Effects of Polychlorinated Biphenyls (PCBs) on Root Meristem Cells of Common Onion (Allium cepa L.) and on Early Life Stages of Zebrafish (Danio rerio)

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Polychlorinated biphenyls (PCBs) are chemical mixtures that are common environmental pollutants and considered potential carcinogens. In this study, the common onion, Allium cepa, was used to determine the cytotoxicity and genotoxicity of a mixture of PCBs at three concentrations, namely, 10 nM, 100 nM, and 1000 nM. Distilled deionized water and 0.2% dimethylsulfoxide (DMSO) were used as negative controls. Cytotoxicity was observed at the three concentrations tested as shown by a significant reduction in average mitotic indices. Chromosomal aberrations were also observed, which is suggestive of the genotoxicity of these pollutants. The effects of PCBs on the early life stages of zebrafish, Danio rerio, were also investigated. The three levels of PCBs were tested including the embryo medium, 0.2% DMSO, and 2% ethanol that served as controls. Embryos at 6 – 8 hours postfertilization were exposed to the different treatments and were observed everyday for 5 days. Results showed a significant decrease in the mean number of melanocytes and average length at 5 days postfertilization, a delay in hatching of embryos, and a significant increase in average percent of fish with deformities for the 1000 nM PCB concentration. This study demonstrates that the Allium test is a simple and reliable method to assess cytotoxicity and genotoxicity of PCBs. Exposure of zebrafish embryos to high concentrations of PCBs also delays growth and hatching rates and causes teratogenic effects. These results may have implications on the health of humans and other organisms at risk to PCB exposures as these chemicals are known to bioaccumulate.

Key Words: cytotoxicity, developmental defects, genotoxicity, mitotic index

INTRODUCTION

Polychlorinated biphenyls (PCBs) are a group of synthetic organic chemicals that are formed by adding chlorines to biphenyl rings. Since a biphenyl ring has 10 possible positions for chlorine substitution, theoretically, there are 209 possible arrangements or congeners, but only about 60 to 90 congeners are present in commercial mixtures (Kimbrough & Krouskas 2003). PCBs were commercially produced in the US from 1929 to 1977 with the trade names Aroclor 1016, 1242, 1254, 1260, and 1268 (Kimbrough & Krouskas 2003). In the past, commercial PCB mixtures were used in capacitors and transformers, flame retardants, inks, adhesives, microencapsulation of dyes for carbonless duplicating paper, paints, pesticide
extenders, plasticizers, polyolefin catalyst carriers, surface coatings, wire insulators, and metal coatings (Kimbrough & Krouskas 2003). Owing to accumulating evidence that they build up in the environment and have harmful effects, production of PCB mixtures has been stopped in most developed countries. But it is unknown if these are still being produced in other countries.

In the Philippines, when the Manila Electric Company (Meralco) dismantled its diesel power plant in Makati City in the 1990s, it had to acquire a permit from the Department of Environment and Natural Resources - Environmental Management Bureau for the transfer of its remaining PCBs (14,000 L of liquid PCBs, and 5,000 cubic meters of PCB-contaminated soil) for storage in its service station in Pasig City while waiting for these PCBs to be exported to Europe for processing (Concepcion 2006). In the early 1990s, PCBs also leaked into the environment from a transformer when the United States left its airbase in Pampanga that affected residential areas.

PCBs are highly persistent and tend to accumulate progressively in soil, plants, and animals (Apostoli et al. 2005), thus they can still be detected in the environment even if commercial production has been banned. Because of their low solubility in water, direct measurement of PCBs in aquatic ecosystems is tedious and expensive, hence, bioindicator organisms such as squid, mussel, and fish have been utilized (Tanabe & Tatsukawa 1987; Ueno et al. 2003a; Ueno et al. 2003b). Ueno et al. (2003b) determined levels of organochlorines (OCs) that include PCBs in the liver of skipjack tuna Katsuwonus pelamis collected from offshore waters of various regions in the world including the Philippines. Widespread contamination of persistent OCs in the marine environment was noted since OCs were detected in livers of all fish collected from all locations. Among the OCs analyzed, PCBs had the highest concentration at 1,800 ng/g lipid weight from the offshore waters of Taiwan. The average PCB level detected in offshore waters of the Philippines was 190 ng/g lipid weight. Among the PCB congeners detected, hexachlorobiphenyl (PCB-153) was the most prevalent, followed by PCB-138. There was also a high prevalence of octachlorobiphenyls in offshore waters of the Philippines. PCBs have been identified also in the human blood and tissues over several decades (Kimbrough & Krouskas 2003). Human exposure to PCBs occurs through food, air, and water. The US Environmental Protection Agency (EPA) and the International Agency for Research on Cancer (IARC) classified PCBs as carcinogens (Font et al. 1996).

Owing to their persistence, potential for bioaccumulation and biomagnification, monitoring of PCBs is important for conservation of the environment and biota (Font et al. 1996). In addition to monitoring the levels of PCBs in the environment, further studies on their cytotoxicity and genotoxicity are also needed. In vivo and in vitro studies have been done to test the genotoxicity of PCBs, but generally the results were negative (ATSDR 2000). To date, studies on cytotoxicity and genotoxicity of PCBs using plant bioassays are lacking. In plant bioassays, the assumption is made that if a chemical can cause damage to the chromosomes in a reliable plant assay, then the chemical is considered to have the potency of damaging the chromosomes of other organisms in the environment (Rank & Nielsen 1997). The common onion, Allium cepa, has been used since the 1930s in plant bioassays in what is called as the Allium test. Being a simple, reliable, and inexpensive test, Grant (1982) proposed that the Allium test be included among those tests routinely used for assessing chromosomal damage induced by chemicals. Fiskesjo (1985) also suggested the Allium test as a standard in environmental monitoring that may be included as part of a test battery. As a short-term test, the Allium test offers a lot of advantages such as ease of handling, low cost, and good chromosome conditions that allow visualization of chromosome damage or disturbance of cell division including the examination of risks of aneuploidy. The Allium test also combines two test targets, namely, cytotoxicity and mutagenicity. Cytotoxicity is measured by growth inhibition, while mutagenicity is correlated to the rate of chromosome breaks. Also, the Allium test is a sensitive test that gives comparable results to other test systems. Hence, positive results in the Allium test may be taken as a warning and an indication that the chemical being tested may pose risk to humans and other organisms, and to the environment. Some recent studies in the literature that utilized the Allium test include those of Chandra et al. (2005) to determine the possible genotoxic effects of leachates from solid waste of a metal and dye industry; Crebelli et al. (2005) to examine the genotoxicity of disinfection by-products resulting from perchloric acid- or hypochlorite-disinfected sewage wastewater; and, Evandi & Bolle (2001) who used the Allium test in pharaco-toxicological screening of 14 commercially available Italian natural mineral waters.

Studies on the teratogenic effects of PCBs are also needed. The zebrafish, Danio rerio, which has been extensively used in developmental biology for several years (Teraoka et al. 2002), is a good organism to investigate possible teratogenic effects of PCBs. The zebrafish is a freshwater fish that is easy to grow and is sexually mature in three months. It spawns a large number of fish, and spawning is triggered by light. Each female may produce 100 large translucent eggs per day (Herrmann 1993; Samson & Shenker 2000). Most organs
develop within one day at 26°C and the embryos hatch after 3 to 4 days. All these features make the species an excellent model for studying embryotoxic effects of pollutants or toxic substances (Samson & Shenker 2000; Hallare et al. 2004). A number of endpoints are used in evaluating the toxicity and teratogenicity of chemicals on zebrafish embryo. Among these are egg and embryo mortality, pigmentation, heartbeat, hatching success, larval viability, larval length, and malformations (Black et al. 1988; Sinha & Kanamadi 2000; Cheng et al. 2000; Todd & Van Leeuwen 2002; Hallare et al. 2004; Tiedeken et al. 2005).

A number of studies on teratogenic effects of toxicants on zebrafish have been undertaken. Samson & Shenker (2000) performed chronic bioassays to evaluate the concentration and exposure duration of methylmercury that resulted in specific teratogenic defects in zebrafish embryos at different developmental stages. Sinha & Kanamadi (2000) treated zebrafish embryos with mercurial fungicide, Emisan®-6. Todd & Van Leeuwen (2002) studied the effects of Sevin (carbaryl insecticide) on early life stages of zebrafish. The effects of maternal exposure to various PCB congeners on different life stages of zebrafish and trout have been investigated (Matta et al. 2002) studied the effects of Sevin (carbaryl insecticide) on early life stages of zebrafish. The effects of maternal exposure to various PCB congeners on different life stages of zebrafish and trout have been investigated (Matta et al. 1997; Billson et al. 1998; Olsson et al. 1999; Westerlund et al. 2000). To date, there is no study yet on the possible effects of direct exposure of zebrafish embryos to a mixture of PCBs.

This study was conducted to assess the cytotoxicity and genotoxicity of a mixture of PCBs at three different concentrations namely, 10 nM, 100 nM, and 1000 nM using the Allium test, and also to determine if direct exposure of zebrafish embryos to this mixture of PCBs will result in developmental defects, and to compare the effects of these three PCB concentrations vis-à-vis the negative controls (embryo medium and 0.2% DMSO) and positive control (2% ethanol) on the early life stages of zebrafish.

**MATERIALS AND METHODS**

**Allium cepa chromosome aberration assay**

Onion bulbs of the desired size and from the same batch were procured from the local market. After removing the loose outer scales and old roots and scraping the bulbs to expose the root primordia, they were suspended in basins containing clean tap water. The onions were kept in the basins for two days to produce roots. Water was replenished every day. Only onions that produced roots of approximately the same lengths were used in the experiment. The onions selected were exposed for 24 h to three different nominal concentrations of Supelco PCB mixture (10 nM, 100 nM, and 1000 nM) and two negative control groups (distilled deionized water and 0.2% DMSO). The PCB mixture that was dissolved in 0.2% DMSO to get the desired concentration consisted of the following congeners: (1) 2,4,4’ trichlorobiphenyl (PCB-28); (2) 3,3’,4,4’ tetrachlorobiphenyl (PCB-77); (3) 2,2’,5,5’ tetrachlorobiphenyl (PCB- 52); (4) 2,2’,4,5,5’ pentachlorobiphenyl (PCB-101); (5) 2,2’,4,4’,5,5’ hexachlorobiphenyl (PCB-153); (6) 2,2’,3,4,4’,5’ hexachlorobiphenyl (PCB-138); and, (7) 2,2’,3,4,4’,5,5’ heptachlorobiphenyl (PCB-180).

Four replicates were done for each treatment. After 24-h exposure to the treatments and control groups, the root tips were cut and fixed in aceto-ethanol (1:3) and stored in the refrigerator until further use. Slides were prepared following the procedure of El-Shahaby et al. (2003) with some modifications. Root tips were macerated using 1N HCl for 3 min followed by staining in Carbol fuchsin for 3 min and passing over the flame in an alcohol lamp twice. After 3 min, excess Carbol fuchsin was removed by blotting off with tissue paper, and exposing the root tips to drops of 2% aceto-orcein stain in 45% acetic acid for 3 min, and passing over the flame again twice. Then the root tips were squashed under the cover glass. Following Grover & Kaur (1999), further squashing was carried out by striking the cover glass with a flat pencil eraser with a bouncing action, which was also done to spread the cells evenly on the surface of the slide. The slides were then examined under the microscope and photographed. For each replicate, 1000 cells from 2 to 3 root tips were examined. The chromosome aberrations scored were bridges in anaphase and disturbed metaphase, anaphase, and telophase. The mitotic index for each treatment was also calculated. The Allium cepa chromosome aberration assay was conducted from August to October 2005.

**Danio rerio assay**

Thirty adult zebrafish (20 females and 10 males) were acclimatized separately for each sex in a 5-L aquarium containing water with the following characteristics: temperature (26.1–26.8°C), salinity (0 ppt), total dissolved solids (110–112 ppm), and conductivity (218–225 μS). Aeration was provided at all times. The tap water used in rearing the fish was aged by storing in an aerated container for at least 2 wk to remove chlorine. The fish were fed with commercial fish flake and frozen blood worm at least twice a day. For spawning, the zebrafish were reared together in a 10-L aquarium and were kept in a 12-h/12-h light/dark cycle. Spawning is triggered once the light is turned on and it usually lasts from 30 min to 1 h.
All the eggs used in the experiment were laid by the same group of adults. The eggs were collected, rinsed in dechlorinated tap water, and placed in an aerated plastic tray containing embryo medium (294 mg CaCl$_2$, 123.25 mg MgSO$_4$, 64.75 mg NaHCO$_3$, and 5.75 mg KCl dissolved in 1 L of distilled water). Unfertilized eggs (opaque or with an irregularly shaped first blastomere) were identified and discarded. Embryos (6 – 8 h postfertilization) were selected for the experiment. One embryo was placed in each well of a 96-well culture plate containing 250 µL of treatment or control. To replace losses by volatilization, equal volumes of fresh solutions were added to each well daily. The treatments include 10 nM, 100 nM, and 1000 nM of PCB mixture (nominal concentrations) and the control groups include embryo medium (negative control), 0.2% DMSO (negative control), and 2% ethanol (positive control). Twelve to 15 embryos were used for each treatment or control. Three trials of the experiment were done. The following data were recorded: (1) body length (from the tip of the head to the tip of the tail) and number of melanocytes on the left side of the body at 5 days postfertilization (dpf); (2) heart beat per minute and hatching rate at 3 dpf; (3) survival rate at 2, 3, and 5 dpf; and, (3) daily observations for abnormalities. The body length was measured under a stereomicroscope using a dial caliper. A stereomicroscope was also used in counting the number of melanocytes and in observing for abnormalities. The abnormalities looked for include tail turned upwards, curved spine, yolk sac edema, and fish swimming on one side of the body. The Danio rerio assay was conducted from November 2005 to March 2006.

Data analysis
For the Allium cepa chromosome aberration assay, mitotic index was calculated as the total number of mitotic cells divided by the total number of cells counted and expressed in percent. Analysis of variance (ANOVA) was done to compare all the treatments simultaneously. Prior to ANOVA, percent values were arcsin square-root transformed. Duncan’s Multiple Range Test (DMRT) was conducted for treatments that showed significant difference in the ANOVA. Data were analyzed using SPSS version 10.

RESULTS
The average mitotic indices for the 3 different PCB concentrations were not significantly different from each other (Table 1), but these were statistically significantly lower compared to the 2 negative control groups ($\alpha = 0.05$). The average mitotic index for DMSO (10%) was also statistically lower compared to water (13%). There was a significantly higher percentage of cells at prophase for root meristem cells exposed to the 3 different PCB concentrations compared to the 2 control groups (Table 1). PCB-exposed meristem cells had generally lower percentage of cells at metaphase and anaphase. There was no significant difference in percentage of cells at telophase for the root meristem cells treated with the 3 concentrations of PCBs compared to cells placed in water. In terms of average percent total chromosomal aberrations, root meristem cells treated with the 3 PCB concentrations were not significantly different from each other ($\alpha = 0.05$). As expected, no chromosomal aberrations were observed in root meristems placed in water and in DMSO. There were also no significant differences in terms of percentage aberrant cells among the plants exposed to the 3 PCB concentrations at metaphase and anaphase (Table 2). This suggests that the 3 different PCB concentrations have the same effect in terms of genotoxicity. The chromosomal aberrations observed were disturbed metaphase, disturbed anaphase, and disturbed telophase (Figure 1).

Fish larvae exposed to 100 nM and 1000 nM PCBs had the lowest mean length at 5 dpf that was significantly different from the control (Table 3). Larvae exposed to 1000 nM PCB had the lowest mean number of

| Table 1. Mitotic index ± standard deviation and percentage of cells ± standard deviation at each phase |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Treatment | Mitotic index** ± SD | Prophase** | Metaphase** | Anaphase** | Telophase** |
| H$_2$O | 13 ± 1.0 | 42 ± 0.9 | 26 ± 1.3 | 19 ± 0.6 | 14 ± 1.7 |
| 0.2% DMSO | 10 ± 1.0 | 29 ± 0.3 | 32 ± 0.9 | 29 ± 1.0 | 10 ± 1.6 |
| 10 nM | 6 ± 1.6 | 59 ± 2.3 | 18 ± 4.4 | 10 ± 3.4 | 14 ± 3.3 |
| 100 nM | 4 ± 1.6 | 57 ± 6.0 | 14 ± 6.2 | 16 ± 7.1 | 13 ± 4.2 |
| 1000 nM | 6 ± 1.4 | 56 ± 4.1 | 17 ± 4.7 | 12 ± 2.8 | 16 ± 2.1 |

For each column, means followed by the same lower case bold letter are not significantly different. Statistically significant differences were determined by one-way ANOVA (**p<0.01) followed by Duncan’s Multiple Range Test and are indicated in the table by lower case bold letters.

For each treatment, values in each column are means of 4 replicates and for each replicate, 1000 embryos were counted. The percentage of cells at each mitotic phase was computed by dividing the number of cells at that phase by the total number of mitotic cells. Mitotic index was computed by dividing the total number of mitotic cells by 1000.
Table 2. Chromosomal aberrations at each phase (% of total dividing cells ± standard deviation)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total % aberrant cells** ± SD</th>
<th>Percentage of aberrant cells at each phase (± SD)</th>
<th>Metaphase**</th>
<th>Anaphase**</th>
<th>Telophase**</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>0 a</td>
<td>0 a</td>
<td>0 a</td>
<td>0 a</td>
<td></td>
</tr>
<tr>
<td>0.2% DMSO</td>
<td>0 a</td>
<td>0 a</td>
<td>0 a</td>
<td>0 a</td>
<td></td>
</tr>
<tr>
<td>10 nM</td>
<td>19 ± 3.1 b</td>
<td>5 ± 2.2 b</td>
<td>11 ± 6.2 b</td>
<td>6 ± 3.8 c</td>
<td></td>
</tr>
<tr>
<td>100 nM</td>
<td>20 ± 6.1 b</td>
<td>5 ± 2.0 b</td>
<td>4 ± 1.2 bc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000 nM</td>
<td>16 ± 7.6 b</td>
<td>6 ± 3.2 b</td>
<td>8 ± 5.8 b</td>
<td>2 ± 1.5 ab</td>
<td></td>
</tr>
</tbody>
</table>

For each column, means followed by the same lower case bold letter are not significantly different. Statistically significant differences were determined by one-way ANOVA (**p<0.01) followed by Duncan’s Multiple Range Test and are indicated in the table by lower case bold letters.

Table 3. Mean length in mm ± standard deviation at 5 days post-fertilization (dpf), mean number of melanocytes ± standard deviation along the lateral line at 5 dpf, and mean heart rate per minute ± standard deviation at 3 dpf

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean length** (mm)</th>
<th>Mean no. of melanocytes*</th>
<th>Mean heart rate (beats/minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo medium</td>
<td>3.2±0.2 a (N=31)</td>
<td>17±1.8 a (N=25)</td>
<td>163±13.6 a (N=24)</td>
</tr>
<tr>
<td>2% ethanol</td>
<td>2.9±0.4 b (N=34)</td>
<td>16±1.9 ab (N=28)</td>
<td>159±14.6 a (N=25)</td>
</tr>
<tr>
<td>0.2% DMSO</td>
<td>2.8±0.6 b (N=31)</td>
<td>16±2.7 b (N=22)</td>
<td>168±14.2 a (N=20)</td>
</tr>
<tr>
<td>10 nM PCB</td>
<td>2.8±0.6 b (N=31)</td>
<td>17±2.2 ab (N=21)</td>
<td>159±22.7 a (N=22)</td>
</tr>
<tr>
<td>100 nM PCB</td>
<td>2.7±0.5 b (N=38)</td>
<td>16±2.3 ab (N=27)</td>
<td>167±19.1 a (N=27)</td>
</tr>
<tr>
<td>1000 nM PCB</td>
<td>2.7±0.6 b (N=26)</td>
<td>15±2.0 b (N=18)</td>
<td>166±15.4 a (N=16)</td>
</tr>
</tbody>
</table>

For each column, means followed by the same lower case bold letter are not significantly different. Statistically significant differences were determined by one-way ANOVA (**p<0.05 or **p<0.01) followed by Duncan’s Multiple Range Test and are indicated in the table by lower case bold letters.

Values in parentheses are the sample sizes (N) for each treatment.
melanocytes, which was also significantly different from the control. There were no significant differences among the treatment means in terms of mean heart rate, mean survival, and mean hatching rate ($\alpha=0.05$). Fish exposed to the 1000 nM PCB treatment tended to have the lowest mean survival rate and hatching rate. Fish exposed to the 1000 nM PCB had the highest average percentage of deformities, which was significantly different from the control (Table 4). The abnormalities observed include tail turned upwards, curved spine, yolk sac edema (Figure 2), and fish swimming on one side of the body.

DISCUSSION

The significant decrease in the mitotic indices of *Allium cepa* exposed to the 3 concentrations of PCB shows the cytotoxicity of this environmental pollutant. This is because inhibition of the mitotic activities is often used for evaluating cytotoxic substances (Smaka-Kinecl et al. 1996). Smaka-Kinecl et al. (1996) found that the decrease in mitotic index in onion root meristem is a reliable means for rapid determination of the presence of cytotoxic substances in the environment, monitoring

**Table 4.** Mean percent survival ± standard deviation at 1, 2, and 5 days post-fertilization (dpf), mean percent deformities ± standard deviation at 5 dpf, mean percent hatched ± standard deviation at 3 dpf

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean % survival 1 dpf</th>
<th>Mean % survival 2 dpf</th>
<th>Mean % survival 5 dpf</th>
<th>Mean % deformities**</th>
<th>Mean hatching rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo medium</td>
<td>88±10.7 a</td>
<td>85±13.2 a</td>
<td>83±3.3 a</td>
<td>0±0 a</td>
<td>96±7.7 a</td>
</tr>
<tr>
<td>2% ethanol</td>
<td>87±12.5 a</td>
<td>87±12.5 a</td>
<td>82±21.3 a</td>
<td>14±5.9 bc</td>
<td>84±11.3 a</td>
</tr>
<tr>
<td>0.2% DMSO</td>
<td>93±6.7 a</td>
<td>93±6.7 a</td>
<td>78±20.2 a</td>
<td>7±6.7 ab</td>
<td>83±28.9 a</td>
</tr>
<tr>
<td>10 nM PCB</td>
<td>90±8.8 a</td>
<td>90±8.8 a</td>
<td>72±23.5 a</td>
<td>2±3.9 a</td>
<td>79±21.6 a</td>
</tr>
<tr>
<td>100 nM PCB</td>
<td>98±3.9 a</td>
<td>94±10.8 a</td>
<td>91±10.1 a</td>
<td>12±5.1 bc</td>
<td>93±12.4 a</td>
</tr>
<tr>
<td>1000 nM PCB</td>
<td>80±22.1 a</td>
<td>80±22.1 a</td>
<td>67±26.0 a</td>
<td>17±3.3 c</td>
<td>67±19.2 a</td>
</tr>
</tbody>
</table>

For each column, means followed by the same lower case bold letter are not significantly different. Statistically significant differences were determined by one-way ANOVA (**$p<0.01$) followed by Duncan’s Multiple Range Test and are indicated in the table by lower case bold letters.

Values are means of three trials; 12 to 15 zebrafish embryos were used per treatment per trial.

**Figure 2.** Normal (A) and malformed zebrafish larvae (B-D). Arrow in B points to yolk sac edema observed in zebrafish larvae exposed to 2% ethanol, 0.2% DMSO, 10 nM, 100 nM, and 1000 nM PCBs. Larvae with curved spine (C) were seen in zebrafish embryos exposed to 100 nM PCBs. Larvae with tail flexures (D) were seen in embryos exposed to ethanol, 100 nM, and 1000 nM PCBs. Bar in A = 0.5 mm.
cytotoxic pollution level in the natural environments, and evaluations of water pollution levels. In a similar study, Chandra et al. (2005) attributed the inhibition of mitotic index to the effect of environmental chemicals they tested on DNA/protein synthesis of the biological system. This could also explain the results in this study.

Prominent vacuolization in the cytoplasm and nucleoplasm was observed also in cells exposed to the 3 concentrations of PCBs (Figure 1F). This was also observed by Ateeq et al. (2002) when they used the Allium test to determine the cytotoxicity and genotoxicity of 3 herbicides, namely, pentachlorophenol (PCP), 2,4-dichlorophenoxyacetic acid (2,4-D), and 2-chloro-2,6-diethyl-N-(butoxymethyl) acetonide (butachlor).

The chromosomal aberrations observed suggest that PCB may be genotoxic. This is contrary to what has been stated that PCBs are not mutagenic in bacterial systems, and that most of the other tests for genotoxicity have yielded negative results (Whysner & Wang 2001). Despite earlier reports that PCBs are not genotoxic, there had been studies which showed that PCB metabolites are capable of interacting with DNA and are thus potentially genotoxic. Wyndham et al. (1976) found that 4-chlorobiphenyl was mutagenic to the Salmonella typhimurium strain TA1538 and that the metabolism of this PCB substrate proceeds via an arene oxide intermediate. Wyndham & Safe (1978) demonstrated that incubation of 4-chlorobiphenyl and induced rat liver microsomal enzyme fraction with exogenous nucleic acids such as calf thymus DNA, denatured calf thymus DNA, poly(A), poly(G), poly(C), and poly (U) resulted in the formation of nucleic acid-4-chlorobiphenyl adducts. Wong et al. (1979) also showed that exposure of Chinese hamster ovary cell cultures to 4-chlorobiphenyl caused metabolically mediated DNA damage. Furthermore, Butterworth et al. (1995) showed that it is the bioactivated form of PCB that is genotoxic and the form of genotoxicity is by recombinogenesis, that is, an increase in the rate of recombination in somatic cells.

Larvae exposed to 100 nM and 1000 nM PCBs had the lowest mean length (2.7 mm) at 5 dpf, which was significantly different from the control (3.2 mm). In a related study, Black et al. (1988) investigated the effect of exposure of adult winter flounder, Pseudopleuronectes americanus, to PCB-polluted areas, on winter flounder eggs and larvae. PCB-polluted study areas included Gaspee Point in upper Narragansett Bay, Rhode Island, New Bedford Harbor in Buzzards Bay, Massachusetts, and Apponagansett Bay, Massachusetts. Fox Island, which is a relatively clean area in lower Narragansett Bay, served as control. Larval length showed differences between study areas that varied inversely with the PCB content of the eggs of adult winter flounder caught from the study areas. Eggs of winter flounder caught from New Bedford Harbor had significantly higher levels of PCB (39.6 µg/g dry weight), and larvae that hatched from these eggs were significantly smaller (2.96 mm) than those from Fox Island fish (1.08 µg PCB/g egg dry weight, 3.22 mm larval mean length). Larvae from Apponagansett Bay winter flounder were intermediate in size (15.7 µg PCB/g egg dry weight, 3.08 mm larval mean length) and not significantly different from either Fox Island or New Bedford larvae. Todd & Van Leeuwen (2002) also observed that zebrafish embryos exposed to four different dilutions of the insecticide Sevin consistently had lower length compared to control at 3 dpf, 4 dpf, 5 dpf, and 6 dpf; the length tended to decrease with increasing concentration of Sevin. In the wild, the severe consequence of smaller larvae resulting from exposure to pollutants is that these larvae are inefficient predators and more vulnerable to predation (Black et al. 1988).

There was also a tendency for the embryos treated with 1000 nM PCB to have the lowest survival rate at all time points (at 1 dpf, 2 dpf, and 5 dpf). Billsson et al. (1998) also used survival rate of embryos and larvae as one of the endpoints in investigating the effect of injecting zebrafish females (1 µmol/kg) individually with four PCB congeners (PCB-60, PCB-104, PCB-173, and PCB-190), 17β estradiol, and peanut oil that was used as PCB solvent. Five days after fertilization, larvae from females injected with the carrier-control peanut oil had the highest survival rate (about 98%), followed by PCB-190 (about 90%), PCB-173 (about 80%), PCB-60 (about 60%), PCB-104 (about 40%), and 17β estradiol (25%). Matta et al. (1997) exposed eggs of rainbow trout (Oncorhynchus mykiss) and westslope cutthroat trout (O. clarki lewisi) to 5, 45, and 95 mg/L of hydroxylated PCB (2',4',6'-trichloro-4-biphenylo1) and to water and 0.65% DMSO carrier controls. For the rainbow trout, the mean percent viable larvae was 55.2%, 65.2%, 26.0%, and 11.1% for the combined controls, 5 mg/L-PCB treatment, 45 mg/L-PCB treatment, and 95 mg/L-PCB treatment, respectively. For the westslope cutthroat trout, the values were 80.0%, 63.6%, 28.9%, and 30.7% for the combined controls, 5 mg/L-PCB treatment, 45 mg/L-PCB treatment, and 95 mg/L-PCB treatment, respectively. Thus, for these 2 species, higher concentrations of hydroxylated PCB significantly affected larval survival. Westerlund et al. (2000) also determined zebrafish larval survival rate following maternal injection of 8 PCB congeners (1 µmol/kg of each PCB dissolved in peanut oil). Eight days after fertilization, larvae of zebrafish females injected with PCB-104 had the lowest survival rate (28%) among the 8 congeners. The larval survival rates for the other congeners were 59% for PCB-60, 73% for PCB-143,
74% for PCB-173, 76% for PCB-112, 81% for PCB-126, and 86% for both PCB-184 and PCB-190. Clearly, the foregoing studies show that PCB affects zebrafish larval survival rate and that the severity depends on the congener. In this study, varying concentrations of PCB mixture containing congeners different from the aforementioned studies were used. It should be noted, however, that there are species differences in response to dioxin-like toxicants. Elonen et al. (1998) investigated the toxic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the early life-stage development of seven freshwater fishes. Among the seven fishes, lake herring (Coregonus artedii) had the lowest no-observed-effect concentration (175 pg/g) and lowest-observed-effect concentration (270 pg/g) based on significant decreases in survival and growth as compared to the controls, while zebrafish had the highest values at 424 and 2,000 pg/g, respectively. In addition, fathead minnow (Pimephales promelas) was the most sensitive (539 pg/g) while zebrafish was the least sensitive (2,610 pg/g) based on LC_{50}s (concentrations in eggs causing 50% lethality to fish at termination of experiment). Among 11 fish species including the seven in Elonen et al. (1998), lake trout (Salvelinus namaycush) is the most TCDD-sensitive and in fact the most TCDD-sensitive vertebrate species known, with an LC50 (concentration that results in the death of 50% of the animals) of 47–80 pg/g egg while zebrafish is the least sensitive fish species, with an LC50 of 2.5 ng/g (Hahn 2001).

In this study, there were also no significant differences among the treatment means in terms of mean heart rate. Tiedeken et al. (2005) also found no significant differences in heart rate between control and domoic acid-treated zebrafish embryos although heart rate increased in both treated and non-treated embryos between day two and three from an average of 125 ± 7 to 177 ± 8 beats/ min which is a normal developmental heart rate. Similarly, Hallare et al. (2004) found that the average heart rate at 2 dpf was unaffected by nominal concentrations of diclofenac (1 to 2000 μg/L) and DMSO controls (0.00002 to 0.04%).

Fish exposed to the 1000 nM PCB had the highest average percentage of deformities (17%), which was significantly different from the untreated embryos (0%). Tail turned upwards and curved spine were two of the abnormalities observed. Samson & Shenker (2000) also observed severe flexure of the entire posterior-most tail region of embryos exposed to 20 μg and 30 μg CH\textsubscript{3}H\textsubscript{2}C\textsubscript{6}H\textsubscript{5}Cl/L. Olsson et al. (1999) also observed craniofacial and posterior malformation following maternal exposure of zebrafish to PCB 104 (2,2’,4,6,6’-PeCB). Olsson et al. (1999) injected 4 female zebrafish with individual PCB congeners (1 μmol/kg) and mated with un.injected males.

One group of eggs from the injected females was reared to adult stage. Some of the adult zebrafish produced by females injected with PCB-104 had severe curvature of the tail and caudal fin agenesis, i.e., imperfect development. Radiographic analysis of the deformed individuals showed marked lordosis, i.e., exaggerated curvature of the vertebral column, and scoliosis or lateral bending of the vertebral column of the caudal region of the spine. In another study, Jönsson et al. (2007) exposed zebrafish embryos to 3,3’,4,4’,5-pentachlorobiphenyl (PCB-126) at nominal concentrations ranging from 0.3 nM to 100 nM in 100 ppm acetone and 100 ppm acetonone alone. They found that at 3 dpf, about 25% of the embryos treated with 3 nM of PCB-126 showed pericardial edema, and the incidence of edema was 95-100% at higher concentrations. Embryos exposed to 10-100 nM PCB-126 also showed craniofacial malformations, heart malformations, slower and weaker heartbeats, impaired circulation, and immobility. These effects became more severe at 4 dpf and included yolk sac edema. At 4 dpf, several embryos exposed to lower concentrations of PCB-126 showed reduced swim bladder inflation.

Although the positive control (2% ethanol) had a much lower mean percent deformities (14%) than embryos exposed to 1000 nM PCB, these values are not significantly different. Ethanol–caused deformities coming in second to 1000 nM PCB may suggest that PCB is more teratogenic than ethanol, but both belong to similar grouping based on Duncan’s Multiple Range Test, thus their effectiveness maybe just the same. There are a number of papers describing negative effects of ethanol in zebrafish larvae, that is why it is used as positive control in teratological experiments using zebrafish. Most of the effects in higher vertebrates is on neural development especially the eyes, and it has been suggested that ethanol may disrupt retinoic acid metabolism in developing vertebrate embryos (Deltour et al. 1996). Ethanol consumption by the mother is associated with producing cyclopic embryos. In one study, Matsui et al. (2006) exposed zebrafish embryos to various concentrations of ethanol (1%-2% ethanol by volume) from 2 to 5 dpf. Embryos exposed to 1% ethanol appeared morphologically normal. Embryos treated with 1.25% to 1.5% ethanol had slightly flatter forebrains, swollen hearts, swollen guts, and abnormal craniofacial development. Embryos exposed to 1.75% and 2% ethanol had several morphological defects such as a dorsally curved body, swollen heart, rounded forebrain, irregular jaw, and smaller eyes. Lockwood et al. (2004) also tested the effect of ethanol on melanocyte morphology by exposing 7dpf zebrafish fry to 0% (control), 0.5%, 1.5%, and 3% ethanol for 1, 10, and 20 min. The pigmentary organelles known as melanosomes of the melanocytes were clustered mainly in the center of the cell in larval
zebrafish that were not treated with ethanol. No observable change was noted after exposure to 1.5% ethanol for 1 min, but a marked dispersion of the melanosomes was observed after 20 min of exposure to 1.5% ethanol and at all time points to 3.0% ethanol. Although it was not clear whether such an effect was due to the direct action of ethanol on melanocytes or if it was mediated through the central nervous system, such change in melanocyte morphology provides a visible cellular measure of the biological effects of ethanol in vivo. In this study, melanocyte morphology was not examined, but larvae exposed to 1000 nM PCB had the lowest mean number of melanocytes, which was also significantly different from the control. Although statistical difference was observed, this was not enough to conclude hypopigmentation following the criterion of Cheng et al. (2000) wherein the number of melanocytes should be less than 70% of untreated controls.

The chromosomal aberrations in the common onion as well as the malformations resulting from direct exposure of zebrafish embryos to a mixture of PCBs, are clear indications of the harm that this persistent organic pollutant may cause to organisms. Humans are exposed to PCBs not just from eating contaminated meat and fish, but also through the air and water. PCBs have been detected in human breast milk, blood, and other tissues (Kimbrough & Krouskas 2003). The harm caused by PCB is compounded by the persistency of this pollutant in the environment and by its highly bioaccumulative nature. Since direct studies on the effect of PCB exposure to humans cannot be done, the zebrafish is a good model to study teratogenic effects of PCBs. Being a vertebrate, the developmental principles of zebrafish are strongly applicable to higher vertebrates such as humans.

CONCLUSIONS AND RECOMMENDATIONS

This study demonstrates that the Allium test can be used reliably to assess the cytotoxicity and genotoxicity of PCBs. Polychlorinated biphenyls were found to be cytotoxic at all levels examined as shown by the decrease in the average mitotic indices. The genotoxicity of PCBs was also shown by chromosomal aberrations observed at all concentrations tested. Exposure to PCBs also significantly decreases the mean length of zebrafish larvae at 5 dpf, hence growth is affected. Exposure to high concentrations of PCBs also significantly reduces the number of melanocytes along the lateral line, although this was not enough to conclude hypopigmentation. Although there were no significant differences among the treatment means in terms of survival rate and hatching rate, fish exposed to 1000 nM PCBs had the lowest survival and hatching rate at 3 dpf. Thus, high concentrations of PCBs tend to cause a reduction in survival and a delay in development. Fish exposed to ethanol and to 100 and 1000 nM PCBs also had significantly higher mean percentage of deformities compared to other treatments, which is suggestive of their teratogenic potential.

In this study, the endpoints used for the zebrafish assay were based on the work of Black et al. (1988), Sinha & Kanamadi (2000), Cheng et al. (2000), Todd & Van Leeuwen (2002), Hallare et al. (2004), and Tiedeken et al. (2005). The endpoints used in the more recent study by Jönsson et al. (2007) such as pericardial edema and swim bladder inflation should be also included in future investigations of the toxicity of PCBs. Other assays should also be used to establish the mutagenicity, carcinogenicity, and teratogenicity of individual PCB congeners or mixtures thereof. The molecular mechanisms underlying the malformations observed in zebrafish embryos upon exposure to PCBs as well as the genes or the gene interactions that are disrupted are also worth pursuing in future studies.

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