase the production of certain human breast cancer cells (Perez *et al.*, 1998). Anti-estrogenic effects were observed for BDE153, 166, and 190.

Delays in puberty in male and female rats have been observed after oral exposure to DE-71 (Stoker *et al.,* 2004). Rats orally exposed to single doses of DE-71 at 3, 30, and 60mg/kg were

monitored for preputial separation (PPS), the separation of the foreskin of the penis from the glans penis, as well as vaginal opening, an estrogen-dependent stage of female puberty that indicates initial ovulation and onset of the estrous cycle. In the females, a significant delay (1.8 days) of vaginal opening was observed at the highest dose (60mg/kg), followed by a regular estrous cycle upon vaginal opening. In males, a significant delay in PPS was observed in the 30mg/kg treatment group (1.7 days) and the 60mg/kg treatment group (2.1 days). After 31 days, a decrease in the weight of the ventral prostate and seminal vesicle, androgen-dependent tissues, was also observed in males exposed to 60mg/kg, which might indicate interference with androgen functioning. This result may be related to changes in thyroid homeostasis which could influence steroid production or have a direct effect on the androgen receptor.

In 2005, Stoker *et al.* examined the binding of PBDEs to the rat androgen receptor (AR) as compared to R1881, a synthetic androgen and anabolic steroid that binds strongly to the AR. They found that both DE-71 and some pure congeners competed with R1881 for binding to the AR *in vitro*. BDE100 and DE-71 showed effective inhibition of R1881-AR binding and were characterized as competitors. The other constituents of DE-71, BDE47 and 99 also displayed inhibition of R1881-AR binding. Delayed transcription of genes was also observed for DE-71, BDE47 and BDE100 *in vitro*. In 2004, they also found increases in the pituitary hormone prolactin at the highest dose after 31 days. This may be related to disruptions in thyroid hormone homeostasis. A decrease in thyroid hormones in the blood triggers the hypothalamus to release TRH. TRH stimulates the pituitary to secrete TSH as well as prolactin. BDE99 and 100 have also shown endocrine disrupting properties in arthropods *in vitro*. These congeners showed weak antagonistic activity of ecdysteroids, which regulate larval development and reproduction in arthropods (Wollenberger *et al.*, 2005).

Nakari and Pessala (2005) exposed rainbow trout hepatocytes to BDE47, 99 and 205 and measured the yolk protein precursor vitellogenin (a measure of the estrogenic activity) and EROD activity. All PBDEs induced the production of vitellogenin in a dose-dependent manner, which increased as the bromine content increased; BDE205 exhibited the highest estrogenic activity. Additionally, all PBDEs significantly increased the cell EROD activity with BDE205 again showing the highest induction. At high test concentrations, there was a reduction in EROD

activity as well as vitellogenin concentration, which the authors attributed to cytotoxicity. They did find a discrepancy between estrogenic responses in yeast and human cell lines as compared to the fish hepatocytes, which may have been due to higher metabolic capacity in the fish leading to the production of metabolites. Hamers *et al.* (2008) investigated the endocrine-disrupting behaviour of PBDE metabolites produced by microsomal incubation of parent compounds. They found metabolites of BDE47 to be 2 to 200 times more effective than BDE47 at inhibiting estradiol sulfotransferase, an enzyme that inactivates the endogenous hormone estradiol.

Adult kestrels were exposed via diet to DE-71 for 75 days resulting in PBDE concentrations in their eggs at (low dose) or within an order of magnitude (high dose) of environmentally relevant concentrations (Great Lakes region). The authors observed impacts on reproductive behaviour, including altered timing and frequency of courtship behaviours which resulted in reduced strength of the pair bond (Fernie *et al.*, 2008). Food consumption was also marginally affected in both sexes, with increased consumption by the males and decreased consumption by the low dosed females at specific times. These behavioural changes would likely result in delayed egg-laying, reduced fertility of eggs, and negative impacts on other clutch parameters which determine reproductive success. Neurobehavioural changes occurred in mice when exposed to PBDEs during a critical period of brain development (Eriksson *et al.*, 2001), however, these results show that exposure of adults kestrels to environmentally relevant PBDE concentrations can induce behavioural changes early in the reproductive season.

1.4.5 Immunotoxicity

Fowles *et al.* (1994) examined immunologic acute and subchronic effects of DE-71 on mice. Post exposure to DE-71, they monitored antibody response after immunization with sheep red blood cells (SRBC) as well as cytotoxicity of natural killer cells (NKC). At the highest subchronic dose (72mg/kg bw/day), a moderate but significant suppression of the antibody response was observed. Thymus weight was also significantly decreased after subchronic treatment at the highest dose. Activity of the NK cells was not significantly altered after exposure to DE-71. In contrast, Fernlöf *et al.* (1997) examined the *in vitro* effects of BDE47 and 85 on human lymphocytes (white blood cells) from blood donors. No effects on mitogen-induced DNA

synthesis or immunoglobulin synthesis were observed after exposure of cells to concentrations up to 10^{-5}M. They concluded that certain functions of lymphocytes in humans are not sensitive to the direct action of PBDEs.

Recently, immunotoxic effects have been observed in lake trout thymocytes exposed *in vitro* to PBDEs. In fish, the thymus is located under the gills and therefore has direct exposure to contaminants in the water. Birchmeier *et al.* (2005) exposed thymocytes to concentrations of BDE47 and 99 between 0.010 and 100 mg/L to determine thymocyte viability, cell apoptosis (programmed death), and necrosis (non-programmed death). At 100mg/L, BDE47 significantly decreased thymocyte viability and increased necrosis. BDE99 was less immunotoxic, causing only a slight but significant decrease in thymocyte viability at 100mg/L.

1.5 Toxicity of PBDEs to *Daphnia magna*

An extensive review of the literature resulted in three studies on the toxicity of PBDEs toward *Daphnia magna*. Nakari and Huhtala (2008) compared the toxicity of PCB, Polybrominated biphenyl (PBB), and PBDE congener 153 on *D. magna*. After exposure to these compounds (PCB154: 10.3 and 23.9μg/L; PBB154: 11.3 and 25.9μg/L; PBDE154: 9.1 and 14.6μg/L) for 21 days, a significant decrease in reproduction was observed relative to the control for all three. The impact on reproduction was in the order of PCB153>PBB153>PBDE153.

Toxicity of BDE99 to the green algae *Pseudokirchneriella subcapitata* and *D. magna* was compared to the toxicity of PCB Aroclor 1254 (technical mixture) by Evandri *et al.* (2003). BDE99 was found to be nontoxic and did not inhibit growth in the algae at all nominal concentrations (0.005-100μM = 2.83-56,500μg/L). In the acute toxicity test, the 24 hour EC_{50} value was 1.028μM for BDE99 versus 0.455μM for Aroclor 1254, whereas the 48 hour EC_{50} values were similar between the two compounds (0.044 and 0.070μM, respectively). In addition, bioaccumulation of BDE99 (5 to 100μM) was investigated by feeding *D. magna* pre-dosed algae for nine days. Motility decreased in daphnids at 25, 50 and 100μM starting on the third day but no mortality was observed. For the two highest treatments (50 and 100μM), the total number of offspring was significantly reduced.

Toxicity of BDE47 to *D. magna* was investigated previously by Kallqvist *et al.* (2006). They exposed the marine algae *Skeletonema costatum* and *D. magna* to nominal concentrations ranging between 1.0 and 100μg/L and 3.0 and 100μg/L, respectively. Results showed growth inhibition of *S. costatum* at concentrations above 6.6μg/L (NOEC) with a 48 hour EC_{50} of 70μg/L. As for the *D. magna,* no lethality was observed in any treatments but reproduction was significantly reduced at 45 and 140μg/L. The NOEC was calculated at 14μg/L, although at this concentration there was a large amount of variation among the data relative to the control. In this study, it was concluded that BDE47 was several orders of magnitude more toxic to algae than BDE99 was to a different species of algae (Evandri *et al.* 2003). The NOEC of 14μg/L for *D. magna* determined by Kallqvist *et al.* (2006) was somewhat higher than the NOEC values calculated for the pentaBDE technical mixture (mainly containing BDE47 and BDE99) of 4.9 and 5.3μg/L in previous studies (EU, 2000).

Since the *D. magna* NOEC determined by Kallqvist *et al.* (2006; 14μg/L) was below the water solubility of BDE47 (Sw = 15.0μg/L; Tittlemier *et al.*, 2002), it was chosen as the exposure concentration for the current study. In much of the existing research on aquatic toxicity, exposure levels of PBDEs and other organic compounds significantly exceed the water solubility of the compound (e.g. Lema *et al.*, 2007; Key *et al.*, 2008; Evandri *et al.*, 2003; Nakari and Huhtala, 2008). Due to the high hydrophobicity of BDE47, this low concentration was chosen to ensure accurate water dosing. The impact of water exposure versus food exposure was also determined. Often, laboratory bioassays only take into account bioconcentration of chemicals in aquatic organisms, while ignoring bioaccumulation from food. To increase the predictive power of laboratory bioassays, introducing exposed food into the test can assess the impact of both bioconcentration and bioaccumulation (Podemski & Culp, 2001).

1.6 Test Organisms

1.6.1 *Pseudokirchneriella subcaptitata* and *Chlorella fusca*

Algae are important links to the transport of contaminants to higher trophic levels, such as grazing zooplankton (Stange & Swackhamer, 1994). Vertical transport of algae is also an important transfer mechanism of contaminants to bottom sediments (Swackhamer & Skoglund,

1993). *P. subcapitata* is a unicellular green algal species common to North American freshwaters (EC, 1992a) and often used in toxicity studies. Standard methodologies using this algal species are readily available in the literature and through regulating agencies such as the USEPA and Environment Canada. *C. fusca* is a unicellular green algae that is a preferred food species for larger *D. magna* (Pers. Comm., D. Poirier, OMOE) and will improve the test environment for this crustacean.

1.6.2 *Daphnia magna*

Daphnia magna, commonly known as a "water flea", is a small freshwater crustacean found in most North American waters (EC, 2000). These filter-feeding invertebrates occupy a central position in aquatic food chains by consuming primary producers and being consumed by predators. They are useful for the study of the interactions between trophic levels due to their central position in aquatic food chains (Lampert, 2006). Natural populations consist mainly of females which reproduce by cyclic parthenogenesis. Under favourable conditions, female genetic clones are produced asexually; when conditions become unfavourable (e.g. decreased photoperiod or food concentration or increased density) sexual reproduction is initiated (Kleiven *et al.*, 1992). During these adverse conditions, daphnids produce a sexual (resting) egg encased in an ephippium that is shed with the next exoskeleton molt (Rand, 1995). The ephippia can survive harsh conditions for many years and act as a dispersal agent (Lampert, 2006). During asexual reproduction, the eggs hatch within the brood and are released within approximately two days, during a molt (Rand, 1995). Neonates (<24 hours old) mature in approximately six to ten days and have two to five juvenile instars which are terminated by a molt (Rand, 1995).

D. magna are the most widely used animal in aquatic toxicity bioassays to determine acute and chronic toxicity (Evandri *et al.*, 2003), due to their availability, small size, short life cycle, ease of culturing, and sensitivity to chemical exposure (EC, 2000, Rand, 1995). In addition, these invertebrates are semi-transparent allowing for the observation of the inner organs, including the heart and the brood pouch, which carries the eggs or the newly hatched neonates (Lampert, 2006). Standard methodologies using this crustacean are readily available in the

literature and through regulating agencies such as the USEPA, Environment Canada, and the Organisation for Economic Co-operation and Development (OECD).

1.7 Research Objectives

This literature review shows that a large amount of research has been dedicated to levels of PBDEs in the environment and their toxic effects on laboratory animals. This information has been useful in identifying the potential threats PBDEs pose to human health. However, little research has focused on the toxic effects of PBDEs on aquatic organisms. The research that does exist focuses on fish, with insufficient research on organisms that form the base of the food chain, such as algae and aquatic invertebrates, and which are very important to the health of the ecosystem.

The objectives of this research were to investigate the effects of BDE47 on the survival and reproduction of *Daphnia magna* over two generations. In addition, the impact of water exposure versus food exposure was investigated. It was hypothesized that BDE47 would not cause mortality but would have impacts on reproduction that could be carried over to the second generation. Exposure of BDE47 via food was expected to impact reproduction more profoundly than water exposure because dietary consumption often results in a greater accumulation of contaminants. BDE47 was used for this research because it is one of the most abundant PBDE congeners found in the environment and accumulates in organisms to a significant extent (accounting for approximately 70% of the total PBDE content in biota (Wollenberger *et al.*, 2005)) and is moderately toxic (D'Silva, 2004). Therefore, this congener is particularly bioavailable to organisms in aquatic systems.

2.0 METHODOLOGY

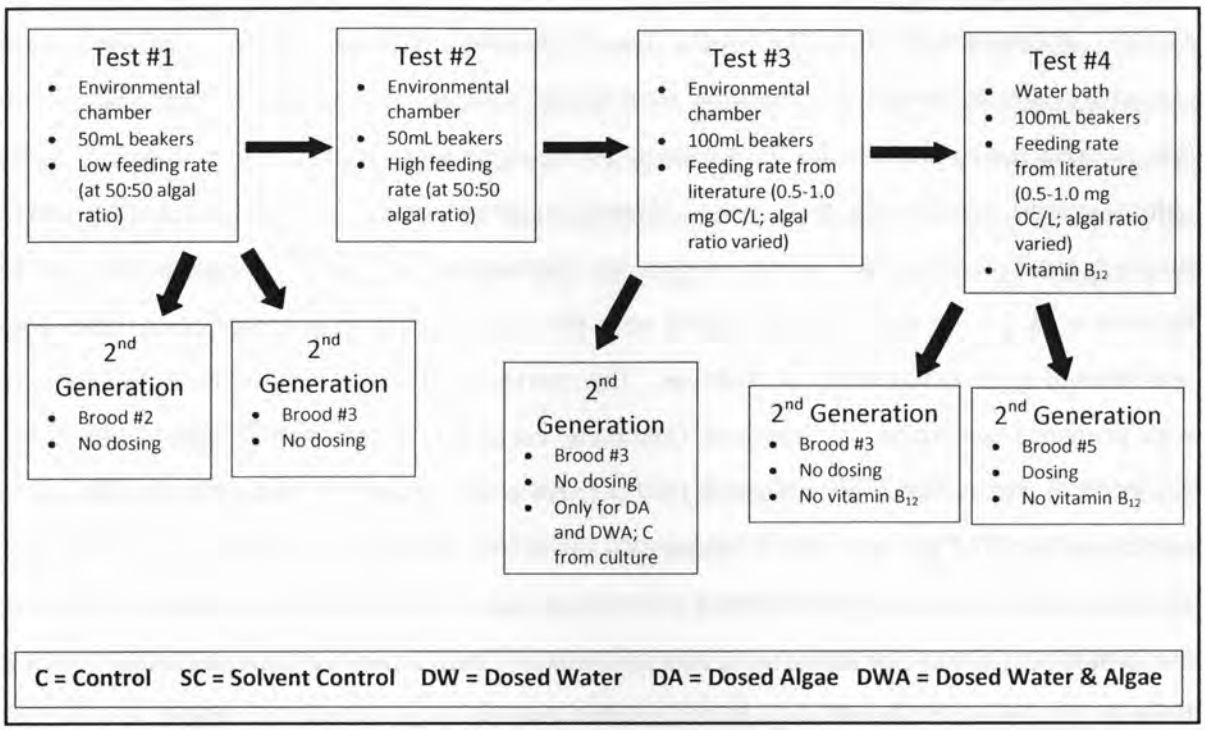


Figure 5. Test protocol development for *Daphnia magna* Reproduction.

In this study, toxicity and bioaccumulation of BDE47 were assessed in a simple laboratory food chain. Impacts on survival and reproduction in *D. magna* exposed to BDE47 via water and food were investigated. Reproduction tests on second generation daphnids were also completed, with and without subsequent BDE47 exposure, to assess the impacts. Figure 5 details the main experimental setup, which is discussed in Section 2.8. Experiments were completed at the Ontario Ministry of Environment Aquatic Toxicology Unit Laboratory. This laboratory is accredited by the Canadian Association of Laboratory Accreditation (CALA) and performs routine auditing for Provincial and Federal regulatory support, contaminated site assessment and enforcement activities.

2.1 Algae Culturing

Pseudokirchneriella *subcapitata* (UTCC (University of Toronto Culture Collection of Algae and Cyanobacteria), 37) and *Chlorella fusca* (var. *fusca*, UTCC, 394) were cultured at the Ontario Ministry of Environment (OMOE) Aquatic Toxicology Unit (ATU) Laboratory. Pure algae were cultured in sterile vented (hydrophobic membrane, 0.22µm) tissue culture flasks (BD Falcon flasks) using pre-autoclaved nutrient medium. The algae were transferred to 4 or 6 Litre (L) culture vessels using aseptic techniques. Algae at an initial density of 10^5 cells/mL was grown in nutrient medium at 20±2°C under continuous illumination of cool fluorescent light (50 to 66µmol·m^{-2}·s^{-1}). The culture was aerated with filtered air (0.45µm) through glass tubing and was housed in an environmental chamber. The nutrient medium (Modified Bristol's Medium) was prepared according to Standard Operating Procedure (SOP) AL1 (OMOE, 2008a; see Appendix I, Table A1-1), pH adjusted to 7.0 (with the addition of sodium hydroxide), and autoclaved at 121.4°C and 120.7 kilopascals (kPa) for 20 to 40 minutes. All glassware, aluminum foil, and tygon tubing were also autoclaved before use. Daily algal counts were performed with a haemocytometer to determine when the culture was growing exponentially. Once in logarithmic phase, the algae was used for the BDE47 exposure experiment.

2.2 *Daphnia magna* Culturing

D. *magna* were cultured at the OMOE ATU Laboratory according to SOP DM1 (OMOE, 2008b). Cultures were grown in pre-aerated dechlorinated water and fed a 50:50 mixture of *P. subcapitata* and *C. fusca.* Neonates (<24 hours old) were fed once at culture initiation and then starting at day seven were fed five to six times a week. A portion (25-50%) of the culture water was strained through nitex screens to capture the organisms. Fresh algae was then added and the organisms were transferred back to the 1.2L culture vessels. The temperature of the culture medium was kept constant at 20±2°C in a water bath under a 16:8 hour photoperiod from cool white fluorescent lights at 7 to 13µmol·m^{-2}·s^{-1} with a pH between 6.5 and 8.5 (generally remained within one base unit of 8.0). Cultures were initiated with 20 neonates per vessel (1.2L). At each feeding, the neonates were counted and removed to monitor reproductive success. Neonates from the cultures with the best reproduction were used to

initiate new cultures. Selenium (3µg/L) and vitamin B_{12} (1-3µg/L) were added to the culture to improve vigour if reproduction decreased within a week. Since all the neonates are genetically similar and maintained under the same environmental conditions, a single neonate from each brood was held in a 50mL vessel and served as the health test organism. The culture health criteria for all *D. magna* included the following: no ephippia (resting egg produced during adverse conditions); natural mortality in the brood no greater than 25% in the week prior to testing; first brood produced within 12 days and at least 15 neonates per brood produced in the week prior to testing; and results from the last reference toxicant test (acute exposure to sodium chloride) within control limits.

2.3 Chemicals and BDE47 Standard

BDE47 crystals (Wellington Laboratories, Guelph, Ontario, >98% purity; see Appendix II) were dissolved in dimethyl sulfoxide (DMSO) to prepare a 119mg/L standard. It was necessary to mix the BDE47 with solvent because of its extreme hydrophobicity which prevented it from going into solution. DMSO was chosen because it has been shown to have very low toxicity to aquatic organisms (green algae and *D. magna*) compared to other commonly used solvents such as acetone, ethanol, and methanol (Okumura, 1996; Barbosa *et al.*, 2003; Wisconsin DNR, 2004). The crystals were poured through a glass funnel into a 25mL volumetric flask under a fume hood. Residue was rinsed from the vial with DMSO and the volumetric flask was filled to a volume of 10mL and mixed end-over-end several times. This became the BDE47 "standard". The standard was stored at room temperature in darkness until use. For chemical analysis, 10µL of standard was diluted in 1mL of nonane, as preliminary analysis showed that DMSO damaged the column in the analytical instrument. The sample was then analyzed by Gas Chromatography-High Resolution Mass Spectrometry (OMOE, 2005; see section 2.12) at the OMOE Laboratory. The resulting concentration was corrected for the above dilution step. The actual concentration, rather than the nominal concentration of the standard, was used to calculate the dosing volumes. DMSO (≥99.8%) was purchased from Sigma-Aldrich, and nonane (glass-distilled) and hexane (glass-distilled) were purchased from Caledon Laboratory Chemicals.

2.4 Glassware Washing

At the MOE Laboratory, glassware was washed according to the ATU's SOP. All glassware was washed in a Laboratory Glassware Washer on the intensive cycle, which included: a pre-rinse cycle, main wash cycle (85°C), neutralizer rinse cycle (citric acid; Neodisher Z: free from phosphates and surfactants), two distilled water rinse cycles, followed by one last water rinse cycle at 85°C. Glassware was then rinsed with dechlorinated water for at least 24 hours before use and was air dried. Glassware used for dosed treatments was rinsed with hexane for several minutes before washing.

2.5 Partitioning Experiment

A partitioning experiment was initiated to determine the partitioning behaviour of BDE47 in the laboratory experiment. Beforehand, a wooden platform was built for the shaker table which would accommodate 12-2L glass Erlenmeyer flasks. Plastic rings were constructed to fit around the top of the flasks and were fastened to the wooden platform with elastics to keep the vessels in place. Test vessels (nine replicates) containing reference sediment, nutrient medium and algae (*P. subcapitata* at 5 x 10^6 cells/mL) were dosed with 14µg/L BDE47 and fastened to the shaker table. To collect the BDE47 lost to volatilization, a solvent-rinsed cheesecloth plug containing precleaned Amberlite non-ionic polymeric absorbent (XAD-2 resin) was placed in the flask opening and was covered with solvent-rinsed aluminum foil and tied in place with solvent-rinsed string. Three other test vessels with only *P. subcapitata* and nutrient medium were also dosed and fastened to the shaker table in the environmental chamber. A cheesecloth/XAD plug was placed in the flask opening, covered with aluminum foil and tied in place. Reference sediment (Lake 240, Experimental Lakes Area, Northern Ontario), nutrient medium and glassware were autoclaved at 121.4°C and 120.7kPa in advance. The shaker table was set at 100 rpms to keep the algae suspended and the environmental chamber was maintained at 18 to 22°C under cool fluorescent light (35.8 to 47.5µmol·m^{-2}·s^{-1}) using a 16:8 hour photoperiod.

The intention was to analyze each matrix (sediment, nutrient medium, algae, XAD and glass vessel) for BDE47 on three occasions (days 1, 7 and 21). At each time interval, the three

replicates would be removed from the experiment and the matrices separated for analysis, so that by day 21, all vessels would be removed. Due to financial constraints related to the cost of chemical analysis, this experiment was not completed. However, the information provided above could be useful for further study, since partitioning experiments are essential to understanding PBDE compartmentalization in the environment.

2.6 Algal Exposure to BDE47

Individual Erlenmeyer flasks containing *P. subcapitata* and *C. fusca* were dosed once with 14µg/L BDE47 at the start of a five to seven day exposure. At this concentration, the exposure of the algae to DMSO was kept to within recommended levels (0.01%v/v; Okumura, 1996). A calibrated Eppendorf pipette was initially used to transfer the necessary volume of BDE47 standard to a 2L volumetric flask partially filled with autoclaved nutrient medium. The flask was then topped up with nutrient medium and mixed end-to-end several times before being decanted into the test flask. The test flask was subsequently filled with nutrient medium and inoculated with algae to obtain an initial algal density of 10^5 cells/mL. Preliminary chemical analysis suggested that the BDE47 was binding to the glass of the volumetric flask during the transfer step, so in subsequent experiments the BDE47 was dosed directly into the test flasks without pre-mixing. Two control flasks (one for each algal species) containing the same initial algal density were set up at the initiation of the experiment. The algae were then grown under culture conditions (20±2°C; 50 to 66µmol·m^{-2}·s^{-1}; under aeration) and counted daily to ensure logarithmic growth was achieved. At the termination of the test, the algae were sealed and stored in the dark at 4 to 5°C. To minimize the volume of algal culture medium transferred to *D. magna* test vessels, the algae were concentrated by centrifugation at 1000g for five minutes (USEPA, 2002). After centrifugation, the algae samples were resuspended in distilled water and the cells were counted (in triplicate) under a microscope (40x) by haemocytometer. The organic carbon content of the algae was measured (in triplicate) by high temperature oxidation with a Total Organic Carbon Analyzer (Shimadzu (Mandel) TOC-V CSH). Distilled water blanks were measured and the TOC concentration was deducted from the algal sample TOC concentration. A nomograph, which is a correlation using a surrogate measurement, was

prepared to relate the cell count (cells/mL) to the organic carbon content of the cells, allowing the cell count to be used as a measure of organic carbon concentration. The cell density representing the necessary organic carbon content was used to determine the feeding ratio for the *D. magna* experiment.

The equivalent of one gram (wet weight) of algae (*P. subcapitata* and *C. fusca)* was concentrated and transferred to glass amber sample bottles (individually) for chemical analysis. A 30:70 hexane:acetone mixture (approximately 50mL) was added to the sample to extract the BDE47. Algae samples were stored in the dark at 4 to 5°C until chemical analysis (>60 days).

2.7 *Daphnia magna* Exposure to BDE47

D. magna were dosed with 14µg/L BDE47 during the Reproduction Tests (see section 2.8). At this concentration, the exposure of the *D. magna* to DMSO was kept to within recommended levels (0.01%v/v; OECD, 1998; Palma *et al.*, 2009). A calibrated Eppendorf pipette was initially used to transfer the necessary volume of standard into a 2L volumetric flask partially filled with dechlorinated water. The flask was then topped up with dechlorinated water and mixed end-to-end several times. The stock solution was subsequently poured into the test beakers. The remaining stock water was stored at 4 to 5°C for chemical analysis. At each media renewal, daphnids were dosed using new stock solutions that were prepared from the standard. In subsequent experiments, the BDE47 was dosed directly into the test vessels without pre-mixing to ensure that the compound was present in the test vessels.

2.8 *Daphnia magna* Reproduction Test

D. magna were exposed to BDE47 at 14µg/L according to the OECD 21-day Reproduction Test (Method 211, OECD, 1998) with some modifications (see Appendix I, Table A1-2 for a summary of the test conditions). *D. magna* neonates were exposed to BDE47 for 21 days via water (DW), algae (DA) and both water and algae (DWA). A control (C) containing only dechlorinated water and a solvent control (SC) containing DMSO at the same concentration as the dosed treatments (0.01% v/v) were also included, for a total of five treatments. The DA and DWA treatments were fed with algae that had been pre-dosed with 14µg/L BDE47 (see

Section 2.6). *D. magna* neonates were held individually in 50mL (Tests #1 and 2) or 100mL (Tests #3 and 4) glass beakers for each treatment, with 10 replicates for a total of 50 test vessels. The test beakers were arranged randomly in an environmental chamber in plastic containers (Tests #1 to 3) or a waterbath in wire baskets (Test #4) and maintained at 20±2°C under cool white fluorescent lights (6-13$\mu mol \cdot m^{-2} \cdot s^{-1}$) and a 16:8 hour photoperiod with a one hour "dusk" transition phase. Temperature and light intensity was measured daily. To measure the light intensity reaching the medium, the meter (Control Company – Traceable® Calibration Light Meter) was held just above the vessels (under the nitex screen in Test #4). In the first two tests, the light intensity recommended in the OECD protocol (15-20$\mu mol \cdot m^{-2} \cdot s^{-1}$) was used. However, this light intensity was found to be too high (Test #2), promoting excess growth of the algae and causing an increase in pH. The light intensity was decreased to 6 to 13$\mu mol \cdot m^{-2} \cdot s^{-1}$, which is the recommended intensity in Environment Canada's acute lethality protocol for *D. magna* (EC, 2000) and is also used by the OMOE ATU Laboratory for culturing.

The waterbath used in Test #4 was in an open laboratory so 363µm nitex screens were placed on the wire baskets to protect the vessels from dust and debris. The daphnids were maintained under static-renewal conditions (media changes three times per week) and were fed daily with algae. In Tests #1 and 2, the *D. magna* were fed at a 50:50 ratio of *P. subcapitata* and *C. fusca*. Daphnids were fed approximately 0.27mg OC/L (organic carbon equivalent) in Test #1; this feeding rate was determined to be too low. In Test #2, the daphnids were fed the same feeding rate used by the OMOE ATU Laboratory for daphnid culturing (*ad libitum* (i.e. as much as desired); approximately 4.5mg OC/L). This feeding rate was determined to be too high and the build-up of algae caused a shift in pH to above 9.0 (the maximum recommended in the OECD protocol). In Tests #3 and 4, the algae feeding rate was 0.5-1.0 mg OC/L (Sims *et al.*, 1993) and the rations were varied to accommodate the filter-feeding abilities of the animals at different stages of their life cycle. Younger daphnids tend to feed on smaller algae, such as *P. subcapitata,* and the older daphnids prefer the larger *C. fusca* (Pers. Comm., D. Poirier, OMOE). Therefore a mixture of *P. subcapitata* and *C. fusca* was used at the following rations: 75:25 on days 1 to 7, 50:50 on days 8 to 14 and 25:75 on days 15 to 21. Vitamin B_{12} (1µg/L) was added to vessels to increase reproduction in Test #4. The B_{12} was added directly to the medium until the

time of first egg formation. During media changes, neonates were gently transferred to the new vessel with glass pipettes, limiting the amount of medium transferred.

Daphnids were observed daily for: mortality (lack of a heartbeat confirmed by microscopic inspection); immobility (heartbeat observed but inability to swim for at least five seconds); swimming pattern (spiraling in water column, at the water surface); carapace molts or incomplete loss of exoskeleton; and aborted eggs or neonates. The time to egg formation and first brood as well as the number of neonates per brood were recorded. After the neonates were counted (daily), they were removed from the test vessel and discarded. On days when a medium change was not scheduled, the medium was poured through a nitex screen (to separate the neonates) and reused. Water parameters (temperature (T), pH, dissolved oxygen (DO), and conductivity) were measured in the medium (two-day old) after each water change (Radiometer Analytical PHM240 pH/Ion meter, MeterLab®; WTW StirrOxG Profiline DO Meter Oxi197; Radiometer Copenhagen CDM230 Conductivity Meter, MeterLab®). Adult body length was measured after day 21 to compare growth of daphnids between treatments. The adults were immobilized by exposing them to carbon dioxide-charged water for one to two minutes. The body length and apical spine length were measured under a culture microscope with a calibrated micrometer. Apical spines were not always present or complete (i.e. damaged during handling) so only the body length was compared between treatments.

To assess the impacts of BDE47 exposure on the second generation, brood three neonates from each treatment were transferred to clean media and observed for 21 days. Ten neonates per treatment were chosen randomly and transferred to 10 beakers of dechlorinated water. The daphnids were maintained under the same conditions as the first generation, without the dosing (clean algae and clean water) for 21 days (Second Generation - No Dose). The same observations and parameter measurements were completed as above. In addition, neonates from the fifth brood were collected and used for a second generation dosing experiment (Second Generation - Dose). Ten neonates per treatment were chosen randomly and exposed to the same dosing regimen as the first generation (C, SC, DW, DA, DWA). The same observations and parameter measurements were completed as above. In Test #4, vitamin B_{12}

was not added to the Second Generation - No Dose or Second Generation - Dose tests. Figure 5 summarizes the test protocol development.

2.9 *Daphnia magna* Feeding Experiment

After Test #2, a feeding experiment was conducted to determine the optimum algal feeding rate for *D. magna*. The neonates were held individually in 100mL glass beakers and fed at five different feeding rates (0.2, 0.4, 0.8, 1.6, and 3.2mg OC/L) with varying media exchange rates for 17 days. At each feeding rate, the media in three replicate test vessels was exchanged using the following rates: daily, three days per week and three days per week with the addition of 2µg/L of selenium and vitamin B_{12} (for a total of nine replicates per feeding rate). Survival and reproduction were assessed to establish optimal experimental conditions and the pH of the media was monitored two to three times daily.

2.10 Culture Experiment

After Test #3, a study was conducted to compare reproduction in *D. magna* cultured in an environmental chamber versus a water bath. The neonates were held individually in 100mL glass beakers with 10 replicates in an environmental chamber and a water bath for 10 days (to monitor the time to first brood). Vitamin B_{12} (1µg/L) was added to the vessels after medium exchanges until the first brood was released. Survival and reproduction were assessed to establish optimal experimental conditions and the pH of the media was monitored every 1-2 days throughout the test.

2.11 Fathead Minnow (*Pimephales promelas)* Experiment

Four month old *Pimephales promelas* were exposed via water to 14µg/L BDE47. The fish were separated from the ATU Laboratory culture and ten replicates were weighed and measured and placed into 20L glass tanks. Three treatments were tested, a control (dechlorinated water), solvent control (0.01%v/v DMSO), and a dosed treatment (14µg/L BDE47). Three replicate tanks for each treatment were used for a total of nine tanks and 90 fish. Tanks were filled with filtered (10µm) dechlorinated water that had been aged for several

days. Tank water was aerated gently through glass tubing and each tank was covered with a plexiglass lid. The temperature was maintained between 23 and 25°C under cool white fluorescent lights (8.25-16.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with a 16:8-hour photoperiod. Fish were fed frozen brine shrimp two times daily and approximately one hour after the second feeding, uneaten food was siphoned out, filtered and the filtrate was returned to the tank. Half of the tank water was renewed once per week; newly dosed water was added to the dosed treatments. Due to general culturing difficulties, this experiment was not completed. The methodologies are included here for reference and future research.

2.12 Chemical Analysis

At the beginning of Test #4, 800mL of dechlorinated water were added to a glass amber sample bottle and dosed directly with BDE47 to produce a ten-fold dilution, which was necessary to avoid supersaturation of the instrument. After water exchanges, the medium (two-day old) from the test beakers (first generation) was filtered through a nitex screen (to remove eggs/debris) and transferred to a glass amber sample bottle. A composite sample from two water changes was used to obtain the minimum sample volume (1L) for analysis. Test beakers were then air dried and soaked with hexane for 30-60 minutes to capture any BDE47 bound to the sides of the vessel. The hexane from each treatment was transferred to a glass amber sample bottle. Water samples were stored in the dark at 4 to 5°C until chemical analysis.

Samples were analyzed by Gas Chromatography-High Resolution Mass Spectrometry (GC-HRMS) at OMOEs Dioxin and Toxic Organics Laboratory according to method E3430 (OMOE, 2005; Kolic *et al.*, 2009). Water samples were acidified (to pH < 3) and fortified with [$^{13}C_{12}$] surrogate standards. Samples were then filtered using a C18 Solid Phase absorption disk. A 70:30 ethanol/toluene solution was used to extract the PBDEs from the disk and particulate matter. Concentrated extracts were further "cleaned" from bulk co-extractables using a two-stage cleanup. First the sample was eluted through a column containing $AgNO_3$-silica-NaOH-silica-H_2SO_4-silica with hexane and a 50:50 dichloromethane-hexane solution. The eluate was then concentrated and put through an additional alumina cleanup. Samples were eluted

through the alumina column with hexane and 10% CCl_4-hexane, followed by 20% methanol-DCM. The final eluate was evaporated to dryness and transferred to a conical vial for analysis. Samples were separated and detected with a Hewlett-Packard 6890-Plus GC (with splitless injection system and temperature programming) coupled to a VG Autospec-Ultima NT High Resolution MS which operates in EI (Electron Ionization) using selected ion monitoring and isotope dilution. Tri to deca-brominated diphenyl ethers were analyzed and quantified on a DB-5HT 15m x 0.25mm x 0.10μm column. Table 4 lists the GC operating conditions.

Table 4. HP 6890 plus GC Operating Conditions (Kolic et al., 2009).

Operating Conditions	
He carrier gas flowrate: 1.0mL/min (constant flow) Injector temperature: 270°C Transferline temperature: 290 - 300°C Splitless injection purge on at 1 minute DB-5HT column: 15m x 0.25mm x 0.10μm	
Run Conditions	
Oven Temperature Program	**GC Conditions**
Initial conditions	110°C hold 1 min
1st Ramp Rate	110°C to 200°C at 40°C/min
2nd Ramp Rate	200°C to 330°C at 10°C/min
Holding Time Rate	~5.5 min (until DecaBDE elutes)

Isotopically-labeled [$^{13}C_{12}$] internal standards from each homologue group, including the decaBDE, were added to all samples to monitor and correct for any losses during preparation and/or instrument and matrix effects. A Spiked Procedure Blank (SPB) and a Precision and Recovery Sample (PAR) were prepared and processed with each set of samples. Standard peak definition criteria were used and a signal to noise ratio for the most abundant ion was at least 5:1. The blanks were monitored for PBDEs and sample results were only reported if the levels were at least double the blank concentrations.

2.13 Statistical Analysis

Exploratory analysis was performed to screen the data for normality and outliers. Dixon's Test (Sokal and Rohlf, 1998) was performed to further detect any outliers. To determine significant differences between treatments for all experiments, a one way Analysis of Variance (ANOVA) followed by a post hoc multiple comparison test (Tukey's Honestly Significant Differences (HSD) Test or, if the sample sizes were unequal, Gabriel's Test which employs the studentized maximum modulus distribution) was used (Sokal & Rohlf, 1995; SPSS 16.0, 2007). When the variances were unequal (as determined by Levene's, Brown-Forsythe, and Welch Tests), a non-parametric Kruskal-Wallis test was performed followed by a multiple comparison test (Statistica 7.0, 2004). For the algae nomograph, a linear regression was calculated (SPSS 16.0, 2007) to determine the relationship between algal density (cells/mL) and TOC. For the culture experiment, Student's t-tests were performed on reproduction parameters to determine differences between the treatments (SPSS 16.0, 2007). The power of the ANOVA was calculated and was deemed acceptable if it was at least 80%.

3.0 RESULTS AND DISCUSSION

This research examined the toxic effects of BDE47 on *Daphnia magna* exposed via water and dietary routes. The *D. magna* diet consisted of the algae *Pseudokirchneriella subcapitata* and *Chlorella fusca*. Impacts on survival and reproduction were assessed in a 21-day experiment. Reproduction tests on second generation daphnids were also completed, with and without subsequent BDE47 exposure, to assess the impacts of the exposure.

3.1 Algal Exposure to BDE47

3.1.1 Algal Growth

Although a formal growth inhibition test was not performed on the algae, algal density was counted daily to determine if any significant impacts on growth were occurring. Figure 6 shows the growth of the control and dosed cultures of *P. subcapitata* and *C. fusca*.

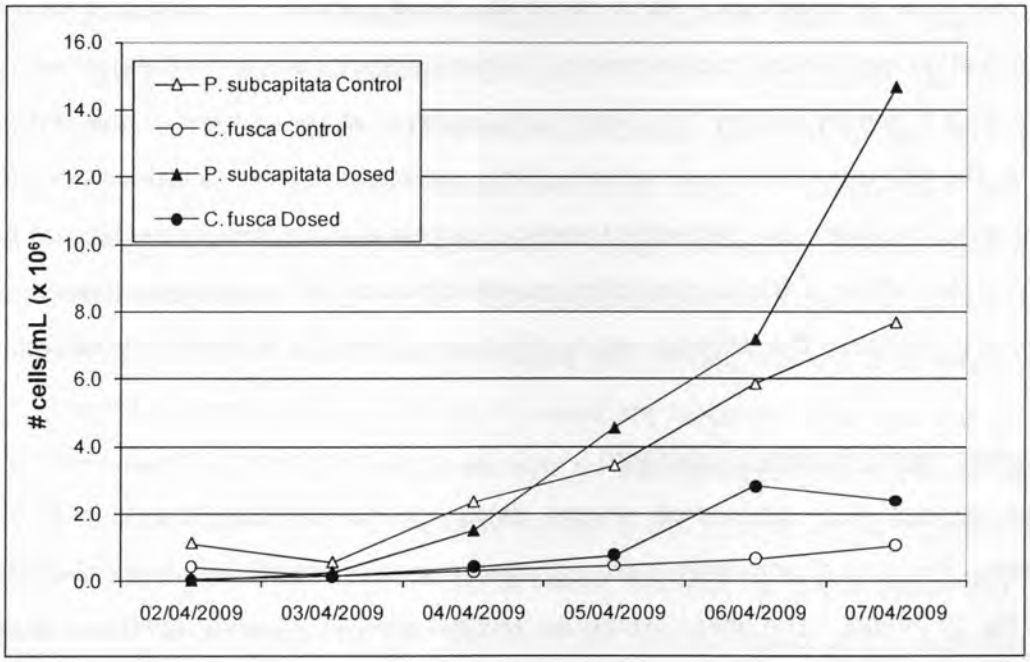


Figure 6. Growth of algal cells during a BDE47 dosing experiment using 14µg/L. Daily samples were counted with a haemocytometer.

It appears from the cell counts that cell growth was not inhibited at 14µg/L BDE47. Regression analysis of algal growth over time was completed on the natural log transformed counts. Cell counts that did not fit the linear pattern were removed beforehand and the slopes of the lines were compared using a Student's t-test (SPSS 16.0, 2007). A significant difference was detected between the slopes of the control and dosed growth curves for both *P. subcapitata* and *C. fusca* ($p < 0.05$). The dosing of BDE47 appears to have enhanced the growth of both algal species. The reason for this apparent enhancement is unknown but may be due to impacts of the DMSO on bacterial growth in the algal culture. Although toxicity information for green algae could not be found for BDE47, Kallqvist *et al.* (2006) determined a No Observed Effect Concentration (NOEC) of 6.6µg/L for BDE47 on the diatom *Skeletonema Costatum* and a 48 hour EC_{50} of 70µg/L. The concentration used in this experiment falls just above this NOEC.

The cell growth for the dosed *C. fusca* appears to have decreased near the end of the exposure. In order to minimize the chance of contamination of the algal culture, samples were taken through the glass air tube using a pipette bulb. In this way, the seal on the culture vessel did not have to be removed under the laminar-flow hood each day. However, in some cases the air circulation was too low to cause mixing of the whole culture and the *C. fusca* cells, which are large with a density greater than water, accumulated at the bottom of the test vessel. Therefore, the cell counts for these samples likely underestimate the actual cell density. In addition, cell counts were only performed once per sample and are therefore subject to human error. As stated above, a formal controlled growth inhibition test was not performed, as this was beyond the scope of the research, so any effects on growth could not be fully determined.

3.1.2 *Daphnia magna* Feeding Calculations

Algal dosings were performed several times to accommodate the four *D. magna* experiments. Two sets of nomographs were prepared to calculate the required cell density for feeding the *D. magna.* The linear regression analysis showed a highly significant increasing relationship between total organic carbon (TOC) and cell density for *P. subcapitata* ($r^2=0.993$, $p < 0.0001$) and *C. fusca* ($r^2=0.99$, $p < 0.0001$). For the fourth set of dosings, the *C. fusca* did not appear to be growing as well. The Ontario Ministry of Environment (OMOE) Aquatic Toxicity

Unit (ATU) had also been having trouble culturing the *C. fusca* and it was unknown what was causing the reduced growth. Since the algae did not appear to be growing at the same rate as in previous cultures, the TOC analysis was repeated and a new nomograph was prepared. Once again the linear regression analysis showed a significant increasing relationship between TOC and cell density for *P. subcapitata* (r^2=0.99, $p < 0.0001$) and *C. fusca* (r^2=0.88, $p < 0.0001$). The TOC analysis, however, showed a reduction in organic carbon content in the *C. fusca* culture compared to the previous analysis, which confirmed the observed reduction in growth. *P. subcapitata* showed a slight increase in organic carbon content relative to the previous analysis. Appendix III, Figures A3-1 and A3-2 show the two sets of nomographs.

3.2 *Daphnia magna* Reproduction Test

Due to a series of difficulties with feeding and culturing of the *D. magna* during the 21-day reproduction test, the experiment was repeated four times. The feeding rate used in Test #1 (0.27mg OC/L) was determined to be too low. In Test #2, the feeding rate was increased to the level used by the OMOE ATU laboratory for daphnid culturing (*ad libitum*; approximately 4.5mg OC/L). This rate of feeding proved to be too high in the small 50mL vessels, and resulted in a large increase in pH, which exceeded the maximum criteria (pH=9.0) for the OECD protocol (OECD, 1998). A feeding experiment was performed at this point to determine the optimal feeding rate and medium renewal rate (see Section 3.2.1). The optimal feeding rate was in line with recommended feeding rates in the OECD protocol and from the literature (0.5-1.0mg OC/L; Sims *et al.*, 1993) and it was decided to apply this feeding rate and to continue with the medium renewal of three times per week. To avoid any large pH variations, the size of the vessel was increased to 100mL for Test #3, allowing for more gas exchange due to the increased water surface area. Unfortunately, the temperature in the environmental chamber was not constant and fell below the minimum temperature allowed by the protocol (18°C). To determine if the environmental chamber was having an adverse impact on the daphnids (either by temperature shifts, evaporation of the media or vibration/noise), a control experiment was conducted comparing reproductive success of daphnids (under control conditions) in the chamber versus a waterbath (see Section 3.2.2). Daphnid survival and mobility were improved

in the waterbath likely due to the consistent temperature and reduced noise and/or vibration. Therefore, the fourth experiment was conducted in the waterbath.

3.2.1 *Daphnia magna* Feeding Experiment

Due to difficulties finding an optimal *D. magna* feeding rate during the first two attempts at the *D. magna* reproduction test, a feeding study was conducted (Table 5).

Table 5. Observations from the *Daphnia magna* Feeding Experiment; means (standard deviation – SD) are listed. The medium was changed at three different intervals: MWF = Monday, Wednesday, Friday; Daily; and MWF + Se/B12 = Monday, Wednesday, Friday with the addition of selenium and vitamin B_{12}.

	Feeding Rate #1 0.2mg OC/L			Feeding Rate #2 0.4mg OC/L			Feeding Rate #3 0.8mg OC/L		
Media Exchanges	**MWF**	**Daily**	**MWF + Se/B12**	**MWF**	**Daily**	**MWF + Se/B12**	**MWF**	**Daily**	**MWF + Se/B12**
pH Range	8.2-8.5	7.9-8.3	7.9-8.4	7.9-8.5	7.8-8.3	7.9-8.4	7.9-8.6	7.8-8.4	7.9-8.6
Time to first brood (days)	-	13.0 (-)	13.0 (-)	11.7 (0.6)	13.0 (2.8)	13.0 (-)	10.0 (0)	10.5 (0.7)	10.0 (0)
Reproduction (n's/brood)	-	2.0 (-)	2.0 (-)	4.7 (2.0)	4.0 (1.4)	3.0 (-)	10.8 (3.1)	10.8 (1.6)	8.7 (1.4)
	Feeding Rate #4 1.6mg OC/L			**Feeding Rate #5 3.2mg OC/L**					
Media Exchanges	**MWF**	**Daily**	**MWF + Se/B12**	**MWF**	**Daily**	**MWF + Se/B12**			
pH Range	8.1-8.8	7.9-8.5	8.0-8.7	8.0-8.9	8.0-8.6	8.2-9.0			
Time to first brood (days)	-	9.5 (0.7)	10.5 (2.1)	10.0 (0)	9.5 (0.7)	10.0 (0)			
Reproduction (n's/brood)	-	14.3 (4.7)	13.5 (6.4)	3.5 (3.5)	1.5 (0.7)	18.8 (9.7)			

Different media exchange rates were also tested (three times per week (Monday, Wednesday, Friday (MWF)) or daily) and selenium and vitamin B_{12} were added to some of the test vessels to determine optimum conditions. The pH was measured one to three times per day to closely monitor any increases. The results showed that at feeding rates 1 and 2 (0.2 and 0.4mg OC/L), daphnids did not meet established criteria for a healthy population: first brood ≤ 12 days and reproduction rates between 15 and 20 neonates per brood (n's/brood) (EC, 1996; Bianchini & Wood, 2002). Feeding rate 5 (3.2mg OC/L) resulted in elevated pH readings (at or near 9.0) and although the first brood was released within 9.0 to 10.0 days, reproduction was very low in two of the three replicates. Mean reproduction was the highest for the MWF +

Se/B_{12} treatment (18.8 n's/brood) but the pH did reach 9.0 during the experiment. Conditions appeared to be optimum for feeding rate 4 (1.6mg OC/L), as the first broods were released within an average of 9.5 to 10.5 days and mean reproduction was high (13.5 to 14.3 n's/brood). At feeding rate 3 (0.8mg OC/L), daphnid performance was also relatively good, with the first brood released between 10.0 and 10.5 days and the mean reproduction ranging from 8.7 to 10.8 n's/brood. Although the reproduction rate for feeding rate 3 was not as high as feeding rate 4, the pH range was somewhat lower. For all of the treatments, there did not appear to be a significant difference between the MWF and daily water exchanges or the addition of selenium and B_{12}, so the MWF water exchange rate was deemed appropriate. The results show an optimal feeding rate between 0.8 and 1.6mg OC/L, which corresponds well with the literature value of 0.5 to 1.0mg OC/L (Sims *et al.*, 1993). Due to the fact that the number of replicates was small for this preliminary experiment and that the pH was slightly elevated at feeding rate 4, it was decided to default to the literature value (0.5-1.0mg OC/L), which falls between feeding rates 3 and 4.

3.2.2 *Daphnia magna* Culture Experiment

After temperature fluctuations in the environmental chamber during the *D. magna* reproduction test #3, a culture experiment was initiated to determine if housing the test vessels in a waterbath would result in better reproduction than in the environmental chamber. Besides the temperature fluctuations, significant evaporation of test medium was noted during *D. magna* Reproduction Test #3 (12-19% over the two days between media exchanges). In addition, the environmental chamber contained fans that produced a loud noise and vibrated, which may also have impacted daphnid performance. In each of the locations (environmental chamber and waterbath), reproduction and survival was monitored until the first brood was released for all replicates (10 days). Mortality was 30% in daphnids in the environmental chamber whereas there was no mortality observed in those in the waterbath. Daphnid mobility appeared to be impacted in the chamber, resulting in daphnids with slower swimming patterns than those in the waterbath. For the daphnids in the chamber, eggs were visible within 6.0 to 7.0 days, while in the waterbath, eggs were visible within 5.0 to 7.0 days, although these did

not differ significantly (p = 0.205). For both treatments, neonates were produced in 8.0 to 10.0 days which is within the acceptable range for a healthy population (EC, 1996) and there were no significant differences between treatments (p = 0.435). Although it appeared that daphnids in the waterbath produced more neonates (mean = 15.6) than the daphnids in the chamber (mean = 12.7), they did not differ significantly (p = 0.126). Due to the higher mortality and the apparent reduction in mobility in the daphnids from the environmental chamber treatment, the waterbath was used to house subsequent experiments (Test #4, first and second generation).

3.2.3 *Daphnia magna* Reproduction Test #1 (see Figure 5)

D. magna neonates were exposed to 14µg/L BDE47 for 21 days via water (DW), algae (DA) and both water and algae (DWA). Two control treatments, the control (C) containing only dechlorinated water and the solvent control (SC) containing DMSO at the same concentration as the dosed treatments (0.01% v/v), were also included, for a total of five treatments. The DA and DWA treatments were fed with algae that had been pre-dosed with 14µg/L BDE47.

Throughout the test, the temperature in the environmental chamber ranged from 16.0 to 21.0°C and the initial light intensity was 15.5µmol·m^{-2}·s^{-1}. The drop in temperature below the required minimum (18°C) occurred on days 12, 19, and 21, but ranged between 19.0 to 20.0°C (within the acceptable range (OECD, 1998)) throughout the rest of the experiment. Daphnids were fed a mixture of algae daily which consisted of a 50:50 ratio of *P. subcapitata* and *C. fusca* at 0.27mg OC/L. After medium exchanges, pH, dissolved oxygen (DO), and conductivity were measured; however, in some cases these parameters were measured one to four days after the exchange, and may not accurately represent conditions during the experiment. The pH was within the recommended levels, ranging between 7.7 and 8.6. DO remained well over the recommended minimum of 3mg/L (OECD, 1998) ranging from 8.2 to 10.7mg/L, which is 91 to 117% of DO at saturation in freshwater at 20°C (9mg/L; APHA, 1989). Conductivity ranged between 255.9 and 413.0µS/cm. Water chemistry results are presented in Appendix IV, Table A4-1.

Table 6. Observations from the *D. magna* Test #1, first generation. Means (standard deviations) are shown, with the exception of mortality, which is an overall value.

First Generation		C	SC	DW	DA	DWA
Mortality	%	0.0	0.0	10.0	20.0	10.0
Time to egg formation (first)	days	7.0 (1.0)	7.9 (1.3)	8.7 (2.2)	7.8 (1.3)	8.8 (0.7)
Time to 1st brood	days	13.4 (2.2)	14.4 (1.9)	15.8 (2.2)	12.7 (0.8)	14.8 (1.8)
Time between brood 1 & 2	days	3.6 (0.5)	4.0 (0.7)	4.1 (0.8)	3.9 (0.4)	5.2 (1.6)
Time between brood 2 & 3	days	4.6 (0.5)	4.2 (1.0)	4.0 (-)	4.3 (0.8)	5.0 (-)
Mean time between broods	days	4.1 (0.2)	4.1 (0.7)	4.2 (0.8)	4.1 (0.4)	5.3 (1.5)
# of broods		2.7 (0.7)	2.5 (0.7)	2.0 (0.5)	2.9 (0.4)	2.1 (0.4)
Cumulative Reproduction (total neonates)		18.1 (5.4)	14.0 (4.4)	11.8 (4.4)	13.9 (2.2)	10.6 (1.9)
Reproduction (n's/brood)		6.5 (1.2)	5.9 (2.2)	6.0 (1.9)	4.9 (0.7)	5.0 (0.9)

Survival and reproduction parameters from the first generation *D. magna* test are listed in Table 6. Mortality was low in all treatments with no deaths in both control treatments. Mobility seemed to be reduced in all daphnids and some, particularly in the DA treatment, displayed a spinning behaviour at the vessel bottom, possibly to stir up any remaining food that had settled. Egg formation took between 7.0 and 8.8 days with marginally significant differences between treatments ($F(4,38) = 2.3$, $p = 0.078$). Time to egg formation was found to be significantly longer in daphnids from the DWA treatment compared to the C treatment only when an alpha level of 0.15 was used (Gabriel's Test; $p = 0.137$). Release of the first brood took between 12.7 and 15.8 days and significant differences between treatments were detected ($F(4,38) = 3.2$, $p = 0.025$).

The DA treatment had a significantly shorter time to first brood ($p = 0.026$; Figure 7) and had significantly more broods than the DW treatment but not the control treatments (C & SC; $F(4,38)= 3.3$, $p = 0.021$). The pooled time between broods was significantly longer for the DWA treatment than the rest of the treatments ($F(4,36) = 3.4$, $p = 0.019$). In addition, the DWA treatment had significantly less cumulative reproduction (total neonates; log transformed due to unequal variances) than the C treatment ($F(4,38) = 2.8$, $p = 0.037$; Figure 7). There was only a marginally significant difference between treatments for reproduction (n's/brood) ($F(4,38) = 2.3$, $p = 0.081$); daphnids from the DA treatment had significantly lower reproduction than the C treatment but only at an alpha level of 0.1 ($p = 0.094$). Throughout the reproduction test, no

male daphnids were observed and no ephippia were present indicating that conditions were not poor enough to trigger a change in reproductive strategy.

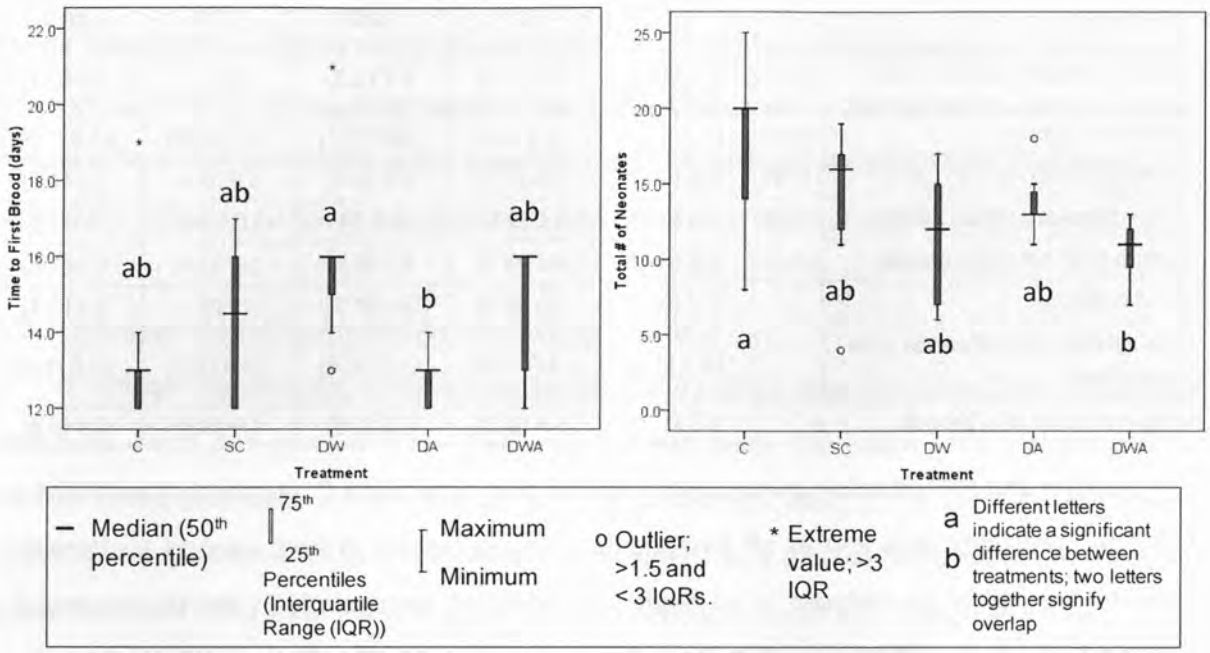


Figure 7. Boxplots showing the time to first brood and cumulative reproduction in daphnids from Test #1 (parent generation).

The results show that reproductive impacts occurred mainly on daphnids from the DWA treatment. This indicates that exposure of BDE47 at 14µg/L via both water and food had the most impact on *D. magna* reproduction. However, in the control treatments, reproduction did not meet established criteria for a healthy daphnid population (EC, 1996; Bianchini & Wood, 2002) and when a subsequent 21-day reproduction test was run without BDE47 dosing on offspring from the first generation, mortality was very high (80% in C, 30% in SC, 50% in DW, no mortality in DA, and 70% in DWA). Due to this high mortality, low reproduction in the first generation, and spinning behaviour, it was concluded that the feeding rate was too low in this test. It is likely that the low feeding rate was a confounding factor on the reproductive impacts.

3.2.4 *Daphnia magna* Reproduction Test #2 (See Figure 5)

During the second experiment, the temperature in the environmental chamber ranged from 18.3 to 20.8°C and the light intensity ranged from 13.7 to 17.5µmol·m^{-2}·s^{-1}, with the exception of one day when the light intensity dropped to 9.7µmol·m^{-2}·s^{-1}. Daphnids were fed a mixture of algae daily (50:50 ratio of *P. subcapitata* and *C. fusca* at 4.5mg OC/L, *ad libitum*). After medium exchanges, pH, DO and conductivity were measured; in some cases these parameters were measured the day after the exchange, and they may not represent conditions during the experiment. The pH was high and exceeded the recommended maximum of 9.0 on several occasions, ranging from 8.1 to 9.5. When pH was measured immediately, it tended to be higher, and therefore the pH measurements that were delayed may have underestimated the actual pH in the test vessel. In addition, DO ranged between 8.2 and 12.6mg/L (90-139% saturation), higher than in the previous experiment, and conductivity ranged from 314.8 to 448.0µS/cm. Photosynthetic activity likely began to increase due to the fact that there was a large amount of unconsumed algae remaining in the test vessels and the light intensity was relatively high. During photosynthesis, carbon dioxide (CO_2) is taken up by algae; this reduces the concentration of carbonic acid in the medium and in turn, raises the pH (Keating *et al.*, 1996). Due to this increase in photosynthetic activity, the medium can become supersaturated with oxygen, which was confirmed by the elevated DO levels. This shift in pH likely impacted the results of the reproduction test. Water chemistry results are presented in Appendix IV, Table A4-2.

Survival and reproduction parameters from the *D. magna* test are listed in Table 7. Mortality was low in most treatments, with no deaths in the DA and DWA treatments. Mortality reached 30% in the SC treatment, exceeding Environment Canada's test validity criteria for *Ceriodaphnia* of 20% mortality (EC, 1992b). Egg formation took between 6.3 and 6.8 days, with no significant differences between the treatments ($F(4,37) = 1.7$, $p = 0.164$). Statistical power was low for this test (~30%) and may have prevented the detection of a significant difference. Due to unequal variances for time between broods, a data transformation (1/x + 1) was necessary. The subsequent ANOVA failed to detect a statistical

difference between treatments ($F(4,37) = 0.7$, $p = 0.611$), but again, this may have been due to very low statistical power.

Table 7. Observations from the *D. magna* Test #2, first generation. Means (standard deviations) are shown, with the exception of mortality, which is an overall value.

First Generation		C	SC	DW	DA	DWA
Mortality	%	20.0	30.0	20.0	0.0	0.0
Time to egg formation (first)	days	6.4 (0.5)	6.3 (0.5)	6.8 (0.5)	6.8 (0.4)	6.7 (0.5)
Time to 1st brood	days	13.1 (0.7)	13.0 (1.6)	13.4 (0.5)	9.6 (0.5)	9.7 (0.5)
Time between brood 1 & 2	days	3.6 (0.5)	3.4 (0.5)	3.5 (0.5)	3.3 (0.5)	3.1 (0.3)
Time between brood 2 & 3	days	3.3 (0.5)	3.8 (0.4)	3.5 (0.5)	3.4 (0.5)	3.8 (0.4)
Time between brood 3 & 4	days	-	4.0 (-)	-	3.8 (0.4)	3.6 (0.5)
Mean time between broods	days	3.4 (0.2)	3.6 (0.2)	3.5 (0)	3.5 (0.2)	3.5 (0.2)
# of broods		3.0 (0)	3.0 (0.6)	3.0 (0)	4.0 (0)	4.0 (0)
Cumulative Reproduction (total neonates)		59.9 (6.9)	62.7 (16.5)	61.5 (8.6)	86.2 (8.6)	89.8 (7.6)
Reproduction (n's/brood)		20.0 (2.3)	20.7 (3.2)	20.5 (2.9)	21.6 (2.2)	22.4 (1.9)

The variances were also unequal for time to first brood and number of broods, and after unsuccessful transformations, the data were analyzed with the nonparametric Kruskal-Wallis test followed by a multiple comparison test of the mean ranks for all groups. The DA and DWA treatments were found to produce significantly more broods ($H(4,42) = 36.5$, $p < 0.0001$) and have a significantly shorter time to first brood ($H(4, 42) = 31.0$, $p < 0.0001$) than daphnids in the C, SC and DW treatments (Figure 8). Cumulative reproduction (total n's) was significantly higher for daphnids in the DA and DWA treatments ($F(4,37) = 19.2$, $p < 0.0001$; Figure 8). This result is due to the fact that reproduction occurred earlier in these two treatments and therefore one extra brood was released before the end of the experiment. When the number of broods was taken into account (n's/brood), there was no significant difference between treatments ($F(4,37)=1.362$, $p=0.266$).

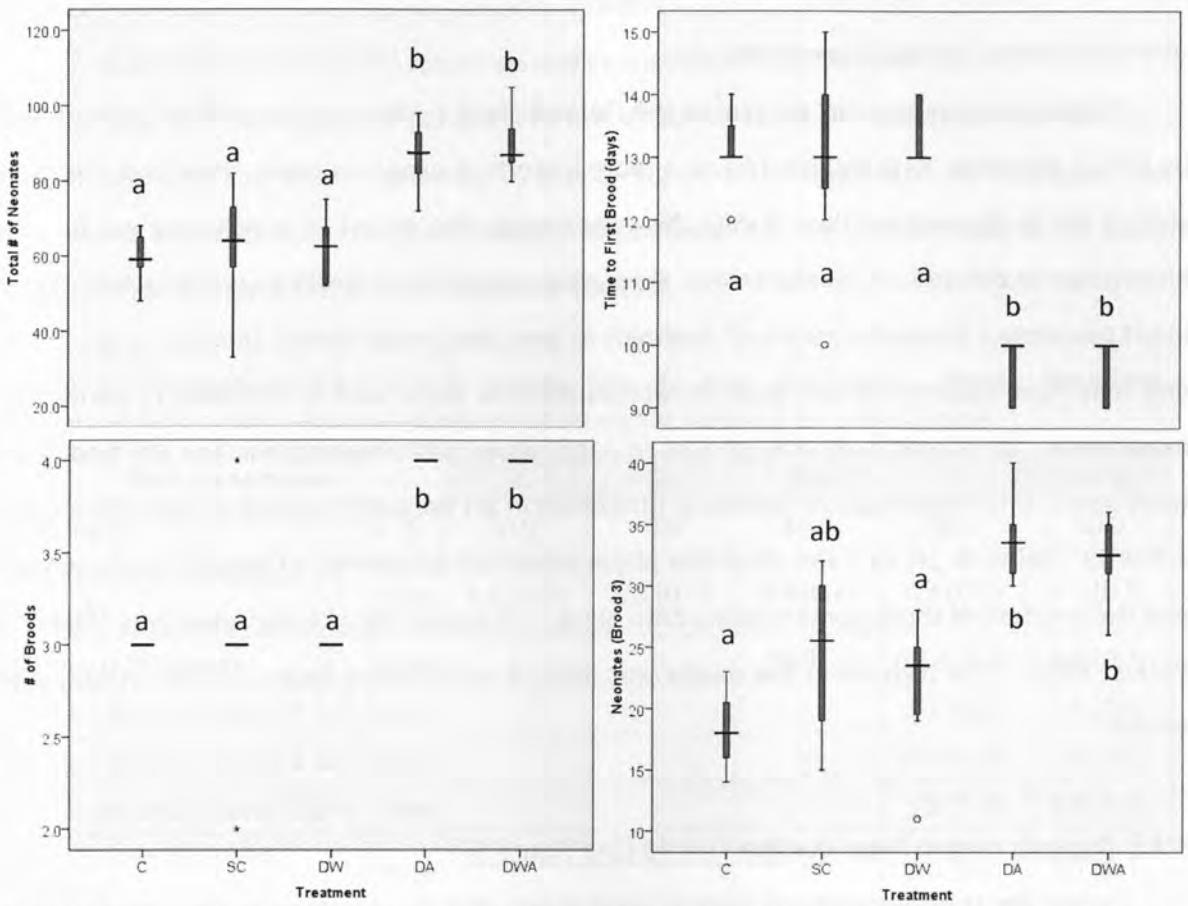


Figure 8. Boxplots showing the cumulative reproduction, time to first brood, number of broods and neonate production from brood 3 in daphnids from Test #2. The letters indicate statistically significant differences; those marked with the same letter, are not significantly different.

The number of neonates produced in each brood was analyzed to detect differences between treatments. In the first brood, the DW treatment produced significantly more neonates than the DA and DWA treatments ($F(4,37) = 5.2$, $p = 0.002$). No differences were detected between treatments for broods 2 ($F(4,37) = 0.7$, $p = 0.619$) and 4 ($F(2,18) = 1.9$, $p = 0.177$), but this may have also been due to low statistical power (<30%). The variances were unequal for the third brood, and after unsuccessful data transformations, the data were analyzed with the nonparametric Kruskal-Wallis test followed by a multiple comparison test. A significant difference between treatments was detected ($H(4,41) = 28.7$, $p < 0.0001$) and the multiple comparison test showed that neonate production was significantly higher for the DA and DWA treatments (Figure 8) compared to the C and DW treatments, but not significantly

higher than the SC treatment. Throughout the reproduction test, no male daphnids were observed and no ephippia were present.

In this experiment, reproduction in the DA and DWA treatments seemed to be enhanced by BDE47 exposure, with the food being a more important exposure route. However, the large shift in pH in the test medium during this experiment, the extent of which may not be fully known due to delayed pH measurement, likely confounded the impacts on reproduction. Shifts in pH can impact the performance of daphnids in laboratory experiments (Keating *et al.*, 1996), and may have caused the decrease in reproduction in the C and SC treatments during this experiment. It is unknown why similar impacts were not observed for the DA and DWA treatments, since there was no statistical difference in pH between treatments ($F(4,35) = 0.3$, $p = 0.901$). Shifts in pH can also alter the physicochemical properties of organic contaminants and the sorption of those contaminants onto algae and impact their biological activity (Richer & Peters, 1993). The high pH in the media was likely a confounding factor on the reproductive effects.

3.2.5 *Daphnia magna* Reproduction Test #3 (See Figure 5)

During the third experiment (parent generation), the temperature in the environmental chamber ranged between 18.0 and 20.0°C. The temperature was not measured in the test medium, but recorded from a thermometer in the environmental chamber. It was determined near the end of the experiment that the water temperature varied between test vessels with some dropping to 17.0°C, however, this drop was not consistent throughout the experiment. Since the water temperature was not measured consistently during the experiment, the full extent of the temperature variation is unknown. Considerable evaporation of the test medium in some vessels at the back of the environmental chamber was also noted. To limit photosynthetic activity and concomitant pH increases, the light intensity was reduced and ranged from 7.2 to 8.9$\mu mol \cdot m^{-2} \cdot s^{-1}$. Daphnids were fed varying rations of a mixture of *P. subcapitata* and *C. fusca* daily (week 1: 75:25, week 2: 50:50 and week 3: 25:75) at 0.5 to 1.0 mg OC/L to further prevent pH increases. Immediately after medium exchanges were complete, pH, DO and conductivity were measured. The pH ranged from 8.3 to 9.0, DO ranged

from 9.1 to 11.6mg/L (101-127% saturation), and conductivity ranged from 293.0 to 460.0 µS/cm. Water chemistry results are presented in Appendix IV, Table A4-3.

Survival and reproduction parameters are listed in Table 8. Mortality was low in all treatments, with no deaths observed in the C, SC, or DA treatments. As the experiment progressed, daphnids from the control treatments became less active while those in the DA and DWA treatments remained active throughout the test.

Table 8. Observations from the *D. magna* Test #3, first generation. Means (standard deviation) are shown, with the exception of mortality, which is an overall value.

First Generation		C	SC	DW	DA	DWA
Mortality	%	0.0	0.0	10.0	0.0	10.0
Time to egg formation (first)	days	6.6 (0.5)	6.8 (0.4)	6.8 (0.4)	6.4 (0.5)	6.3 (0.5)
Time to 1st brood	days	-	-	19.0 (-)	10.4 (1.3)	9.9 (0.3)
Time between brood 1 & 2	days	-	-	-	3.7 (0.5)	3.5 (0.5)
Time between brood 2 & 3	days	-	-	-	3.9 (0.3)	3.8 (0.6)
Time between brood 3 & 4	days	-	-	-	3.8 (0.4)	3.9 (0.4)
Mean time between broods	days	-	-	-	3.8 (0.2)	3.7 (0.1)
# of broods		-	-	1.0 (-)	3.4 (0.7)	3.8 (0.4)
Cumulative Reproduction (total neonates)		-	-	23.0 (-)	52.3 (14.5)	56.2 (13.0)
Reproduction (n's/brood)		-	-	23.0 (-)	15.3 (2.1)	14.7 (2.8)
Body Length (without tail spine)	mm	3.6 (0.1)	3.6 (0.1)	3.5 (0.1)	3.6 (0.1)	3.5 (0.2)

Egg formation occurred within 6.3 to 6.8 days and there was no difference detected between the treatments ($F(4,45) = 2.3$, $p = 0.072$); however, the statistical power for this test was very low (~35%). During the 21-day experiment, no daphnids from the C and SC treatments produced any neonates, and only one daphnid produced neonates from the DW treatment (on day 19). In both the DA and DWA treatments, neonate production met the criteria for a healthy population (i.e. first brood within 12 days and approximately 15 to 20 neonates per brood). There was no significant difference between reproduction in the

daphnids from DA and DWA, however statistical power was also low (~30-40%) and may have prevented the detection of significant differences. Throughout this reproduction test, no male daphnids were observed and no ephippia were present. As in Test #2, it appears that exposure to BDE47 via food had some protective effect on the daphnids, since reproduction was within accepted criteria in the DA and DWA treatments. Because reproduction was so poor in the control treatments, it is clear that experimental conditions were inadequate and these results are not reliable.

Since the C, SC and DW treatments did not produce any neonates, they could not be used for the second generation test. Neonates from the DA and DWA treatments were used in a second generation experiment (no dose) and neonates from the OMOE ATU laboratory culture were used for the C treatment (hereafter referred to as C* to differentiate it from the first generation C treatment). The temperature of the environmental chamber was the same as in the first generation, and therefore there were periods when the temperature dropped below 18°C.

Survival and reproduction parameters from the *D. magna* test are listed in Appendix IV, Table A4-4. Mortality was low in all treatments during the second generation test. The times to egg formation and first brood had unequal variances and after data transformation was unsuccessful, the nonparametric Kruskal-Wallis test followed by multiple comparison tests were used to determine significant differences between treatments. Egg formation took significantly longer for the DWA treatment than the DA and C* treatments ($H(2,25) = 16.1$, $p = 0.0003$). Daphnids from both the DA and DWA treatments released their first brood sooner than those in the C* treatment ($H(2,25) = 10.8$, $p = 0.0045$). The C* treatment also had fewer broods than the DA and DWA treatments ($F(2,25) = 32.4$, $p < 0.0001$) and lower cumulative reproduction (total neonates) than the DWA treatment ($F(2,25) = 4.8$, $p = 0.017$). Conversely, the daphnids from the C* treatment were significantly larger in size than those from the DA and DWA treatments ($F(2,24) = 37.0$, $p < 0.0001$). Throughout this reproduction test, no male daphnids were observed and no ephippia were present.

In all three treatments, release of the first broods took longer than 12 days and was significantly longer in the C* treatment (17.8 days; $p < 0.001$). Reproduction was also low in all

treatments; DA and DWA produced less than 15 neonates per brood, and although C* produced more than this, the daphnids only produced one brood throughout the whole experiment. The variation in temperature during the experiment may have resulted in low reproduction rates, as occurred during the first generation experiment for the C, SC and DW treatments. It is unknown why reproduction was acceptable in daphnids from the DA and DWA treatments during the first generation, but this apparent protective quality of BDE47 exposure via food did not continue for the second generation, where reproduction results were not acceptable. Due to poor performance of the control daphnids in both the first and second generation experiments, these results are questionable.

3.2.6 *Daphnia magna* Reproduction Test #4 (See Figure 5)

During the first generation Reproduction Test (GEN 1), the temperature and light intensity remained stable and were within recommended levels (20.1 to 20.7°C; 7.1 to 9.8µmol·m^{-2}·s^{-1}). Because daphnids initially consume less food, they were fed 0.5mg OC/L of a 75:25 mixture of *P. subcapitata:C. fusca* for the first week. This was also done to avoid large variations in pH. The feeding rate was then increased to 1.0 mg OC/L and a 50:50 mixture (week 2) followed by a 25:75 mixture (week 3) of *P. subcapitata: C. fusca* was fed to the daphnids. At every medium exchange, pH, DO and conductivity were measured immediately. The pH did not vary significantly throughout the experiment, ranging from 8.1 to 8.4 ($F(4,25) = 0.3$, $p = 0.880$). DO remained well above the recommended 3mg/L (OECD, 1998) ranging from 8.2 to 9.2mg/L (90-101% saturation) and did not differ significantly between treatments ($F(4,25) = 1.7$, $p = 0.179$). Conductivity ranged between 271.6 and 395.0µS/cm and was similar between treatments ($F(4,25) = 0.9$, $p = 0.486$).

Hardness was not monitored until the last day of the experiment but since the conductivity was consistently above 100µS/cm, hardness was likely above 100mg/L (as $CaCO_3$) as well (OMOE, 2008c). On the last day of the second generation, dose test (Section 3.2.6.2), the hardness of the medium was measured and ranged from 180mg/L (as $CaCO_3$) in the SC vessels to 400mg/L (as $CaCO_3$) in the DA vessels. Hardness measurements of dechlorinated laboratory water used in ATU laboratory tests during the same time period (April to May, 2009) ranged

from 128 to 144mg/L (as $CaCO_3$), near the minimum requirement for this protocol (140mg/L (as $CaCO_3$); OECD, 1998). Although, the water hardness may have been slightly lower than recommended, the performance of the *D. magna* culture always met the validity criteria, and was considered acceptable (OECD, 1998). Water chemistry results measured at medium exchanges are presented in Appendix IV, Table A4-5.

Survival and reproduction parameters from the first generation *D. magna* test are listed in Table 9 and a selection of these parameters is shown in Figure 9. Throughout the test, no male daphnids were observed and no ephippia were present. The mortality was very low in all treatments and only 10% of the C and SC treatment animals did not survive.

Table 9. Observations from the *D. magna* Test #4, First Generation. Means (standard deviation) are shown, with the exception of mortality, which is an overall value.

First Generation		C	SC	DW	DA	DWA
Mortality	%	10.0	10.0	0.0	0.0	0.0
Time to egg formation (first)	days	5.3 (0.5)	5.3 (0.5)	5.4 (0.5)	6.0 (0)	5.9 (0.3)
Time to 1st brood	days	8.0 (0)	8.0 (0)	8.0 (0)	8.6 (0.5)	8.2 (0.4)
Time between brood 1 & 2	days	3.0 (0)	3.0 (0)	3.0 (0)	2.4 (0.5)	3.0 (0)
Time between brood 2 & 3	days	3.0 (0)	3.0 (0)	3.0 (0)	3.0 (0)	3.0 (0)
Time between brood 3 & 4	days	3.0 (0)	3.0 (0)	3.0 (0)	3.0 (0)	3.0 (0)
Time between brood 4 & 5	days	3.0 (0)	3.0 (0)	3.0 (0)	3.0 (0)	3.0 (0)
Mean time between broods	days	3.0 (0)	3.0 (0)	3.0 (0)	2.9 (0.1)	3.0 (0)
# of broods		5.0 (0)	5.0 (0)	5.0 (0)	5.0 (0)	5.0 (0)
Cumulative Reproduction (total neonates)		124.0 (8.0)	115.9 (10.2)	119.9 (7.0)	89.6 (9.3)	100.0 (9.2)
Reproduction (n's/brood)		24.8 (1.6)	23.2 (2.0)	24.0 (1.4)	17.9 (1.8)	20.0 (1.8)
Body Length (without tail spine)	mm	4.3 (0.1)	4.3 (0.1)	4.3 (0.1)	4.2 (0.1)	4.2 (0.1)

Eggs were visible in the brood pouch within 5.0 to 6.0 days for all treatments. Daphnids from the C, SC, and DW treatments released their first brood within 8.0 days while daphnids from the DA and DWA treatments took somewhat longer (8.6 and 8.2 days, respectively). These results show a slightly shorter time to first brood compared to the daphnid culture

animals, which generally release their first brood between 9.0 and 12.0 days (OMOE, unpublished data). This is likely due to the addition of the vitamin B_{12} (first generation only), which has been shown to be necessary for normal reproduction in *Daphnia pulex* (Keating, 1985). Deficiencies can cause delayed reproduction and infrequent molting, and can disrupt the reproduction of the second generation (Keating, 1985). In the second generation experiments, vitamin B_{12} was not added as it was thought that the benefits provided to the first generation would be passed on to the second generation, improving their reproduction. Without the addition of vitamin B_{12} in the second generation experiments, the release of the first broods generally took longer than in the first generation.

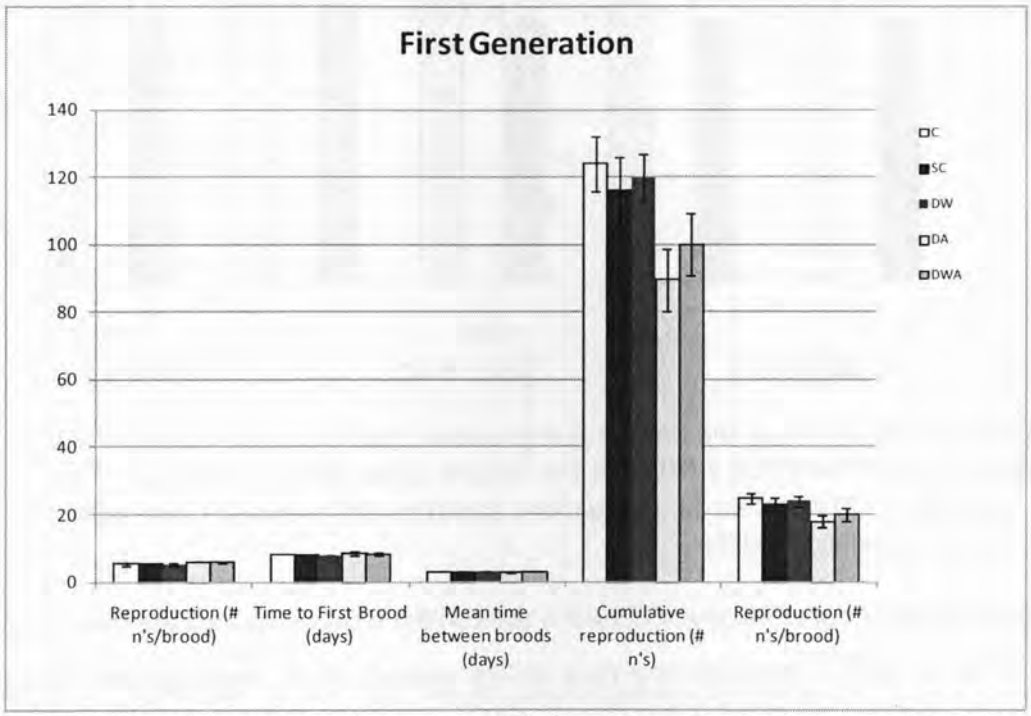


Figure 9. Observations (means ± SD) from the *D. magna* Test #4, First Generation.

In Figure 10, time to first brood is compared in the First Generation (GEN 1), the Second Generation - No Dose (GEN 2-ND), and the Second Generation - Dose (GEN 2-D) Tests. An ANOVA followed by a Tukey's post-hoc test was completed on the DWA treatment and due to unequal variances, the nonparametric Kruskal-Wallis test followed by multiple comparison tests were performed on the other treatments (C: $H(2,27) = 24.1$, $p < 0.0001$; SC: $H(2,24) = 21.7$, $p <$

0.0001; DW: H(2,25) = 23.2, $p < 0.0001$; DA: H(2,28) = 23.3, $p < 0.0001$; DWA: F(2,18) = 52.9, $p < 0.0001$). The first brood was released significantly later in GEN 2-D than in GEN 1 for all treatments ($p < 0.0001$). In addition, the first brood was released significantly later in the GEN 2-D test than in the GEN 2-ND test for the DA ($p = 0.016$) and DWA treatments ($p < 0.0001$).

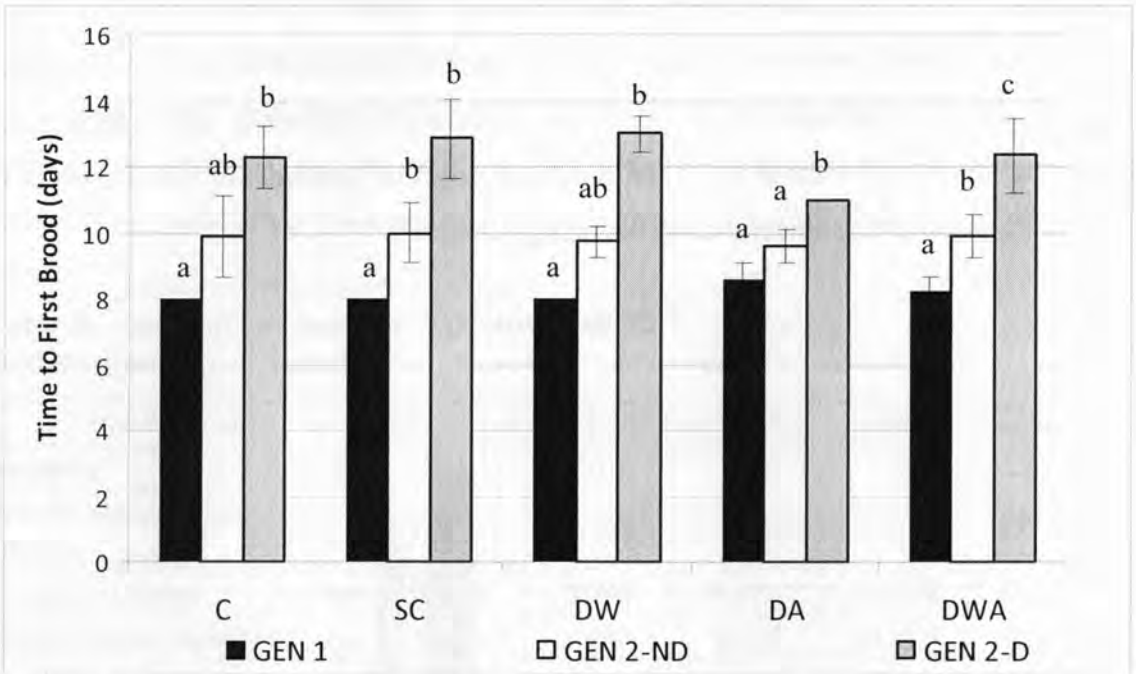


Figure 10. A comparison of the time to first brood for the First Generation (GEN 1), Second Generation - No Dose (GEN 2-ND) and the Second Generation - Dose (GEN 2-D) tests for each treatment. The letters (a,b,c) identify statistical differences; those with the same letter are not significantly different.

Significant differences observed between the control treatments indicate that the addition of vitamin B_{12} to GEN 1 improved the time to first reproduction. Reproductive delays in the second generation tests may have resulted from the lack of vitamin B_{12} but impacts caused by the BDE47 exposure cannot be distinguished in the dosed treatments. In addition, other factors relating to food resources and environmental conditions that were not considered here may have also led to differences between the generations. Although the algae feed used in the first and second generation experiments likely contained some level of vitamin B_{12}, it appears that the supplement given to the first generation did improve reproduction.

Of the observations listed in Table 9, only reproduction (both total neonates and neonates per brood; $F(4,41) = 24.8$, $p < 0.0001$) and body length ($F(4,42) = 6.2$, $p = 0.001$) showed significant differences between treatments. The boxplot in Figure 11i shows that reproduction was significantly lower in daphnids from the DA and DWA treatments than the other treatments (C, SC, DW). Cumulative reproduction was also significantly lower in the DA and DWA treatments compared to the C, SC and DW treatments ($F(4,41) = 24.8$, $p < 0.0001$). At the end of the 21-day experiment, the daphnids from the DA treatment were significantly smaller than those in the C, SC, and DW treatments while daphnids from the DWA treatment were significantly smaller than the C daphnids only (Figure 11ii). It was noted during the experiment that the neonates produced by daphnids in the DA treatment were light in colour and were less mobile than in the other treatments.

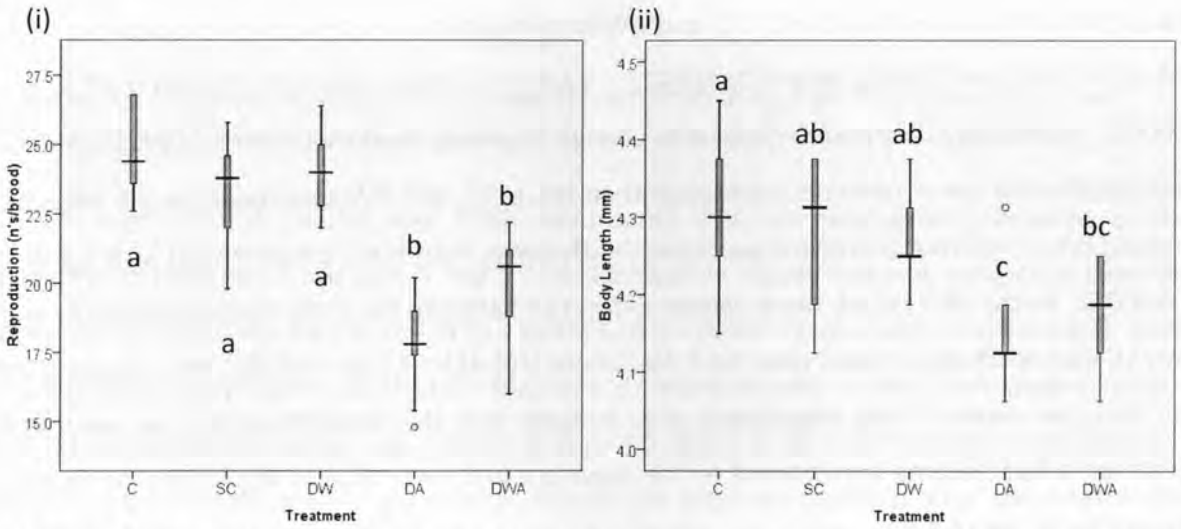


Figure 11. Reproduction (i) and body length (ii) in daphnids from the first generation (Test #4). The letters indicate statistically significant differences; those marked with the same letter, are not significantly different.

Statistical analysis of some of the other parameters (time to egg formation, time to first brood, and time between broods) showed unequal variances. When transformations were unsuccessful, the nonparametric Kruskal-Wallis test detected significant differences between treatments for time to egg formation ($H(4, 47) = 16.3$, $p = 0.0026$), time to first brood ($H(4, 37) = 16.5$, $p = 0.0024$), and time between broods ($H(4, 47) = 23.1$, $p = 0.0001$). However, the

multiple comparison tests did not show significant differences between treatments for these three parameters. Unplanned multiple comparison tests, as were used here, utilize error rates which are conservative and insensitive to differences of individual means or differences within small subsets (Sokal & Rohlf, 1995) and can therefore fail to detect differences. Yet by simply examining the data it appears that daphnids in the DA treatment had a significantly longer time to egg formation and first brood than the C, SC, and DW treatments.

Time between broods was generally three days for all treatments. In previous experiments, neonates were not released at such regular intervals; neonate production instead varied in the replicates and split broods (broods released over two consecutive days) were often observed. It appears that the addition of vitamin B_{12} has caused a very consistent reproductive cycle in these treatments. A comparison of the total neonates from each brood was made between treatments and the number of neonates from the DA and DWA treatments was significantly lower than the C, SC, and DW treatments for the first three broods (brood 1: $F(4,42) = 20.6$, $p < 0.0001$, brood 2: $F(4,42) = 23.8$, $p < 0.0001$, brood 3: $F(4,42) = 27.4$, $p < 0.0001$). By brood 4 the pattern began to change, and only daphnids from the DA treatment had significantly lower neonate production than the C, SC and DW treatments, while neonate production in the DWA treatment was significantly lower than the C treatment ($F(4,42) = 9.2$, $p < 0.0001$). By the fifth brood, there was no difference between the three dosed treatments and only SC was significantly lower than the C treatment ($F(4,41) = 3.7$, $p = 0.012$). This change after the first two weeks of the experiment may indicate that the daphnids in the DA and DWA treatments had become accustomed to the exposure and were able to adjust and/or recover (Appendix IV, Figure A4-1).

The number of neonates between broods was compared in each treatment separately to determine when neonate production was highest. Figure 12 shows that for the C, SC, and DW treatments, broods 3 and 4 produced the most neonates and there was a significant decline in neonate production between broods 4 and 5 (C: $p = 0.002$; SC: $p < 0.001$; DW: $p < 0.001$).

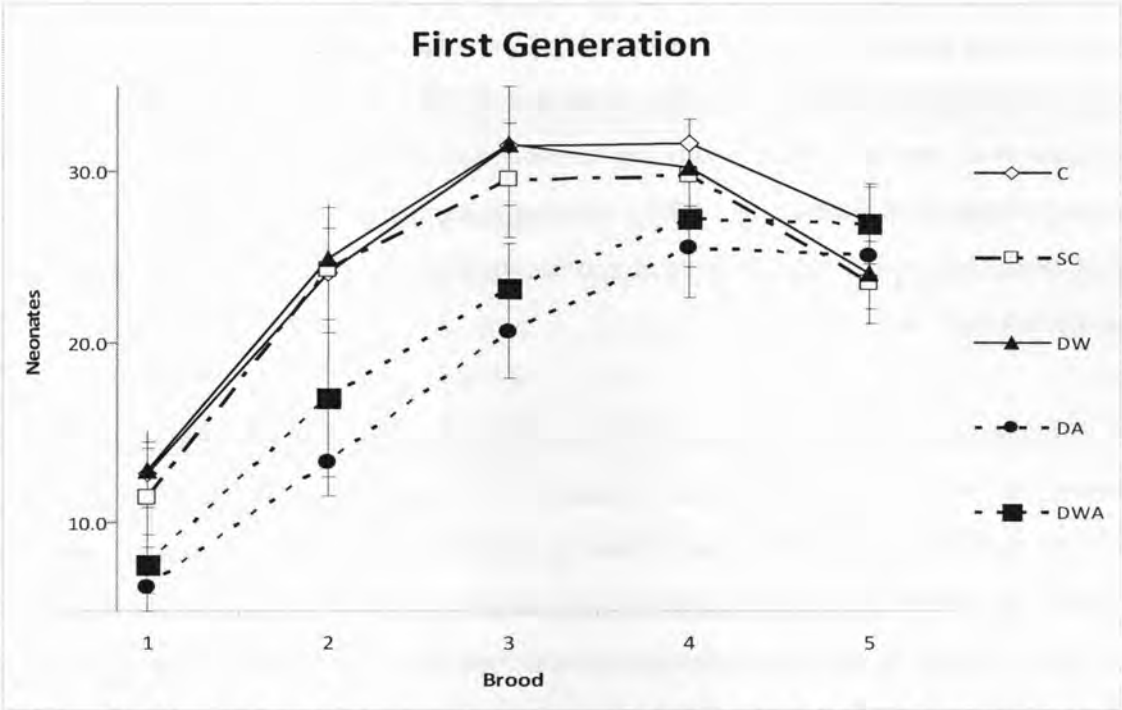


Figure 12. Neonate production per brood (mean ± SD) in Test #4, First Generation. In the C, SC and DW treatments, neonate production in brood 5 decreased significantly.

In contrast, for the DA and DWA treatments, neonate production increased as the experiment progressed. Broods 4 and 5 had significantly higher neonate production than the previous broods in the DA treatment ($p = 0.002$ and $p = 0.005$, respectively) and broods 3, 4 and 5 had significantly higher production than the previous broods in the DWA treatment ($p < 0.001$). The reduced neonate production in brood 5 of the C, SC and DW treatments may have been a result of a decline in food quality. Due to the apparent degradation of the control food between days 16 to 19, the algae from week two was used instead. In the interim, a decrease in food quality may have impacted neonate production in these treatments.

The results of the first generation test show that reproduction was significantly reduced in daphnids from the DA and DWA treatments as compared to those from the C, SC, and DW treatments. In addition, growth was reduced in daphnids from the DA and DWA treatments compared to those in the C treatment. For the first three broods, neonate production was significantly lower in daphnids from the DA and DWA treatments as compared to the other three treatments. As time progressed, neonate production in the DA and DWA treatments

appeared to recover and, by brood 5, was not significantly different between treatments. For most parameters, daphnids from the DW treatment were not significantly different from the C and SC treatments indicating that water exposure of BDE47 at 14µg/L does not impact reproduction in *D. magna*. These results confirm the identification of 14µg/L as the NOEC for BDE47 in *D. magna* by Kallqvist *et al.* (2006). Instead, it is apparent that *D. magna* exposure to BDE47 at 14µg/L via food is more important and has significant impacts on reproduction over a 21-day reproduction test.

3.2.6.1 *Daphnia magna* Reproduction Test #4, Second Generation - No Dose (See Figure 5)

Neonates from the first generation (brood 3) were used for a second generation test without dosing (Gen 2-ND). In this test, vitamin B_{12} was not added to the vessels as was done with the first generation and this is apparent in the longer time to first brood, lower number of broods and variation in the time between broods (see Table 10 below). This variation in reproduction is in contrast to the very regular reproduction cycles in the first generation (Table 9). Despite this change, reproduction still meets the accepted criteria for a healthy population. Throughout the duration of this test, the temperature of the medium remained stable between 19.8 and 20.4°C and the light intensity ranged from 8.0 to 9.6µmol·m^{-2}·s^{-1}. Due to some difficulties culturing *C. fusca*, the daphnids were fed 100% *P. subcapitata* for the first week (0.5mg OC/L for the first day and then increased to 1.0mg OC/L). The feeding rate was maintained at 1.0 mg OC/L and a 50:50 mixture (week 2) followed by a 25:75 mixture (week 3) of *P. subcapitata: C. fusca* was fed to the daphnids. The pH did not vary significantly throughout the experiment, ranging from 8.0 to 8.5 ($F(4,25) = 0.1$, $p = 0.973$). DO remained well over the recommended 3mg/L (OECD, 1998) ranging from 8.0 to 9.2mg/L (88-101% saturation) and did not differ between treatments ($F(4,25) = 0.3$, $p = 0.853$). Conductivity ranged between 268.0 and 408.0µS/cm and also did not differ between treatments ($F(4,25) = 1.3$, $p = 0.288$). Water chemistry results measured at medium exchanges are presented in Appendix IV, Table A4-5.

Survival and reproduction parameters from the Second Generation - No Dose test are listed in Table 10 and a selection of these parameters is shown in Figure 13.

Table 10. Observations from the *D. magna* Test #4, Second Generation – No Dose. Means (standard deviation) are shown, with the exception of mortality, which is an overall value.

Second Generation - No Dose		C	SC	DW	DA	DWA
Mortality	%	20.0	20.0	30.0	0.0	10.0
Time to egg formation (first)	days	6.4 (0.8)	7.1 (1.1)	6.4 (0.5)	6.2 (0.4)	6.5 (0.8)
Time to 1st brood	days	9.9 (1.2)	10.0 (0.9)	9.7 (0.5)	9.6 (0.5)	9.9 (0.6)
Time between brood 1 & 2	days	3.2 (0.7)	3.4 (0.5)	3.2 (0.5)	3.4 (0.5)	3.0 (0)
Time between brood 2 & 3	days	2.5 (0.5)	2.5 (0.5)	2.4 (0.5)	2.5 (0.5)	2.8 (0.5)
Time between brood 3 & 4	days	3.1 (0.4)	3.4 (0.5)	3.3 (0.5)	3.3 (0.7)	3.1 (0.4)
Time between brood 4 & 5	days	3.0 (0)	3.0 (-)	3.0 (0)	-	3.0 (0)
Mean time between broods	days	3.0 (0.3)	3.1 (0.2)	3.0 (0.2)	3.1 (0.1)	3.0 (0.1)
# of broods		4.2 (0.5)	4.1 (0.4)	4.3 (0.5)	4.0 (0)	4.2 (0.5)
Cumulative Reproduction (total neonates)		93.1 (16.5)	86.4 (14.9)	92.3 (17.5)	83.4 (4.1)	86.0 (12.2)
Reproduction (n's/brood)		21.9 (2.9)	20.9 (2.2)	21.4 (2.1)	20.8 (1.0)	18.7 (2.7)
Body Length (without tail spine)	mm	4.3 (0.2)	4.1 (0.1)	4.3 (0.1)	4.3 (0.1)	4.2 (0.2)

The mortality was within the accepted 20% in the C and SC treatments, but was somewhat elevated (30%) in the DW treatment. Maternal exposure to BDE47 via water may have weakened the neonates causing this increase in mortality in the second generation. Time to egg formation ranged from 6.2 to 7.1 days and was not significantly different between treatments ($F(4,34) = 1.8$, $p = 0.145$). For the time to first brood, the ANOVA failed to detect a difference between the treatments ($F(4,36) = 0.3$, $p = 0.857$). Although mean cumulative reproduction (total neonates) was lower in the DA and DWA treatments than in the C treatment, no statistically significant difference was detected ($F(4,36) = 0.8$, $p = 0.511$). Statistical analysis of reproduction (n's/brood) showed no significant difference between treatments ($F(4,36) = 2.5$, $p = 0.063$), although the statistical power was low (~35%). A difference was detected at an alpha level of 0.10, which allows for a compromise between a type I and type II error. In this case, a marginal significant difference between reproduction of daphnids from the DWA treatment and those from the C treatment ($p = 0.056$) was detected.

The statistical power was very low for the other parameters as well (<30%), however, the *p* values are much greater than 0.10 and therefore a significant difference was still not detected.

Daphnids from the DW treatment were significantly larger than in the SC treatment ($F(4,36) = 3.2$, $p = 0.024$). In addition, neonate production in the first brood was significantly higher in daphnids from the SC treatment versus the DWA treatment ($F(4,36) = 3.1$, $p = 0.026$) while in the second brood, neonate production was significantly higher in the C treatment compared to the DW treatment ($F(4,36) = 2.6$, $p = 0.049$).

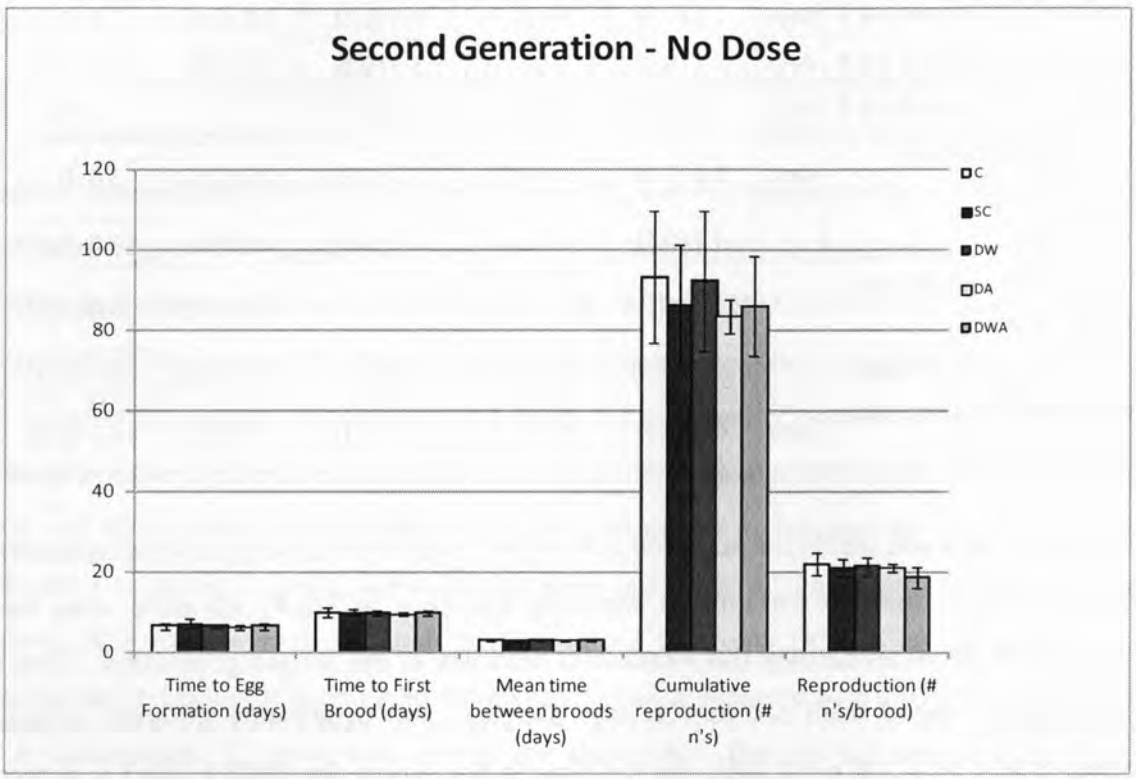


Figure 13. Observations (mean ± SD) from the *D. magna* Test #4, Second Generation – No Dose.

Figure 14 shows the neonate production for each treatment over time (brood). The general pattern shows an increase in the neonate production over time, which is different from the pattern in the first generation for the C, SC and DW treatments (see Figure 12).

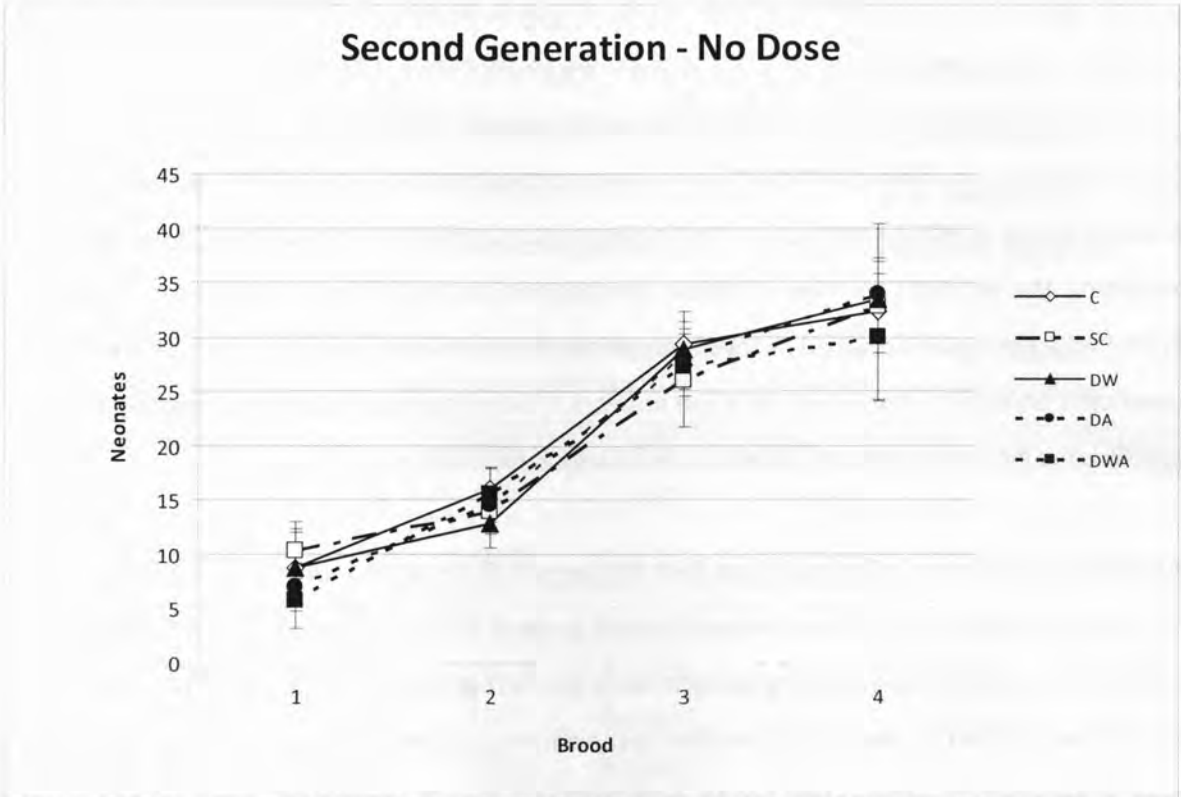


Figure 14. Neonate production per brood (mean ± SD) in Test #4, Second Generation - No Dose. Only broods 1 to 4 are shown due to low/no production of the fifth brood in the SC and DA treatments.

For the DW and DWA treatments, neonate production increased over time and was significantly higher in broods 3, 4 and 5 than in broods 1 and 2 (DW: $F(4,25) = 95.6$; $p < 0.0001$; DWA: $F(4,29) = 65.8$, $p < 0.0001$). This also occurred in the C treatment; however, the variances were unequal so the data had to be transformed (squareroot) beforehand ($F(4,29) = 30.5$, $p < 0.0001$). In the DA treatment, neonate production increased significantly for each brood ($F(3,28) = 74.3$, $p < 0.0001$) while in the SC treatment, neonate production increased after brood 2 and was significantly higher in brood 4 than brood 3 ($F(3,36) = 189.4$, $p < 0.0001$).

Significant impacts on reproduction in the dosed treatments were not observed in the Second Generation - No Dose test. Although for broods 1 and 2, neonate production was significantly lower in the DWA and DW treatments, respectively, this was not the case overall and only a very marginal difference was found for reproduction (n's/brood) between the DWA and the C treatments. These results show that maternal exposure to 14µg/L BDE47 does not

lead to significant reproductive effects in the offspring, as long as there is no further exposure to BDE47. Maternal transfer of BDEs was shown in zebrafish (*Danio rerio*) that had been fed food dosed (at high and low concentrations) with several different congeners (BDE28, 183, and 209; Nyholm *et al.*, 2008). Although toxicity was not addressed in this study, the lipid adjusted concentrations of BDE28 and 183 in the fish eggs was significantly higher than in the fish tissue, indicating the efficient transfer of these compounds to the offspring. Maternal transfer of BDE47 may have also occurred in these daphnids, although it appears that the transfer was not significant enough to cause reproductive impacts. The offspring of daphnids exposed to BDE47 appeared to be able to recover after maternal exposure via food and water.

3.2.6.2 *Daphnia magna* Reproduction Test #4, Second Generation – Dose (See Figure 5)

Neonates from the first generation (brood 5) were used for a second generation test (GEN 2-D) with the same BDE47 dosing scheme as in the first generation. Like in GEN 2-ND, vitamin B_{12} was not added to the vessels in this test and impacts on reproduction were apparent in several parameters: time to first brood, time between broods (increased variation) and number of broods (see Table 11 below). In this test, the first brood took between 11 and 13 days to be released, exceeding the 12-day maximum for all treatments except for the DA treatment. However, reproduction in all treatments still meets the accepted criteria for a healthy population (15-20 neonates/brood). Throughout the duration of this test, the medium remained stable between 19.8 and 20.4°C and light intensity ranged from 8.0 to 9.6 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Due to difficulties with growth of the *C. fusca* culture, the daphnids in the DA and DWA treatments were fed 100% *P. subcapitata* for the first week at 0.5mg OC/L until day 3 and then increased to 1.0mg OC/L. The feeding rate was then maintained at 1.0 mg OC/L and a 50:50 mixture (week 2) followed by a 25:75 mixture (week 3) of *P. subcapitata: C. fusca* was provided to the daphnids. Daphnids from C, SC, and DW were fed a 75:25 mixture of *P. subcapitata:C. fusca* at 0.5mg OC/L which was increased to 1mg OC/L on day 3 to maintain the feeding rate of the other treatments. The food ration was then changed to a 50:50 mixture (week 2) and then a 25:75 mixture (week 3) of *P. subcapitata: C. fusca*. The pH did not vary significantly throughout the experiment, ranging from 8.1 to 8.5 ($F(4,35) = 2.1$, $p = 0.099$). DO

remained well over the recommended 3mg/L (OECD, 1998) and did not vary significantly between treatments ($F(4,30) = 1.3$, $p = 0.280$). DO ranged from 8.2 to 10.1mg/L (91-111% saturation), a range somewhat higher than in the GEN 1 or GEN 2-ND. Conductivity also did not vary significantly ($F(4,30) = 0.6$, $p = 0.636$) and ranged between 279.5 and 464.8µS/cm. Water chemistry results measured at medium exchanges are presented in Appendix IV, Table A4-5.

Table 11. Observations from the *D. magna* Test #4, Second Generation – Dose. Means (standard deviation) are shown, with the exception of mortality, which is an overall value.

Second Generation - Dose		C	SC	DW	DA	DWA
Mortality	%	0.0	20.0	20.0	10.0	70.0
Time to egg formation (first)	days	6.0 (0)	6.1 (0.4)	6.9 (0.4)	7.0 (0)	10.0 (1.7)
Time to 1st brood	days	12.3 (1.0)	12.9 (1.2)	13.0 (0.5)	11.0 (0)	12.3 (1.2)
Time between brood 1 & 2	days	2.9 (0.3)	2.7 (0.5)	2.9 (0.4)	2.4 (0.5)	3.0 (0)
Time between brood 2 & 3	days	2.8 (0.6)	3.0 (0.6)	2.9 (0.4)	3.2 (0.4)	3.0 (0)
Time between brood 3 & 4	days	3.3 (0.5)	3.2 (0.5)	3.0 (-)	2.6 (0.5)	3.0 (-)
Time between brood 4 & 5	days	-	-	-	-	-
Mean time between broods	days	3.0 (0)	3.0 (0.1)	2.9 (0.2)	2.7 (0.2)	3.0 (0)
# of broods		3.9 (0.3)	3.6 (0.5)	3.1 (0.4)	4.0 (0)	3.3 (0.6)
Cumulative Reproduction (total neonates)		93.8 (8.3)	89.3 (13.0)	64.9 (15.2)	68.9 (5.1)	59.3 (3.8)
Reproduction (n's/brood)		24.2 (2.7)	25.3 (3.5)	20.6 (3.1)	17.2 (1.3)	18.1 (2.7)
Body Length (without tail spine)	mm	4.2 (0.1)	4.2 (0.0)	4.0 (0.1)	4.1 (0.1)	3.8 (0.1)

Observations from the dosed second generation are listed in Table 11 and a selection of the parameters is shown in Figure 15. Mortality was relatively low in all treatments except in the DWA treatment where only 30% of the daphnids survived. The exceptionally high mortality in the DWA treatment after 3 days was unexpected. High mortality has generally not been observed in previous studies on the impacts of BDEs on *D. magna* although effects on the second generation have not been investigated. For example, Evandri *et al.* (2003) dosed BDE99 to *D. magna* via food for nine days at much higher concentrations (5–100µM = 2,830-

56,500µg/L) and did not observe any mortality but did observe a significant reduction in mobility and decreased reproduction. In studies on the pentaBDE technical mixture (which contains mostly BDE47 and BDE99) detailed in EU (2000), a similar mortality rate (72.5%) was observed in *D. magna* after four days at 20µg/L. Technical mixtures can often contain impurities which may have caused the observed mortality (Stoker *et al.*, 2005; Birnbaum *et al.*, 2003). Conditions in the DWA media (pH, DO, and conductivity) were similar to all other treatments for the duration of the experiment. In addition, the daphnids in the DA treatment received exactly the same food and had a low mortality rate (10%), and therefore it is unlikely that feeding rate or food quality caused the high mortality. It is possible that the exposure of the parent generation to BDE47 via water plus food weakened the neonates, causing mortality after a subsequent exposure.

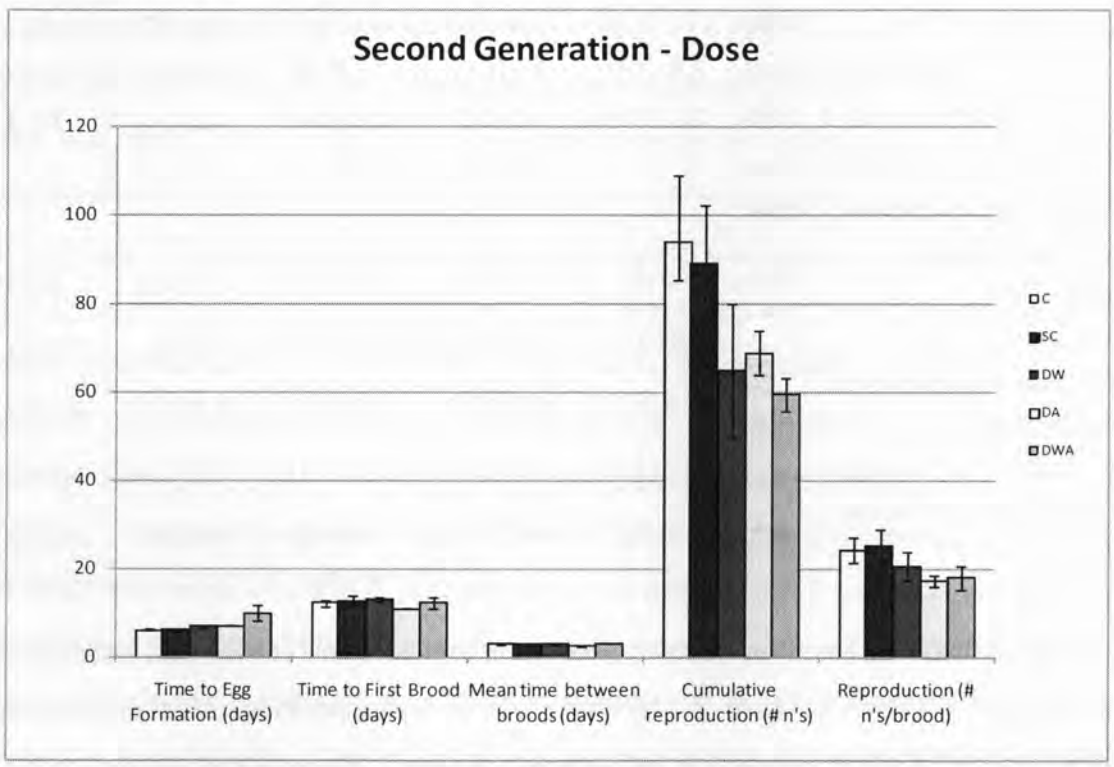


Figure 15. Observations (mean ± SD) from the *D. magna* Test #4, Second Generation – Dose.

A recent study has shown that BDE47 exposure impacted two life stages of the grass shrimp (*Palaemonetes pugio)* differently (Key *et al.*, 2008). Adult and larval grass shrimp were

exposed via water to a range of BDE47 concentrations (6.25 to 100µg/L) in 96-hour toxicity tests. BDE47 was over three times more toxic to larval grass shrimp (LC_{50} = 23.6µg/L) than adults (LC_{50}=78.1µg/L). Daphnid neonates may be more sensitive to BDE47 exposure, and this may have resulted in high mortality in the already weakened DWA neonates. Neonates may also have a reduced detoxification capacity than adults, and their greater surface–area-to-volume ratio may increase the uptake of toxicants, as was shown for metals in chironomids (Krantzberg, 1989). However, this size-to-volume difference may be more important for metals which are mainly adsorbed to the surface of the organism (Krantzberg, 1989). The neonates produced by the surviving daphnids in the DWA treatment had low mobility and did not appear as healthy as those in the other treatments.

Statistical analysis of time to egg formation, time to first brood, and time between broods showed unequal variances. After data transformations were unsuccessful, the non-parametric Kruskal-Wallis test was performed followed by a multiple comparison test. Egg formation was significantly shorter in the C treatment than all dosed treatments (DW, DA, and DWA; $H(4,37) = 30.7$, $p < 0.0001$). Time to first brood in daphnids from the DA treatment was not statistically different from the C treatment, but was significantly shorter than those from the SC and DW treatments ($H(4,37) = 23.7$, $p = 0.001$). Time between broods was significantly shorter in the DA treatment compared to daphnids from the C treatment ($H(4,37) = 15.6$, $p = 0.0035$). Cumulative reproduction (total neonates) was significantly higher in daphnids from both control treatments (C and SC) than the dosed treatments (DW, DA, DWA; $F(4,32) = 15.1$, $p < 0.0001$). Reproduction (n's/brood), however, was significantly reduced in the DA and DWA treatments only, as compared to the control treatments ($F(4,32) = 12.7$, $p < 0.0001$). Daphnids in both control treatments were significantly larger in size than in the DW treatment, which were significantly larger than those in the DWA treatment ($F(4,32) = 15.3$, $p < 0.0001$). Impacts on body length were also observed in the first generation DA and DWA treatments (Section 3.2.6). A similar impact on growth was seen in *D. magna* exposed to 9.8µg/L of pentaBDE technical mixture via water (EU, 2000).

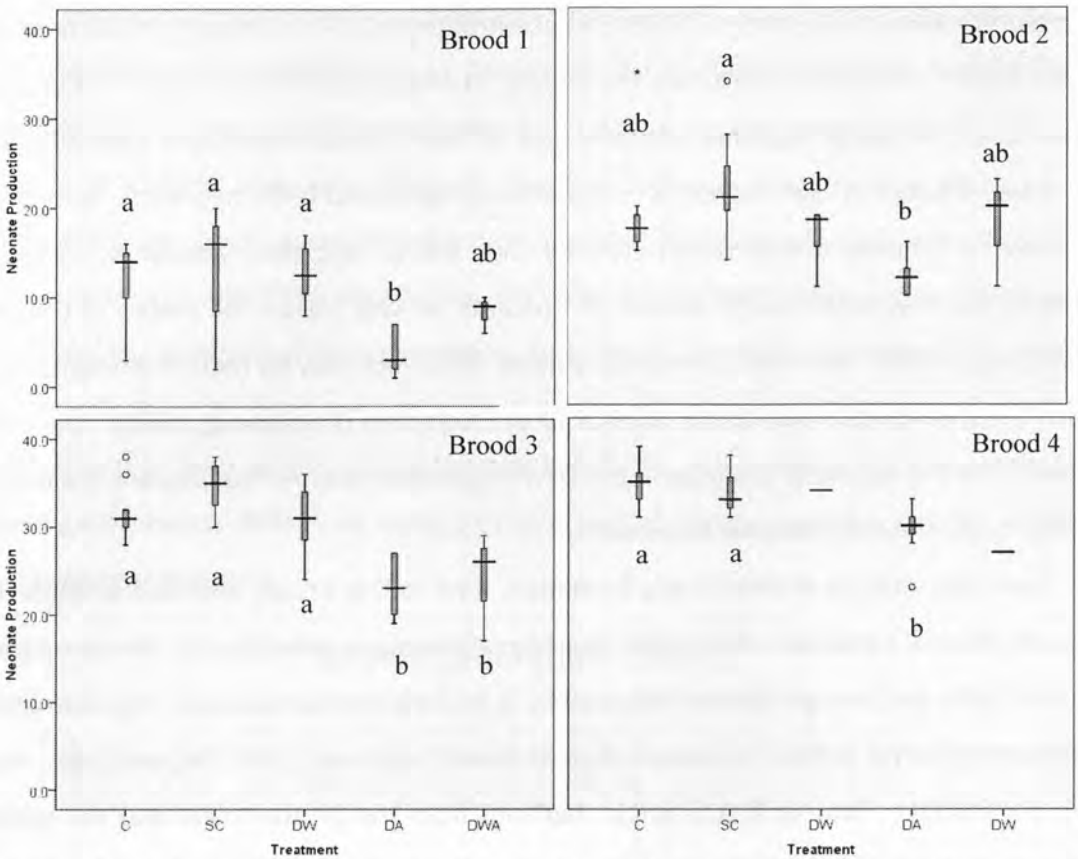


Figure 16. Neonate production in broods 1 to 4 from Test #4, Second Generation - Dose. Significant differences (identified by different letters) were detected between treatments for each brood.

After maternal exposure to BDE47, it appears that the daphnids from the DW treatment are not significantly different from the other dosed treatments (DA and DWA). During the first generation dosing, it did not appear that BDE47 exposure via water (DW) had a significant impact on reproduction. However, this exposure may have weakened the neonates to the point where reproduction was impaired in the second generation after subsequent dosing. Evidence of this weakening was seen in the elevated mortality rate in the DW treatment from the Gen 2-ND test. The daphnids from the DA treatment had the lowest neonate production in all broods, while both DA and DWA had significantly lower neonate production in the third brood (brood 1: $H(4,37) = 14.7$, $p = 0.0054$; brood 2: $F(4,32) = 5.1$, $p = 0.003$; brood 3: $F(4,32) = 13.4$, $p < 0.0001$; brood 4: $F(2,19) = 8.5$, $p = 0.002$; Figure 16). Although reproductive impacts were observed on all three dosed treatments in this test, it appears that neonate production

was lowest in the DA treatment. When neonate production was compared within a treatment, there was a general increase over time (Figure 17), in contrast to the pattern seen in the first generation for the C, SC and DW treatments but similar to the pattern seen in Gen 2-ND.

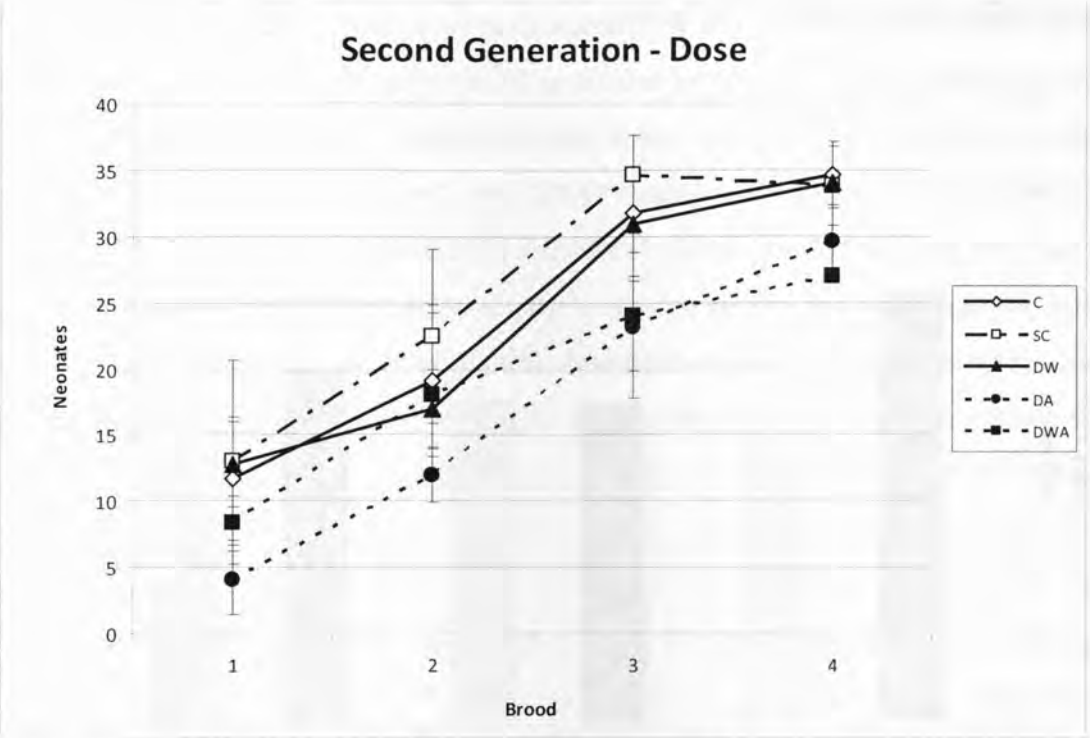


Figure 17. Neonate production per brood (mean ± SD) in Test #4, Second Generation – Dose.

Impacts on daphnid reproduction and growth were observed in the first generation after food and food plus water exposure to BDE47. Neonates from the first generation were able to recover when the exposure was not continued into the second generation. In a subsequent exposure to BDE47, impacts on reproduction and growth were observed once again and significant impacts on survival were identified in the DWA treatment. Figure 18 shows neonate production in the dosed treatments as a percentage of the control treatment (C only). Significant differences are evident in the DA and DWA treatments during the initial (first generation) exposure as well as the second generation exposure (GEN 2-D). Neonate production in the DW treatment was significantly lower than in the control only in the GEN 2-D

test. This indicates that although water exposure of BDE47 at 14µg/L does not significantly impact the first generation, it may weaken neonates in the second generation, causing impacts on reproduction. The tests show that food exposure to BDE47 has more significant impacts on reproduction than water exposure, which only showed significant impacts on the second generation after a second BDE47 exposure.

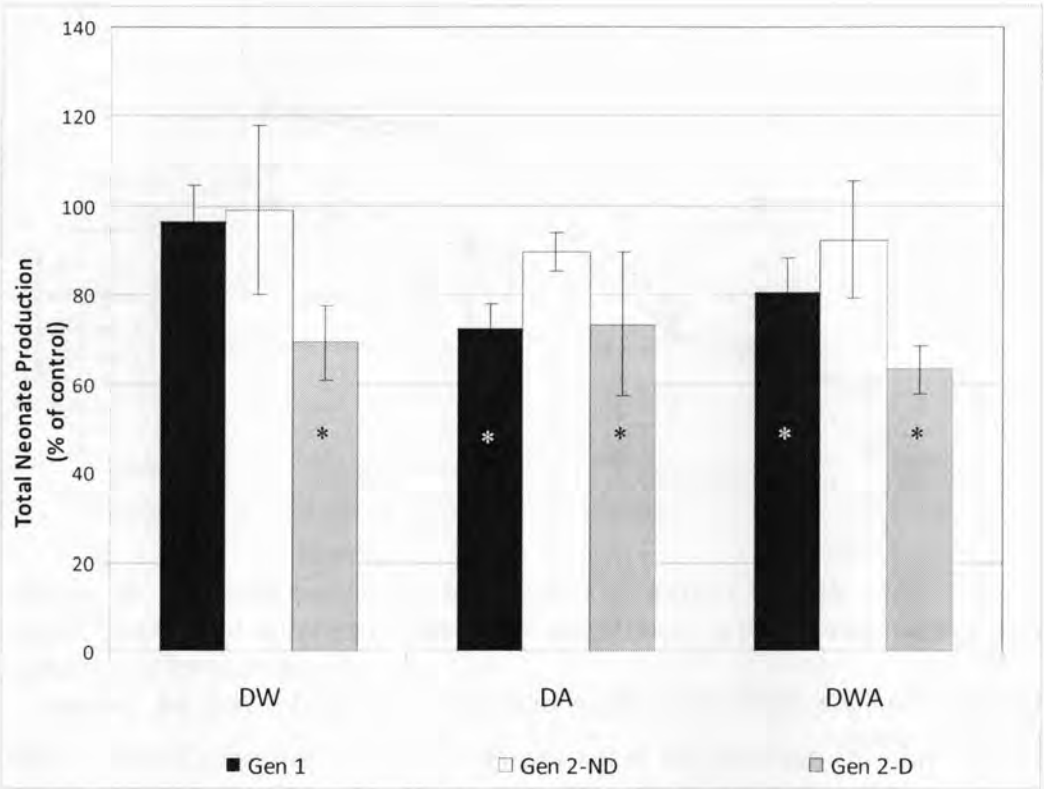


Figure 18. Cumulative neonate production in Test #4, First Generation (Gen 1), Second Generation, No Dose (Gen 2-ND) and Second Generation, Dose (Gen 2-D). * indicates significant difference ($p < 0.05$) from the control treatment (C).

3.3 Chemical Analysis

PBDE chemistry results are listed in Appendix V, Table A5-1. Appendix V, Figure A5-1 shows the chromatograms for the Precision Recovery Standard (PAR) and Figure A5-2 shows the chromatograms for the dosed *C. fusca* sample. All BDEs were below method detection limits in the dechlorinated water used in the *Daphnia magna* reproduction tests. The measured concentration of BDE47 in the dosed water sample was 11.5µg/L, approximately 82% of the

nominal concentration (14.0µg/L). BDE47 made up 99.7% of the total PBDEs in the dosed water sample. Trace amounts of BDE17 and BDE28 were also detected, which may have been due to breakdown of the BDE47, either during storage or sample preparation. BDE71, 77, 119 and 126 were also detected in the dosed water sample at negligible concentrations.

The two-day old water samples were composites of the water from two different media exchanges. No BDEs were detected in the SC water and only trace amounts of BDE138 and 183 were detected in the C water. Water samples from both the DW and DWA treatments contained approximately 4µg/L BDE47. The BDE47 made up more than 99% of the total BDEs. Small amounts of BDE28 (0.19% and 0.28%) and BDE17 (0.05% and 0.07%) were detected in the DW and DWA water samples, respectively. These may be due to degradation of the BDE47 in the water by photolysis. Fang *et al.* (2008) showed that under UV light, BDE47 in hexane degrades mainly into BDE28 as well as BDE17 (Figure 19). They found that bromines in the ortho position (Figure 19) were more reactive than the para-position, which explains why BDE28 was the major debromination product. Fluorescent light was used in the current experiment, and although the UV light emitted is very low (UVA is approximately 3% of visible component (Wilcoxen et al., 2003)), it may be responsible for the debromination. It is also possible that the BDE47 was metabolized within the *D. magna* and excreted, resulting in the detection of the lower congeners in the water. Debromination occurs in other organisms, such as fish (Stapleton *et al.*, 2004a; Stapleton *et al.*, 2004b; Tomy *et al.*, 2004; Kierkegaard *et al.*, 1999) but has never been shown in algae or *D. magna*. As with photolysis, bromines in the ortho and meta positions have been

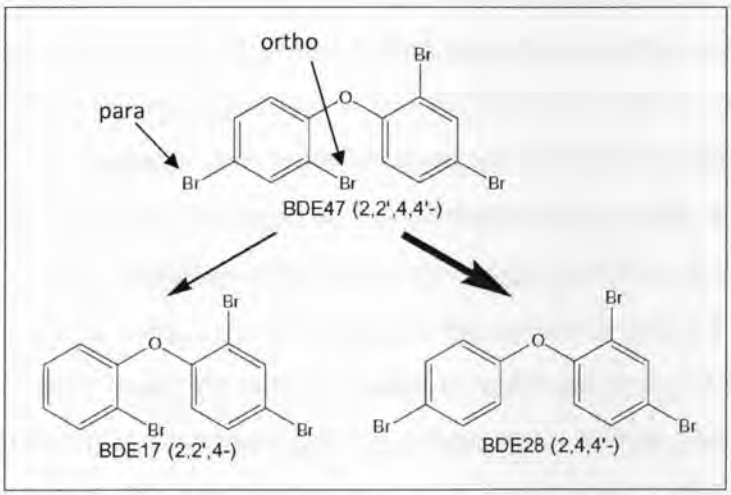


Figure 19. Chemical structure of BDE47, 28, and 17; the ortho and para positions are indicated on the BDE47 molecule. Source: Fang *et al.*, 2008.

shown to be more susceptible to debromination in animals (Stapleton *et al.*, 2004a; Kierkegaard *et al.*, 2007). Trace amounts of other congeners were also detected in the DW sample (BDE66, 85, and 100) and the DWA sample (BDE85, 99, and 100). These congeners are negligible at these low concentrations and may be due to background contamination. In both the DW and DWA samples, BDE17 was in the same range as some of these trace congeners (BDE99 and 100) and could also be due to background contamination. In the water sample from the DA treatment, low levels of BDEs were present, with 0.46µg/L BDE47, and very small amounts of BDE17 and 28. This could be due to partitioning of BDEs from the algae into the water followed by photolysis. Since these compounds are very hydrophobic, it is more likely due to metabolism and excretion by the *D. magna*. Drouillard *et al.* (2007) found that BDE183 and 190 were eliminated very rapidly after dosing the freshwater mussel, *Elliptio complanata*, and speculated that debromination may have occurred in these organisms.

BDE17 and 28 were also present in the dosed algae samples, which is also likely due to photolysis. The algae was exposed to BDE47 under high light intensity for five to seven days which likely led to the debromination of BDE47 into BDE28 and 17 within the nutrient medium. Subsequently, the algae likely accumulated the lower brominated congeners. Alternatively, but less likely, is the possibility that the BDE47 was absorbed into the algal cells and underwent some metabolic processing. Some studies have shown an initial surface sorption of PCBs to algal cells, followed by a slower transfer into the cell (Skoglund *et al.*, 1996) where cell membrane proteins were found to be more important storage sites than the lipids (Skoglund and Swackhamer, 1999). After BDE47, BDE28 made up the highest proportion of total BDEs followed by BDE17, at 3.6 and 0.8%, respectively in *P. subcapitata* and 0.9 and 0.2%, respectively in *C. fusca*. BDE28 made up a higher proportion of the total BDEs in the *P. subcapitata* sample compared to the *C. fusca*. Other BDEs were present in the algal samples (BDE49, 66, 85, 100, 153, 154 and 183 in both algal samples and BDE71 only in the *C. fusca* sample) but were detected at trace levels and are considered negligible. The presence of these congeners at such low levels is likely due to background contamination.

The concentration of BDE47 was much higher (approximately eight times) in the *C. fusca* sample than in the *P. subcapitata* sample (19.4 versus 2.5µg/g), indicating that uptake of BDE47

was greater in the *C. fusca* cells. During sample preparation, a small portion of the *P. subcapitata* sample was spilled however it was less than 10% of the entire sample and would not account for such a large difference. This result may be due to differences in cell shape and density, as *C. fusca* cells are much larger and are circular in shape versus the smaller crescent-shaped *P. subcapitata* cells. Swackhamer and Skoglund (1991) reviewed research showing that different algal cells sorb organic compounds differently indicating that surface type is an important factor in the initial uptake period. The *P. subcapitata* cells had a somewhat higher growth rate (see Figure 7) than the *C. fusca* cells, which may have resulted in growth dilution and a decreased BDE47 concentration in the *P. subcapitata* cells. Growth dilution has also been shown to be an important factor in uptake of PCBs in algal cells (Swackhamer and Skoglund, 1991). In the GEN 2-D experiment, daphnids were initially only fed *P. subcapitata* due to problems culturing the *C. fusca.* Therefore, the daphnids were likely exposed to a lower concentration of BDE47 via food. However, this occurred during the first week of the experiment when the smaller neonates would generally consume the *P. subcapitata* over the *C. fusca* due to its smaller size.

In Appendix V, Table A5-2, levels of BDE47 in the water and beaker rinse (hexane) are presented as masses (μg) for comparison purposes. Approximately 37% of the initial dose of BDE47 was detected in the DW and DWA composite water samples. This reflects the total BDEs contained in both the dissolved and particulate phases. In their partitioning study, Brietholtz and Wollenberger (2003) exposed the particle-feeding crustacean *Nitocra spinipes to* BDE47 and 99 via water. After eight days of exposure, they found that BDE47 partitioned mainly into the particulate matter (49.9%) and the walls of the glass vessel (47%), with much smaller portions dissolved in the water (2.3%) and partitioning into the test animal (0.3%). By day 15, more BDE47 was bound to the particulate matter (58.1%) and to the test animals (3.6%) while less was bound to the walls (38.2%) and in the water (0.5%). In the current study, smaller amounts of BDE47 were detected in the hexane rinse (14 and 24% in the DW and DWA samples, respectively) than in the water sample. This fraction is not bioavailable to the daphnids as it is bound to the glass (Kallqvist *et al.*, 2006). It is possible that this fraction was

underestimated in the current study, as the 30 minute extraction time period may not have been long enough to completely extract the BDEs bound to the glass.

Nakari and Huhtala (2008) exposed daphnids to BDE153 using the same OECD reproduction test used here and found that a low proportion of the BDE partitioned into the daphnids (3.8 to 5.9%), similar to the findings of Brietholtz and Wollenberger (2003). In the current experiment, chemical analysis of the daphnids was not available as the sample mass was too low. It is unknown where the remaining BDE47 partitioned. Volatilization is assumed to be negligible due to the relatively low vapour pressure and Henry's Law Constant of BDE47, especially since the algae were available for binding. Significant loss likely occurred during sample handling in the experiment. The samples were filtered with nitex screens (affixed to plastic) to remove neonates each day. There was likely a high potential for BDE47 loss during the transfer through the nitex filter into a second glass vessel and then back into the original test vessel.

4.0 CONCLUSIONS

After a significant amount of method development, including modifications of feeding rates, light intensity, test vessel size, and test environment, the fourth *Daphnia magna* Reproduction Test produced reliable results. The challenges experienced during this thesis are likely not unique and highlight the difficulties that researchers may encounter when using *D. magna* in toxicity tests. Because aquatic toxicology tests involve living organisms, these challenges can be heightened by the innate variability associated with them. In addition, a lack of experience handling and culturing these organisms may lead to further difficulties.

In the first generation, significant impacts on reproduction were observed in daphnids in the DA and DWA treatments relative to the control treatments and the DW treatment. These results confirmed that at a water exposure of 14µg/L BDE47, impacts on reproduction are not observed, as was found in Kallqvist *et al.* (2006). Based on these observations, it is clear that the impacts of food exposure can occur at a lower concentration than water exposure. This was not considered in the determination of the NOEC for BDE47 by Kallqvist *et al.*, 2006, and shows that using the OECD *Daphnia magna* Reproduction Test without considering all routes of exposure can underestimate the toxicity of a compound. These toxicity values, which are used for regulatory actions, then do not truly represent the impacts that may be occurring on aquatic organisms in the environment.

In the second generation, daphnids were able to recover from maternal exposure to BDE47 as no significant impacts on reproduction were observed. However, when second generation daphnids were exposed to BDE47, high mortality was observed in the DWA treatment and significant impacts on reproduction in all dosed treatments (DW, DA and DWA) were shown. The effects environmental contaminants have on subsequent generations are also important factors to consider when determining the true impacts these compounds have at the population level. Although the concentration used in this experiment is higher than normal environmental levels, which are in the pg/L range in surface waters, results showing impacts of BDE47 via food exposure rather than water exposure is an important conclusion.

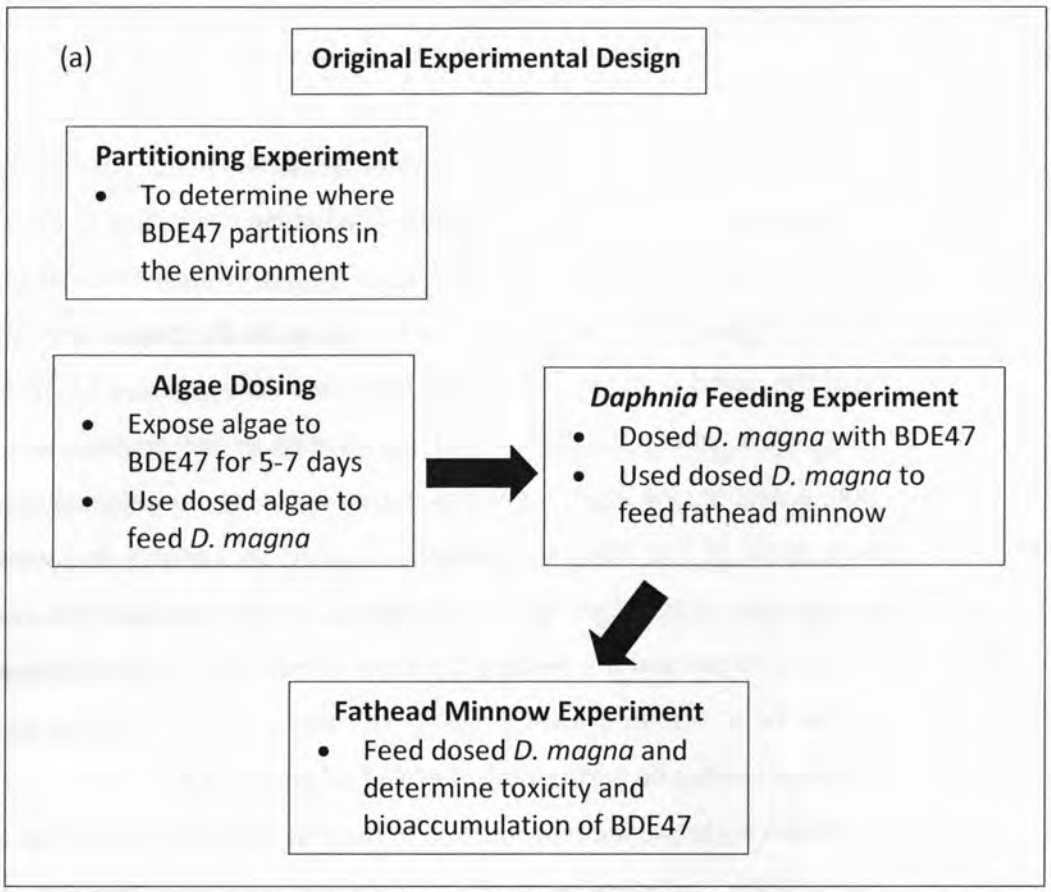
Chemical analysis confirmed that direct dosing of the BDE47 from the stock (in DMSO) resulted in a measured concentration close to the nominal concentration. In addition, detected levels of BDEs in the algae confirm that the *D. magna* were indeed exposed to BDE47 via food. The detection of lower brominated congeners (BDE28 and 17) in the algal samples show that debromination was occurring either in the water (photolysis) or possibly in the algae, which has not been shown before. Results showing higher levels of BDE47 in *C. fusca* versus the *P. subcaptitata* sample are interesting and the BDE47 exposure should be repeated to verify this large difference in uptake.

The *Daphnia magna* Reproduction Test showed that food was an important route of exposure for BDE47. Over the longer term, water exposure of BDE47 impacted daphnid reproduction, indicating that maternal exposure was a factor. These results show the importance of assessing all routes of contaminant exposure over multiple generations in aquatic toxicity tests.

5.0 RECOMMENDATIONS

The initial intent of this project was to trace the movement, partitioning, accumulation, and toxicity of BDE47 through a simple laboratory aquatic food chain (including algae, *Daphnia magna*, and the fathead minnow (*Pimephales promelas*)). The original experimental design (as outlined in Figure 20a) involved the feeding of dosed algae to *D. magna* and then the subsequent feeding of the dosed *D. magna* to fathead minnows. As a first step to this study, it was necessary to determine where the BDE47 was partitioning in the environment. After extensive preparation, a partitioning experiment was initiated, but was terminated soon after due to the excessive costs of the chemical analysis required to address the partitioning question. When it was also determined that a prohibitive number of daphnids would be needed for the chemical analysis and the feeding the fathead minnows, it was decided that it was beyond the scope of a Master's level project. As this research is important, it is recommended that further studies on partitioning of BDE47 be performed.

The addition of vitamin B_{12} improved reproduction in the first generation tests, but was not used in the second generation. In future research, it is recommended that vitamin B_{12} be used in all generations to improve daphnid performance and subsequent comparisons between generations. This research should be continued so that toxicity and bioaccumulation can be determined in higher trophic levels and the bioaccumulation and biomagnification of these compounds can be fully assessed.



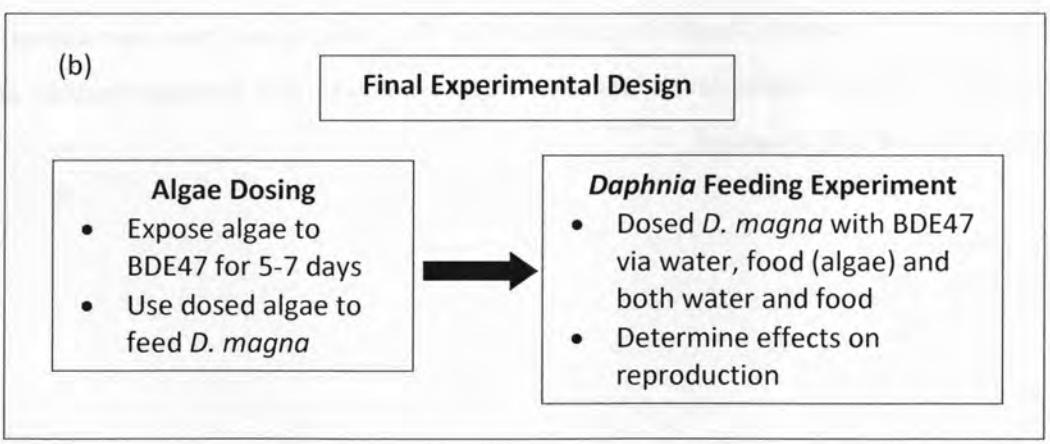


Figure 20. Experimental design for (a) Original project and (b) Final project.

APPENDIX I

Table A1-1. Ingredients used in algae nutrient medium (Modified Bristol's Medium) according to Standard Operating Procedure AL1 (OMOE, 2008a).

Nutrient Stock Solutions	Concentration (mg/L)
$NaNO_3$	125
$CaCl_2 \cdot H_2O$	12.5
$MgSO_4 \cdot 7H_2O$	37.5
K_2HPO_4	37.5
KH_2PO_4	87.5
NaCl	12.5
$EDTA \cdot Na_2$	25
KOH	15.5
$FeCl_3 \cdot 6H_2O$	2.42
H_3BO_3	5.7
$MnCl_2 \cdot 4H_2O$	0.77
$ZnSO_4 \cdot 7H_2O$	4.41
MoO_3	0.36
$CuSO_4 \cdot 5H_2O$	0.79
$Co(NO_3) \cdot 6H_2O$	0.25

Table A1-2. Summary of test conditions used for the *Daphnia magna* Reproduction Test (based on OECD, 1998).

Test Type	Static-renewal, 21 day, chronic/reproduction
Temperature	20±2°C
Light Intensity	5.6-11.2 μmol photons$\cdot m^{-2} \cdot s^{-1}$
Photoperiod	16:8 hour light:dark
Test Solution Volume	50-100mL
Renewal	Static-renewal
Test Organism	*Daphnia magna*
Age of Test Organisms	Neonates, <24 hours
Number/Container	One per vessel
Replicates	10
Feeding	Daily
Aeration	None
Dilution Water	Dechlorinated laboratory water
Test media/leachate concentrations	N/A
Test Duration	21 days
Effects measured	Survival, growth, reproduction

APPENDIX II

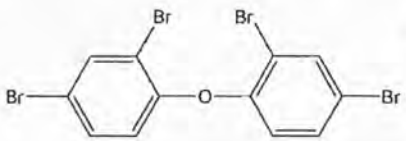
WELLINGTON
LABORATORIES

CERTIFICATE OF ANALYSIS/DOCUMENTATION

PRODUCT CODE: BDE-47 **LOT NUMBER:** AKBD4799

COMPOUND: 2,2',4,4'-Tetrabromodiphenyl ether

STRUCTURE:



CAS #: 5436-43-1

IUPAC #: 47

MOLECULAR FORMULA: $C_{12}H_6Br_4O$ **MOLECULAR WEIGHT:** 485.80

CONCENTRATION: Crystalline

CHEMICAL PURITY: >98%

LAST TESTED: (mm/dd/yyyy) 09/16/2008 (HRGC/LRMS)

RECOMMENDED STORAGE: Store at 4°C protected from light

DETAILS:

- One-product unambiguous synthesis
- Structure confirmed by proton NMR and HRGC/LRMS
- HRGC/LRMS (TIC and mass spectrum) data attached, see Figure 1

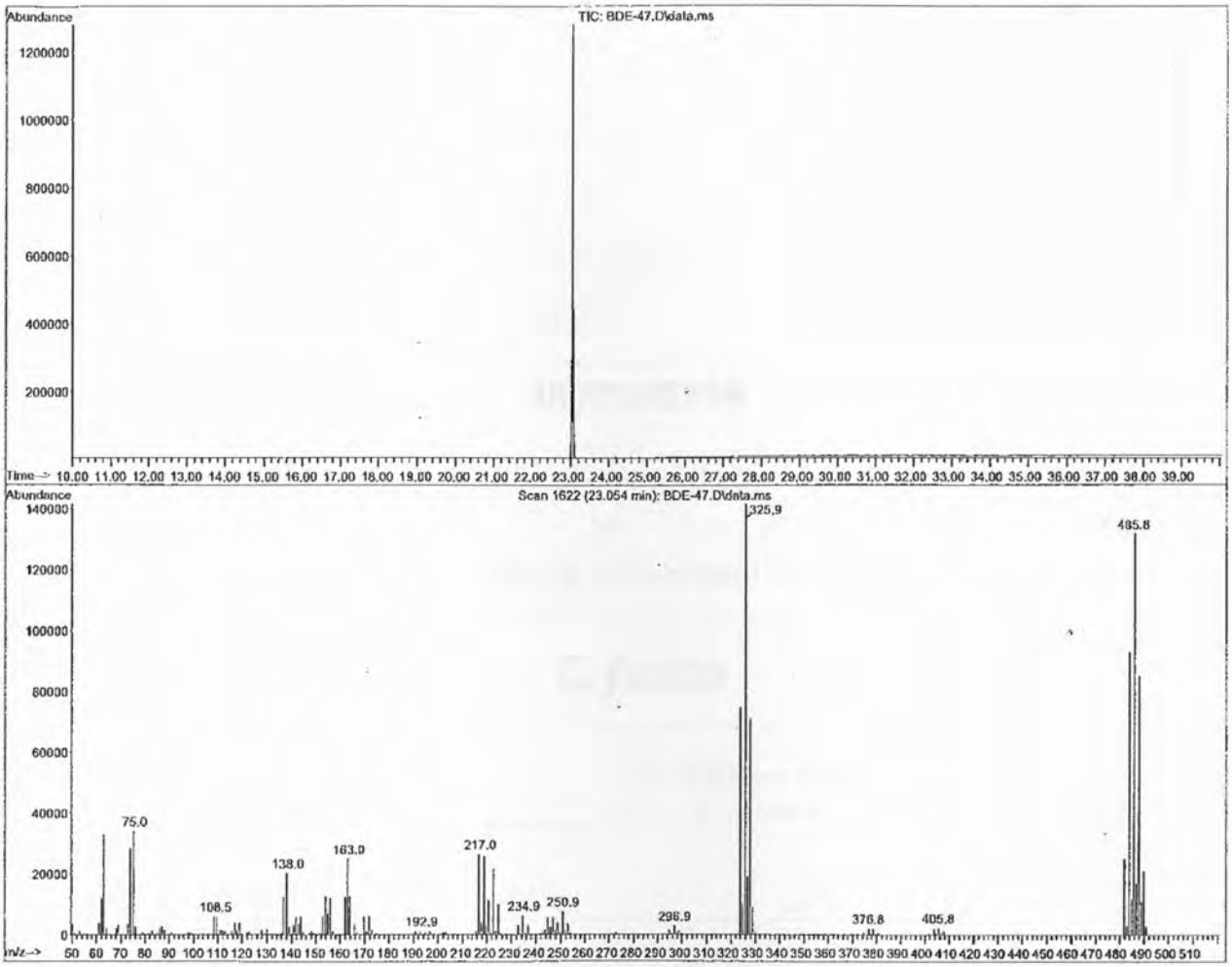
ADDITIONAL INFORMATION:

For additional information including potential hazards, handling/storage, accuracy, and shelf life please request a copy of our Reference and Handling Guide. For your convenience this can be downloaded from our website at www.well-labs.com.

NOT FOR HUMAN/DRUG USE
POTENTIALLY TOXIC TO HUMANS

Certified By: B.G. Chittim **Date:** 01/30/2009 (m/m/dd/yyyy)

Figure 1: **BDE-47; HRGC/LRMS Data (TIC & mass spectrum)**



HRGC/LRMS:

Agilent 7890A (HRGC)
Agilent 5975C (LRMS)

Chromatographic Conditions:

Column:	30 m DB-5 (0.25 mm id, 0.25 µm film thickness) J&W Scientific
Injector:	250 °C (Splitless Injection)
Oven:	100 °C (5 min)
	10°C/min to 325 °C
	325 °C (20 min)
Detector:	250 °C
	Full Scan (50-1000 amu)

APPENDIX III

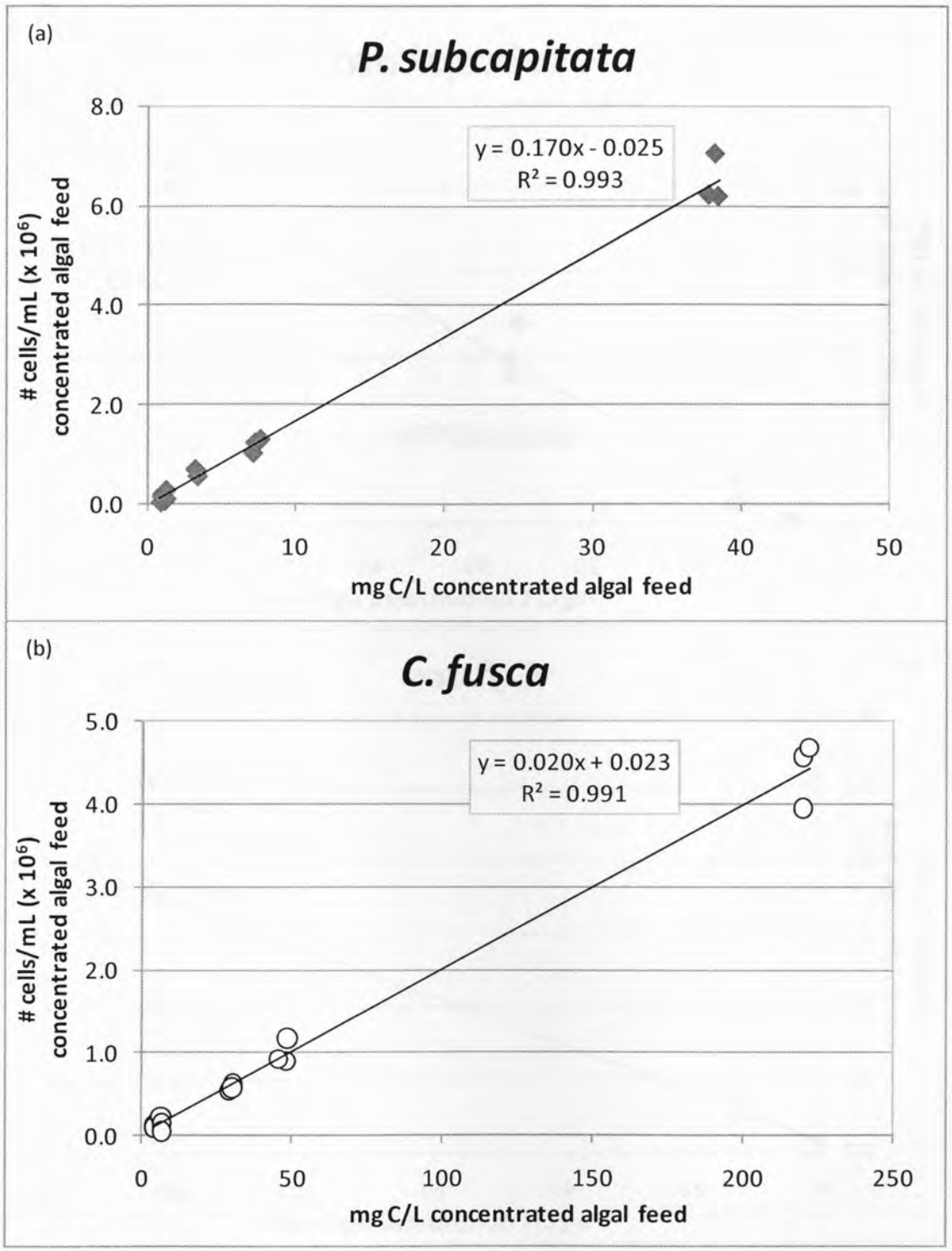


Figure A3-1. Nomographs for (a) *Pseudokirchneriella subcapitata* and (b) *Chlorella fusca* used to calculate the cell density required for *Daphnia magna* feeding in Test #3.

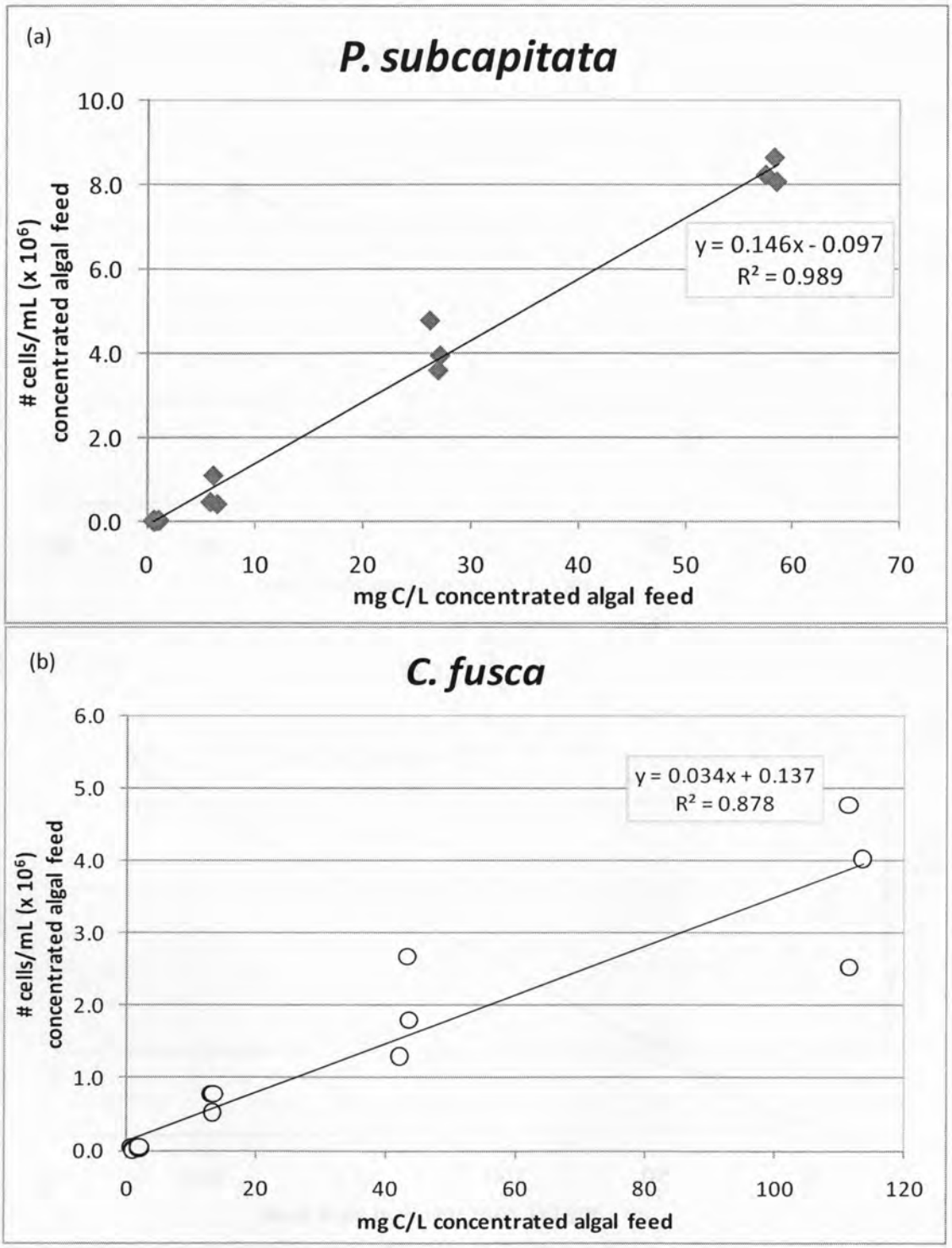


Figure A3-2. Nomographs for (a) *Psuedokirchneriella subcapitata* and (b) *Chlorella fusca* used to calculate the cell density required for *Daphnia magna* feeding in Test #4.

APPENDIX IV

Table A4-1. Water chemistry for the media used in the *D. magna* Reproduction Test #1 (first generation); measured after media exchanges (*i.e.* 2-day-old water). Mean and standard deviation (SD) are indicated in grey. C=Control; SC=Solvent Control; DW=Dosed Water; DA=Dosed Algae; DWA=Dosed Water & Algae.

Date	T	C			SC			DW			DA			DWA		
	(°C)	pH	DO (mg/L)	Conductivity (µS/cm)	pH	DO (mg/L)	Conductivity (µS/cm)	pH	DO (mg/L)	Conductivity (µS/cm)	pH	DO (mg/L)	Conductivity (µS/cm)	pH	DO (mg/L)	Conductivity (µS/cm)
24/10/2007	20.0	7.8	9.2	310.6	7.9	8.9	258.2	8.0	8.9	255.9	-	-	-	-	-	-
26/10/2007	19.0	7.8	8.7	279.2	7.8	8.6	279.4	8.2	8.3	286.3	8.1	8.7	281.4	8.2	8.7	275.6
29/10/2007	19.0	7.9	8.5	312.2	8.0	8.7	303.0	8.2	8.3	284.7	8.2	8.8	293.7	8.3	8.8	308.9
31/10/2007	19.0	7.7	9.2	279.4	8.0	9.0	304.4	-	-	-	8.0	9.4	281.3	7.9	9.2	274.7
02/11/2007	20.0	8.1	9.2	277.8	8.1	9.1	305.8	8.2	9.2	279.9	8.1	9.3	276.0	8.2	9.3	277.3
05/11/2007	20.0	8.0	8.6	309.9	8.2	8.5	413.0	8.3	8.5	313.4	8.2	8.6	314.9	8.3	8.4	318.2
07/11/2007	20.0	8.2	8.8	320.0	8.3	8.7	329.1	8.4	8.9	319.8	8.3	8.9	335.4	8.5	8.7	324.2
09/11/2007	20.0	8.3	8.9	281.3	8.3	8.9	279.5	8.3	8.9	280.6	8.2	9.0	272.7	8.3	9.0	287.4
12/11/2007	17.0	8.3	9.6	337.3	8.4	9.8	318.8	8.4	9.6	341.6	8.6	10.7	319.0	8.4	9.6	322.7
Mean	19.3	8.0	9.0	300.9	8.1	8.9	310.1	8.3	8.8	295.3	8.2	9.2	296.8	8.3	8.9	298.6
SD	1.0	0.2	0.4	21.9	0.2	0.4	44.3	0.1	0.5	27.4	0.2	0.7	23.3	0.2	0.4	22.1

Table A4-2. Water chemistry for the media used in the *D. magna* Reproduction Test #2 (first generation); measured after media exchanges (*i.e.* 2-day-old water). Mean and standard deviation (SD) are indicated in grey.

Date	T	C			SC			DW			DA			DWA		
	(°C)	pH	DO (mg/L)	Conductivity (µS/cm)	pH	DO (mg/L)	Conductivity (µS/cm)	pH	DO (mg/L)	Conductivity (µS/cm)	pH	DO (mg/L)	Conductivity (µS/cm)	pH	DO (mg/L)	Conductivity (µS/cm)
04/02/2008	19.2	8.8	8.6	344.2	8.8	8.8	314.8	8.8	8.7	340.2	8.8	8.7	316.1	8.8	8.7	326.1
06/02/2008	19.1	8.3	8.6	361.0	8.3	8.6	344.0	8.3	8.5	355.0	8.3	8.5	361.0	8.4	8.4	379.0
08/02/2008	19.2	8.1	11.3	341.9	8.3	11.0	361.0	8.4	11.2	365.0	8.4	11.0	339.0	8.5	11.3	377.0
11/02/2008	19.2	8.9	9.4	344.3	9.0	9.5	346.3	**9.1**	9.5	443.0	8.9	9.5	358.2	8.9	9.4	322.8
13/02/2008	19.3	**9.4**	11.7	421.0	**9.4**	11.8	418.0	**9.5**	11.7	411.0	**9.2**	11.4	422.0	**9.3**	11.7	315.0
15/02/2008	19.2	8.7	9.0	345.8	8.9	9.2	346.7	8.8	9.1	448.0	8.6	9.2	368.3	8.7	9.0	415.0
18/02/2008	19.2	8.7	8.2	388.0	8.7	8.2	413.0	8.8	8.4	412.0	8.3	8.3	428.0	8.3	8.4	410.0
20/02/2008	19.1	9.0	11.5	373.9	**9.3**	12.0	385.8	**9.4**	12.6	386.0	**9.2**	11.6	392.6	**9.3**	11.6	394.4
Mean	19.2	8.7	9.8	365.0	8.8	9.9	366.2	8.9	10.0	395.0	8.7	9.8	373.2	8.8	9.8	367.4
SD	0.1	0.4	1.5	28.1	0.4	1.5	36.2	0.4	1.6	40.1	0.4	1.3	38.9	0.4	1.5	40.5

Table A4-3. Water chemistry for the media used in the *D. magna* Reproduction Test #3 (first generation); measured after media changes (*i.e.* 2 day old water). Mean and standard deviation (SD) are indicated in grey. C=Control; SC=Solvent Control; DW=Dosed Water; DA=Dosed Algae; DWA=Dosed Water & Algae.

Date	T	C			SC			DW			DA			DWA		
	(°C)	pH	DO (mg/L)	Conductivity (µS/cm)	pH	DO (mg/L)	Conductivity (µS/cm)	pH	DO (mg/L)	Conductivity (µS/cm)	pH	DO (mg/L)	Conductivity (µS/cm)	pH	DO (mg/L)	Conductivity (µS/cm)
19/02/2009	18.0	8.8	10.3	372.0	8.7	10.3	361.0	8.9	11.1	359.0	8.6	9.9	293.0	8.7	10.3	337.0
21/02/2009	19.0	8.9	11.0	327.5	8.9	11.1	390.0	8.8	10.4	328.0	8.7	10.3	327.3	8.5	9.9	375.0
24/02/2009	19.0	8.8	11.6	448.0	8.9	11.2	374.0	8.9	11.3	435.0	8.6	10.1	381.4	8.6	10.0	368.4
26/02/2009	19.0	8.6	10.4	348.1	8.6	10.4	460.0	8.7	10.3	355.2	8.4	9.7	352.7	8.4	9.7	377.3
28/02/2009	19.0	8.6	10.1	430.0	8.6	10.3	315.2	8.7	10.4	311.1	8.4	9.5	378.7	8.4	9.7	395.0
03/03/2009	20.0	9.0	11.1	374.3	8.9	11.0	357.0	8.7	10.4	380.6	8.6	9.8	396.9	8.5	9.7	364.8
05/03/2009	19.0	8.5	9.4	414.0	8.5	9.5	341.2	8.5	9.6	422.0	8.3	9.2	376.2	8.4	9.2	403.0
07/03/2009	19.0	8.5	9.7	350.9	8.5	9.9	383.2	8.5	9.6	429.0	8.3	9.2	391.1	8.3	9.2	407.1
Mean	19.0	8.7	10.4	383.1	8.7	10.4	372.7	8.7	10.4	377.5	8.5	9.7	362.2	8.5	9.7	378.5
SD	0.5	0.2	0.7	43.0	0.2	0.6	42.6	0.2	0.6	47.3	0.2	0.4	35.8	0.1	0.4	23.0

Table A4-4. Observations from the *D. magna* Test #3 (second generation). Means (standard deviation) are shown, with the exception of mortality, which is an overall value. C*=control from daphnid culture; DA=Dosed Algae; DWA=Dosed Water & Algae.

2nd Generation		C*	DA	DWA
Mortality	%	10.0	10.0	0.0
Time to egg formation (first)	days	7.0 (0)	7.7 (1.0)	9.7 (1.6)
Time to 1st brood	days	17.8 (0.4)	16.1 (1.4)	15.8 (0.4)
Time between brood 1 & 2	days	4.0 (-)	3.5 (0.5)	3.9 (0.3)
Time between brood 2 & 3	days	-	-	-
Time between brood 3 & 4	days	-	-	-
Mean time between broods	days	4.0 (-)	3.5 (0.5)	4.0 (0)
# of broods		1.1 (0.3)	1.9 (0.3)	2.0 (0)
Cumulative Reproduction (total neonates)		18.7 (4.5)	21.3 (5.2)	25.0 (3.4)
Reproduction (n's/brood)		17.2 (2.8)	11.5 (2.7)	12.5 (1.7)
Body Length (without tail spine)	mm	3.7 (0.1)	3.4 (0.1)	3.3 (0.1)

Table A4-5. Water chemistry for the media used in the *D. magna* Reproduction Test #4, first and second generations; measured in conjunction with media exchanges (i.e. 2 day old water). Mean and standard deviation (SD) are indicated in grey. C=Control; SC=Solvent Control; DA=Dosed Algae; DW=Dosed Water; DWA=Dosed Water & Algae.

Date	T	C			SC			DW			DA			DWA		
	(°C)	pH	DO (mg/L)	Conductivity (µS/cm)	pH	DO (mg/L)	Conductivity (µS/cm)	pH	DO (mg/L)	Conductivity (µS/cm)	pH	DO (mg/L)	Conductivity (µS/cm)	pH	DO (mg/L)	Conductivity (µS/cm)
First Generation																
20/04/2009	20.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22/04/2009	20.2	8.3	8.8	376.6	8.4	9.0	365.1	8.4	9.0	296.6	8.2	8.5	363.7	8.2	8.5	367.0
24/04/2009	20.1	8.2	8.8	295.2	8.3	8.8	355.1	8.3	8.9	364.6	8.3	8.7	305.1	8.2	8.7	296.3
27/04/2009	20.4	8.2	8.6	272.5	8.2	8.7	336.4	8.2	8.6	346.3	8.2	8.2	356.3	8.1	8.7	271.6
29/04/2009	20.7	8.1	9.1	287.0	8.2	9.2	286.3	8.1	9.0	289.6	8.2	8.9	289.5	8.1	9.0	289.4
01/05/2009	20.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
04/05/2009	20.2	8.3	8.7	372.0	8.2	8.5	395.0	8.3	8.5	385.0	8.2	8.2	363.9	8.2	8.5	313.4
06/05/2009	20.4	8.3	8.5	292.0	8.3	8.4	357.0	8.2	8.5	324.1	8.2	8.2	323.8	8.2	8.2	346.5
Mean	20.3	8.2	8.8	315.9	8.3	8.8	349.2	8.3	8.8	334.4	8.2	8.4	333.7	8.2	8.6	314.0
SD	0.2	0.1	0.2	45.9	0.1	0.3	36.2	0.1	0.2	37.8	0.0	0.3	32.2	0.1	0.3	36.3
Second Generation - No Dose																
06/05/2009	20.4	8.4	9.2	280.4	8.4	9.1	281.8	8.5	9.2	280.5	8.5	9.2	279.5	8.5	9.2	279.9
08/05/2009	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11/05/2009	20.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13/05/2009	20.2	8.3	9.2	327.9	8.3	9.2	309.1	8.3	9.2	309.7	8.2	8.9	281.7	8.3	9.0	309.8
15/05/2009	20.2	8.3	8.8	319.1	8.2	8.9	307.1	8.3	9.1	306.4	8.2	8.8	302.5	8.3	9.2	297.6
18/05/2009	19.9	8.3	8.7	340.0	8.2	8.8	285.0	8.2	8.6	308.0	8.2	8.7	303.8	8.2	8.8	303.6
20/05/2009	20.0	8.0	9.0	408.0	8.1	8.9	297.7	8.1	9.2	345.3	8.1	9.0	270.3	8.2	9.0	268.0
22/05/2009	19.8	8.1	8.7	304.7	8.0	8.9	308.4	8.1	8.7	288.6	8.1	8.6	301.3	8.0	8.5	407.0
Mean	20.1	8.2	8.9	330.0	8.2	9.0	298.2	8.3	9.0	306.4	8.2	8.8	289.9	8.3	8.9	311.0
SD	0.2	0.2	0.2	43.4	0.1	0.2	12.2	0.2	0.3	22.4	0.1	0.2	14.4	0.2	0.3	49.5
Second Generation - Dose																
12/05/2009	20.3	8.4	9.2	280.4	8.4	9.1	281.8	8.5	9.2	280.5	8.5	9.2	279.5	8.5	9.2	279.9
14/05/2009	20.0	8.4	-	-	8.3	-	-	8.3	-	-	8.4	-	-	8.4	-	-
17/05/2009	20.2	8.1	9.2	453.6	8.3	9.0	442.6	8.4	9.4	464.8	8.3	9.1	303.0	8.3	8.8	447.6
19/05/2009	20.0	8.2	8.8	325.1	8.1	8.7	301.8	8.1	9.4	283.1	8.2	10.1	311.5	8.4	10.1	395.9
21/05/2009	19.8	8.1	8.7	326.4	8.1	8.8	301.4	8.1	8.8	300.9	8.3	9.3	311.5	8.3	9.5	395.1
24/05/2009	20.0	8.2	8.9	378.3	8.3	9.1	360.6	8.4	9.2	359.4	8.3	9.0	363.0	8.5	9.7	352.1
26/05/2009	19.8	8.2	8.8	331.5	8.3	9.0	348.3	8.2	8.7	345.7	8.3	8.8	346.7	8.3	9.0	348.8
28/05/2009	20.4	8.2	8.3	361.1	8.2	8.4	355.3	8.2	8.3	355.7	8.2	8.5	356.5	8.3	8.6	357.0
Mean	20.1	8.2	8.8	350.9	8.3	8.9	341.7	8.3	9.0	341.4	8.3	9.1	324.5	8.4	9.3	368.1
SD	0.2	0.1	0.3	54.8	0.1	0.3	54.1	0.1	0.4	63.9	0.1	0.5	31.2	0.1	0.5	52.2

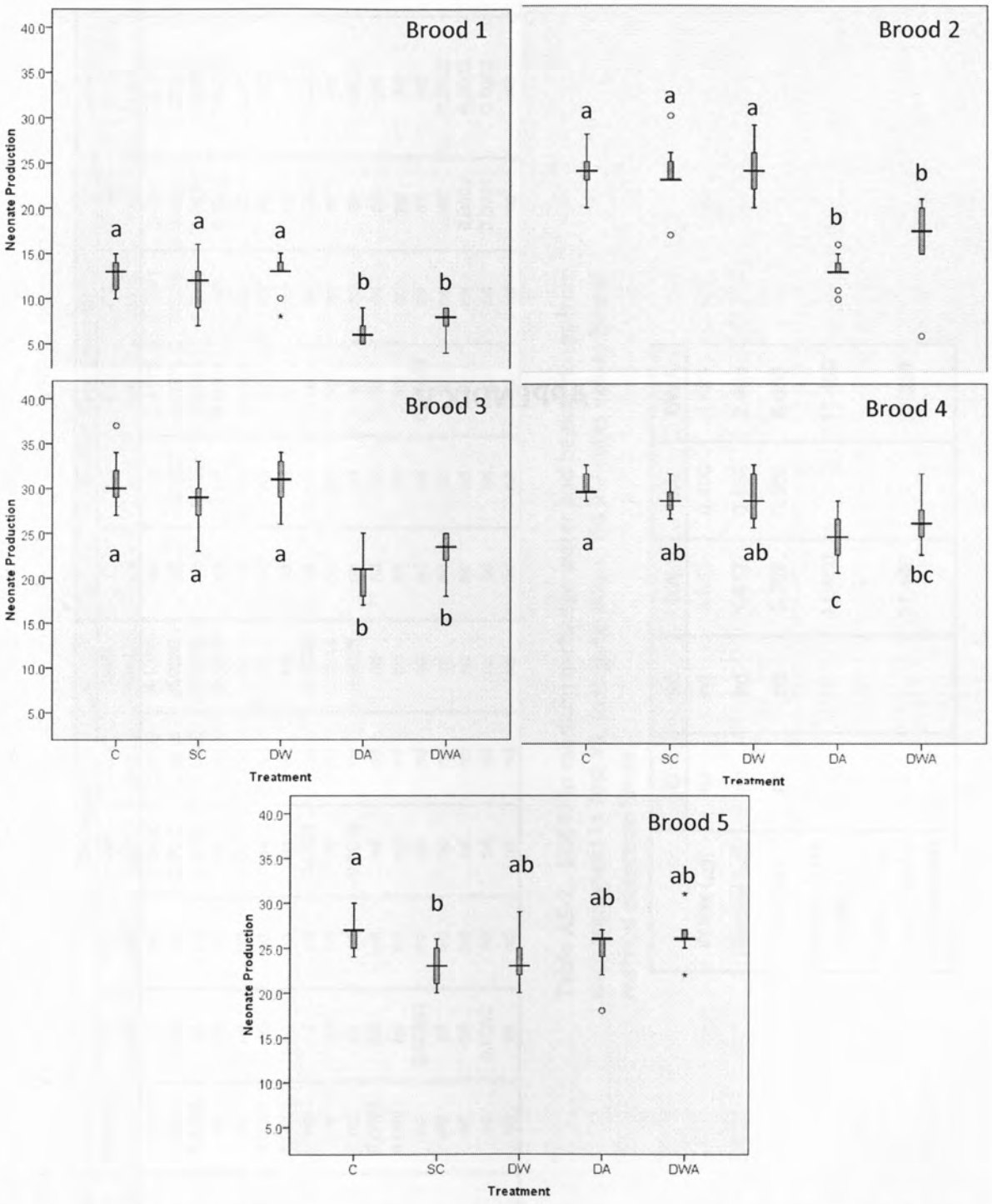


Figure A4-1. A comparison of the number of neonates produced by daphnids from the first generation (Test #4) in each brood. The letters indicate statistically significant differences; those marked with the same letter, are not significantly different.

APPENDIX V

Table A5-1. Chemistry results for the PBDE analysis of background and dosed water, dosed algae, and medium exchange water and beaker rinses from each treatment in Test #4, first generation. Nd indicates values below method detection limits.

	Dechlor. Water (µg/L)	Dosed Water (µg/L)	2 Day Old Water					Beaker Rinse (hexane)					Dosed *P. subcapitata* (µg/g)	Dosed *C. fusca* (µg/g)
			C (µg/L)	SC (µg/L)	DW (µg/L)	DA (µg/L)	DWA (µg/L)	C (µg)	SC (µg)	DW (µg)	DA (µg)	DWA (µg)		
BDE47	nd	11.4648	nd	nd	4.2438	0.4592	4.1381	nd	nd	1.4173	0.1891	2.4031	2.5187	19.4001
BDE17	nd	0.0081	nd	nd	0.0022	0.0010	0.0030	nd	0.0003	0.0008	0.0008	0.0025	0.0200	0.0424
BDE28	nd	0.0203	nd	nd	0.0082	0.0040	0.0115	nd	0.0005	0.0021	0.0019	0.0059	0.0964	0.1762
BDE49	nd	nd	nd	nd	nd	nd	0.0002	nd	nd	0.0001	nd	0.0003	0.0005	0.0027
BDE66	nd	nd	nd	nd	0.0006	nd	nd	nd	nd	0.0002	nd	0.0007	0.0020	0.0027
BDE71	nd	0.0005	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.0003
BDE77	nd	0.0002	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
BDE85	nd	nd	nd	nd	0.0001	nd	0.0002	nd	nd	nd	nd	0.0001	0.0003	0.0002
BDE99	nd	nd	nd	nd	nd	nd	0.0048	nd	nd	nd	nd	nd	nd	nd
BDE100	nd	nd	nd	nd	0.0018	nd	0.0025	nd	nd	nd	nd	nd	0.0033	0.0045
BDE119	nd	0.0002	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
BDE126	nd	0.0001	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
BDE138	nd	nd	0.00002	nd	nd	nd	nd	nd	nd	0.00001	nd	nd	nd	nd
BDE153	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.0002	0.0003
BDE154	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.0002	0.0002	0.0002
BDE183	nd	nd	0.0002	nd	nd	nd	nd	nd	nd	nd	nd	0.0001	0.0002	0.0003
BDE209	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table A5-2. BDE47 in medium exchange water and beaker rinses from each treatment in Test #4, first generation. Nd indicates values below method detection limits.

	C	SC	DW	DA	DWA
Water (µg)	nd	nd	3.692	0.400	3.683
Hexane (µg)	nd	nd	1.417	0.189	2.403
Total (µg)	nd	nd	5.109	0.589	6.086
Original Water Dose (µg)	-	-	11.460	-	11.460
% of original dose in water (bioavailable)	-	-	37.446	-	37.353

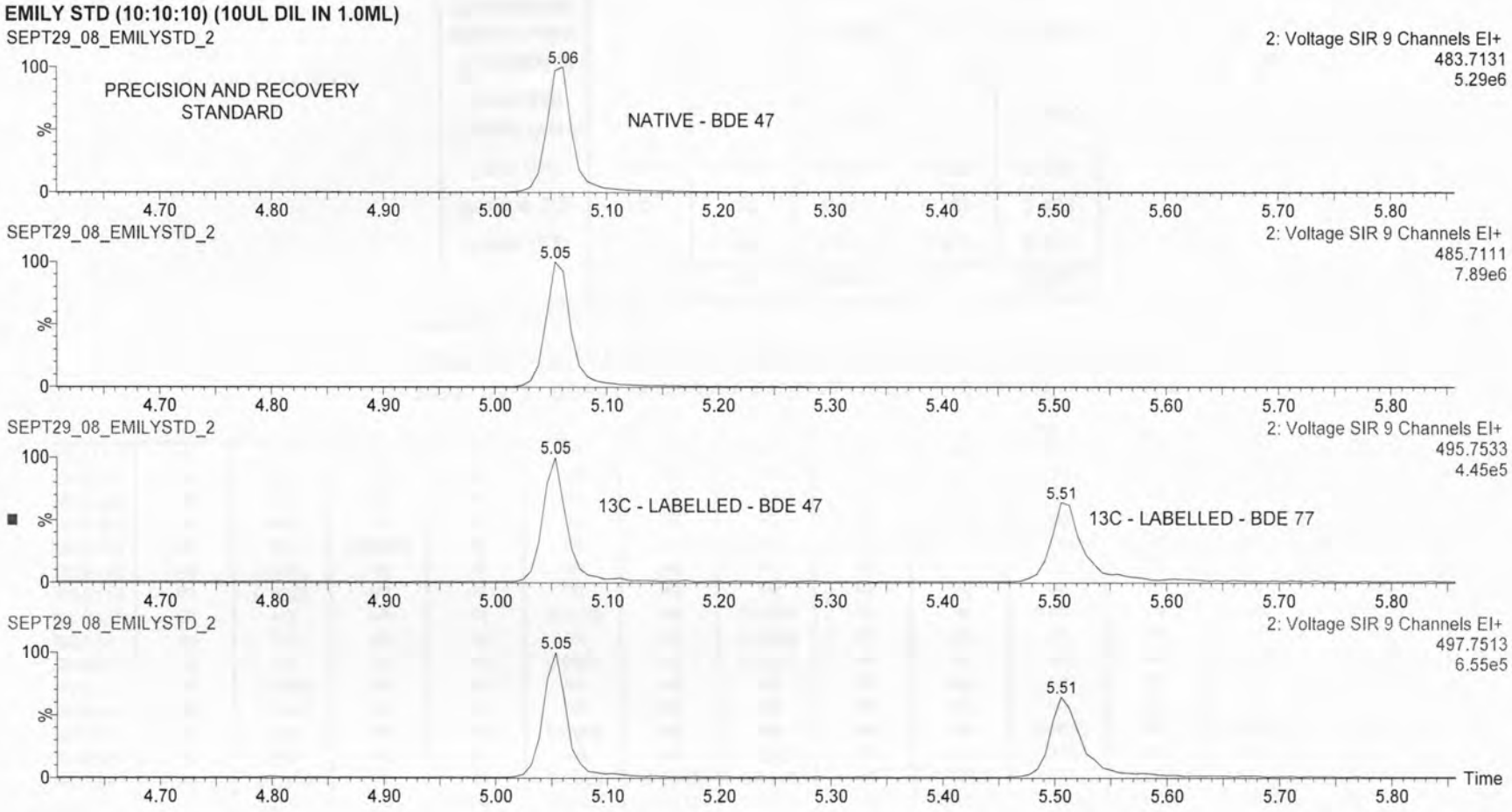


Figure A5-1. Chromatogram for Precision Recovery Standard (PAR) for PBDE chemical analyisis by GC/HRMS.

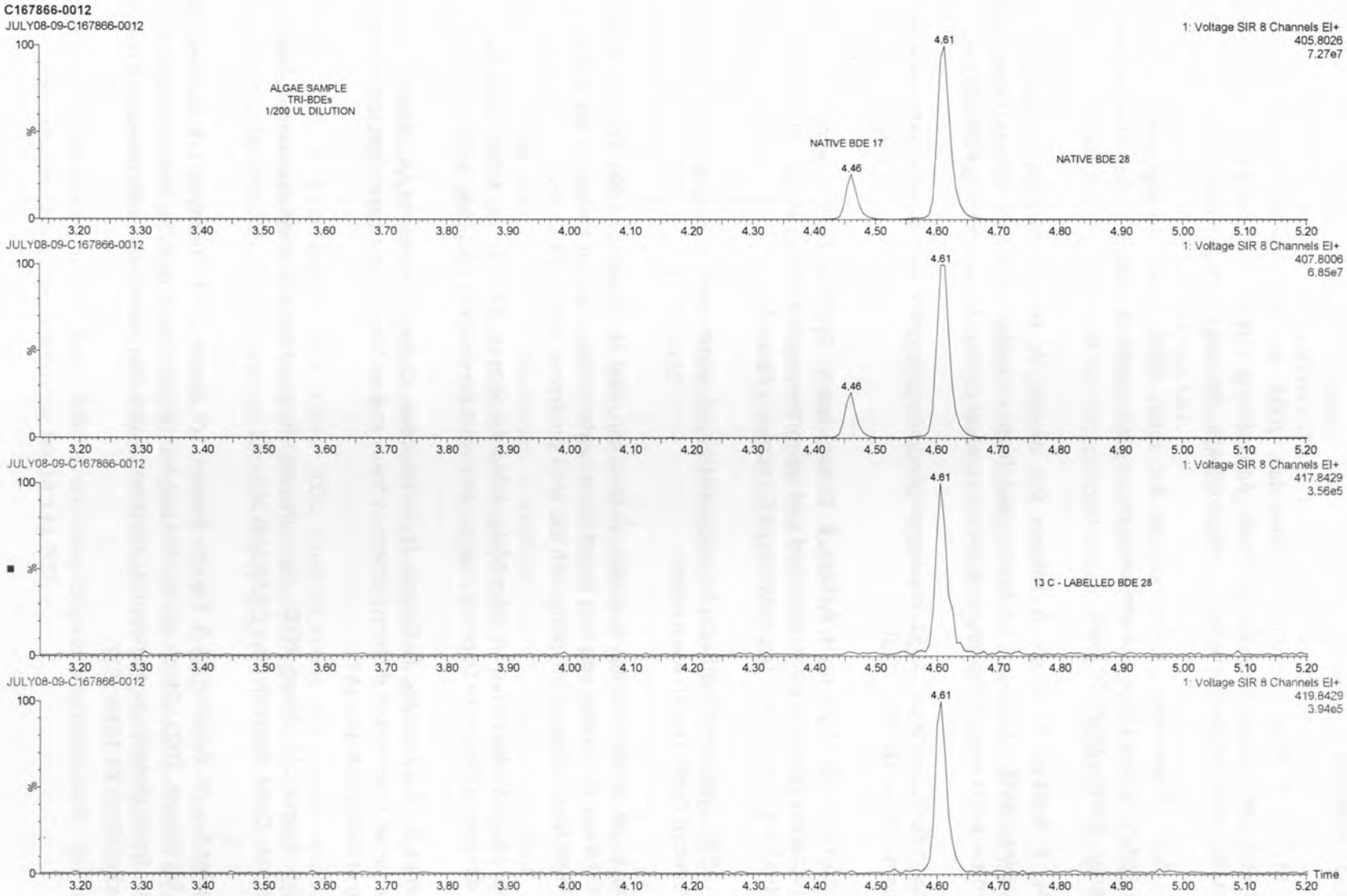


Figure A5-2. Chromatogram for dosed *Chlorella fusca* sample showing the peaks for BDE17 and BDE28.

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