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Part I: The Dangers of Plastic

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**Abstract:** Bisphenol A (BPA) is used to make plastics and has been found to be a xenoestrogen. Planaria (*Girardia tigrina*), regenerating flatworms, were exposed to BPA and deuterated BPA (D8-BPA). Phenotypic effects of BPA on the planaria were recorded during exposure and BPA was then extracted to quantify the amount absorbed by the flatworms. Deuterated BPA (D8-BPA) was used to distinguish added BPA from BPA already present in the organism from supply-chain contamination. A control experiment tested whether the multiple washes performed after incubation removed physisorbed BPA from planaria. Additional analysis was performed using high performance liquid chromatography (HPLC, reverse phase). Improvements in HPLC method to analyze BPA and D8-BPA resulted in better separation between BPA and D8-BPA peaks. Gas chromatography mass spectroscopy (GCMS) was used to quantify BPA/D8 BPA. It is concluded that planarian regeneration is negatively affected by exposure to 20 μM concentrations of BPA/D8-BPA, planaria absorbed BPA and D8-BPA from solution, the washing method removed most physisorbed BPA, and decreasing the HPLC pump flow rate improved peak separation. This section describes studies done by other researchers to explore detrimental effects of BPA on humans, including miscarriage, infertility, obesity, and sexual dysfunction. The detrimental environmental effects of excessive plastic use, plastic alternatives, and solutions to reducing the damage of plastic are also described.
I. Introduction

Approximately 300,000,000 tons of plastic is produced worldwide per year. To put that number in perspective, that is about 820,000 tons each day, and 9.5 tons per second. Only 10 percent of this plastic actually gets recycled per year. This means that 270,000,000 tons of plastic gets dumped into landfills each year. (Wassener). This plastic disposal causes there to be excessive amounts of plastic pollution; because plastic is not biodegradable, it only breaks down into smaller pieces. The plastic in the environment has many adverse direct and indirect effects on life. Plastic is everywhere since it is used for basic everyday items to more important items. These include, “bicycle helmets, child safety seats, airbags in automobiles, cell phones, televisions, computers and other electronic equipment, roofs, walls, flooring and insulation, and in food packaging and utensils (“Plastics: Uses, Benefits, and Chemical Safety Facts”). Almost one-third of plastic is used once and then thrown away, such as water bottles, bags, and straws. Because the physical presence of plastic particles can obstruct animal digestive systems, and the chemicals can cause detrimental health effects, it is a very harmful substance and its use needs to be reduced or hopefully, replaced.

II. What is Plastic, and How is it Made?

Plastic is defined as material that consists of synthetic or semi-synthetic organic compounds that can be molded into shapes or objects. It has become so popular because it is cheap to make, easy to manufacture, very versatile, and resistant to water. The first fully synthetic plastic (the type of plastic used today is fully synthetic), was created in 1907 by Leo Baekeland (Knight). Once plastic started becoming more and more popular in the early 20th century, people became concerned about its slow decomposition rate and so the approach towards recycling came about.
Plastic does not decompose easily because it is made of large molecules. Plastics are organic, meaning they contain compounds made of the element carbon, and also include nitrogen, sulfur, and chlorine. The key ingredients in plastic are derived from petroleum, natural gas, and coal. The way plastic is made consists of multiple steps. First, these ingredients are broken down into monomers, which are the building blocks of polymers. This is done by heating hydrocarbons in what is called a cracking phase. A catalyst helps to break down the large molecules into smaller ones such as ethane, propane, butane, and other small hydrocarbons. These monomers are then joined together in either addition reactions, which include the breaking of a double bond to add another molecule to a carbon, or condensation reactions, which include the loss of a water molecule to add another molecule to a carbon. Then, chemical additives may be introduced into the plastic polymers for certain functions (Craftech Industries). For example, things such as antioxidants to preserve the plastic, flame retardants, lubricants for flexibility, pigments to add color, antistatic chemicals, etc.

III. What is BPA?

Bisphenol A, abbreviated as BPA, is a chemical that is added to plastic to make it more resilient and durable. BPA is also used in canned foods, printer receipts, CDs and DVDs, and more. The problem with BPA is that it is known to be a xenoestrogen, or a chemical that mimics estrogen, a hormone in the female body. This is known to have adverse effects on life. Many brands that claim to be “BPA-free” have replaced BPA with a similar chemical, BPS, or BPF, which both act in the same way as BPA, and thus are not an adequate replacement for the endocrine disrupting chemical (Figure 1). The way BPA makes its way into the human body is mostly through a human’s diet. In containers made with BPA, not all of the BPA is sealed into the product, so the BPA can mix with the fluid or food in the container, and then make its way
into the body. BPA has a very similar chemical structure to the hormone estrogen, which means it can mimic estrogen once in the body (Figure 1). This means it can bind to estrogen receptors in the body. This is why BPA is called an Endocrine Disrupting Compound (Diamanti-Kandarakis, et al.). Endocrine disruptors have been shown to have effects on both human male and female reproduction, breast development and cancer, prostate cancer, thyroid metabolism, and more (Diamanti-Kandarakis, et. al). By binding to estrogen receptors, it can affect human growth, cell repair, fetal development, and reproduction. BPA may also interact with other hormone receptors such as the thyroid, and alter their function. Thus, the presence of BPA in the human body can affect human health.

Figure 1. Structures of BPA and Estrogen. Figure 1a: BPA chemical structure. Figure 1b: Estrogen Chemical structure. 1c: BPS structure. 1d: BPF structure. The similarity of these structures allows them to interact with estrogen receptors.

IV: Research at SUNY New Paltz: BPA and Planaria

There are various organismal studies that have suggested the deleterious effects of BPA on life. At SUNY New Paltz, chemistry and biochemistry students have researched the effects of BPA exposure on planaria, which are a type of aquatic regenerating flatworm. Previously, the detrimental effects of BPA exposure on planaria were studied, and it was found that it hinders planaria cellular regeneration (Figure 2, below). Prior experiments to detect BPA absorbed by planaria using HPLC showed that amino acids tryptophan, tyrosine, and phenylalanine co-elute with the compound and fluoresce in the same region of the UV spectrum and thus may interfere
with BPA signal detection. Previous experiments also indicated that the planaria absorb BPA and D8-BPA from solutions they are incubated in. In the most recent project, planaria were incubated in deuterated BPA as well as BPA, phenotypic effects were measured, and the endocrine-disrupting chemical was extracted and quantified. Deuterated BPA was used to distinguish BPA present in the organisms from supply chain contamination from BPA introduced to the organisms experimentally. D8-BPA was also used to separate the signal from the amino acids that co-elute with BPA.

Our experiments incubated worms in media that contained BPA, D8-BPA, or neither. They incubated for two days, and then were killed, frozen in liquid Nitrogen, and washed ten times using centrifugation and milliQ purified water to remove any residual BPA/D8 BPA that they had not absorbed into their bodies. Then the worm samples were lyophilized, placed in a 50:50 chloroform:methanol solution to extract the BPA or D8-BPA, dried, and analyzed for the presence of BPA and D8-BPA in the extractions. HPLC with fluorescence detection and GCMS were used to quantify the amount of BPA or D8-BPA present in the worm extractions. It was found that the planaria absorbed the endocrine disrupting compound from their solution, and their regeneration was negatively affected when they were exposed to the endocrine disrupting compounds.

Additionally, HPLC methods were implemented to further separate the BPA and D8-BPA signals on HPLC by decreasing the HPLC pump flow rate, which usually runs at 1.0 mL/minute. The peaks were originally only slightly separated, and data would be easiest to read if the peaks were more separated. Decreasing the pump flow rate has separated the peaks of the BPA and the D8, which helped to interpret data more easily.
A negative control experiment was also completed using silica microspheres and diatomaceous earth to test if the washing procedure is effective at removing physisorbed BPA/D8 BPA. The control experiment has suggested that the washing experiments are successful. The control experiments were done using silica beads and diatomaceous earth, both inorganic compounds that would not absorb BPA or D8-BPA. If the extensive washing procedure is effective, the extracts obtained from these compounds should show negligible BPA or D8-BPA signal in HPLC. These are done by incubating a quantity of the inorganic sample with an equivalent weight to 10 worms in media, BPA, or D8-BPA, and following the same procedure the worms are subjected to. The control experiments suggested that the washing experiment removes most physisorbed BPA and D8-BPA. Overall, this experiment is helping to further an understanding of the relationship between the amount of BPA exposure and the phenotype of Planaria. The materials and methods, results, and conclusions of this experiment are outlined in detail in Part II of this paper.

Figure 2. Growth during regeneration of planaria after a transverse excision across the mid-section then incubation in minute BPA concentrations in planarian media. a) Growth curves for 14 days in varying BPA
concentrations. Growth is unitless due to blastema growth being measured relative to total tissue area. BPA exposure shows diminished regeneration relative to water control. b) Images of planaria on Day 0, Day 7, and Day 11 of incubation. Media planaria regenerates a full head, complete with dorsal eyespots and flanking lateral auricles. BPA and D8-BPA planaria both failed to regenerate a head and failed to survive past Day 11.

V: Other Research, Planaria and BPA:

Because aquatic organisms in freshwater are frequently impacted by waste water chemicals such as pharmaceuticals, endocrine disruptors, and other contaminants, researchers developed an analytical technique to analyze over 40 pharmaceuticals and over 20 endocrine disruptors in freshwater invertebrates. The bioaccumulation of these contaminants in three organisms that lived in a waste-water treatment plant impacted river was studied. The contaminants were extracted from the organisms using sonication, purification by removing phospholipids, and Ultra-Performance Liquid Chromatography with a Mass Spectrometer in tandem to analyze the compounds. The goals of this study were to create a way to analyze the PhACs and EDCs in aquatic invertebrates and to assess persistence, distribution, and bioaccumulation of these contaminants in macroinvertebrate communities exposed to wastewater treatment plants. This specifically was in the River Segre in the Pyrenees, Northeast Iberian Peninsula. The water and invertebrate samples were collected at both 500 meters upstream and 1500 meters downstream from the WWTP in the river. Three different organisms from different families were taken from the river: a gastropod Ancylus fluviatilis, a filterer Hydropsyche spp, and a flatworm Phagocata spp. (Huerta et al.). It was found that diclofenac and ibuprofen, which are non-esteroidal anti-inflammatory medications, and four endocrine disrupting chemicals, BPA, estrone, TBEP, and nonylphenol, were measured in at least one of the taxa studied in
concentrations up to 183 ng/g, dry weight. Thus, the aquatic organisms were uptaking the chemicals from their environment.

VI: Effects of BPA on Human Health

A. BPA and Human Miscarriage and Infertility:

A human study done by Sugiura-Ogasawara and Ozaki examined 45 women who had a history of three or more (3-11) consecutive first trimester miscarriages. They were tested for serum BPA levels, among various other chemicals. 32 healthy women with no history of miscarriage were also examined. It was shown that women who had a history of miscarriages had a 2.59+/-5.23 ng/mL BPA levels in their serum as compared to healthy women with 0.77+/-0.38 ng/mL of BPA in their serum. The P value was 0.024, meaning the results were significant. Higher BPA levels were also associated with the presence of antinuclear antibodies, which are autoantibodies produced by the body when the immune system fails to recognize the difference between self and non self. Overall, it was determined that BPA exposure is associated with miscarriage (Sugiura-Ogasawara et al.).

Additionally, it has been found that women with higher BPA concentrations have proportionally lower egg production, and are therefore less likely to get pregnant. At Massachusetts General Hospital, the urine of women undergoing in-vitro fertilization (IVF) was analyzed for BPA concentration and related to their ovarian response in IVF. The BPA concentration levels were measured using online solid phase extraction coupled with isotope dilution High performance Liquid Chromatography tandem mass spectroscopy. Also, peak serum oestradiol was measured by using an Elecsys Estradiol II immunoassay kit. 84 women undergoing 112 IVF cycles were analyzed and 23 of these women contributed to more than one IVF cycle. The BPA levels ranged from <0.4 to 25.5 ug/L+/-3.2. Peak serum estradiol correlated
with the total number of oocytes retrieved per cycle ($r=0.65$, $p=0.007$). Also for each log unit increase of BPA there was an average decrease of 12% in the number of oocytes retrieved and an average decrease of 213 pg/mL in beak estradiol. So, BPA concentration was inversely associated with the number of oocytes retrieved and peak oestradiol levels (Mok-Lin et al.).

Another study also looked at the effect of BPA on human oocyte maturation in vitro. The study found that there is altered human oocyte maturation at a certain dose of BPA. The experiment was done with patients undergoing in-vitro fertilization (IVF) cycles at Brigham and Women’s Hospital from 2011-2012. An oocyte from one of each patient’s cycles was included in the study, and the oocytes were exposed to media containing different concentrations of BPA for 30 hours. These concentrations included 20 ng/mL, 200 ng/mL, and 20 ug/mL. Their meiotic stage was monitored (meiosis is cell replication). Then, the cells at metaphase II (further state of meiosis) were examined and analyzed for their maturation status. The results of this study showed that as the concentration of BPA increased, the amount of oocytes that degenerated or underwent spontaneous activation increased. Then, among oocytes exposed to BPA that made it to Meiosis II, as the concentration of BPA increased, there was a decreased incidence of bipolar spindles and aligned chromosomes, meaning that replication was not occurring or was slowing down (Machtinger et al).

B. BPA and Male reproduction

Another study was done on couples using IVF in Albany, New York. 27 couples were analyzed by providing their blood serum on the day of oocyte retrieval. From the serum, unconjugated BPA was measured by HPLC with Coularray detection. Researchers also measured embryo quality by ECN--embryo cell number, and EFS--embryo fragmentation score. The results showed that there were no significant differences in the amount of BPA in the serum of
men versus women. The results also suggested that the concentration of BPA in women did not affect their embryo EFS or ECN. However, for men, as the amount of BPA increased, the probability for being fertile decreased. Their odds for higher ECN decreased by 30% there was a 46% decrease in EFS for each log-unit increase in male BPA in serum (Bloom et al.).

Next, a cohort study was done in China comparing BPA levels in male urine to their semen quality. This study took men from 4 regions in China that had high BPA workplace exposure. 218 men with and without BPA workplace exposure were used in this study. Their semen was analyzed for BPA levels. Their results showed that an increase in BPA levels in the urine was significantly associated with four factors pertaining to sperm. Increase in BPA urine levels in men is associated with a decrease of: sperm concentration, total sperm count, sperm vitality, and sperm motility. To get more specific, men with detectable BPA in their urine had over three times the risk of lowered sperm concentration and vitality, over four times the risk of lower sperm count, and over two times the risk of lower sperm motility. BPA did not have any effect on semen volume or sperm morphology (Li et al., Urine and Bisphenol A.).

The same researchers as the previous study also examined the relationship between workplace exposure to BPA and erectile dysfunction in males in China. Workers from BPA exposed factories and control factories were used in the study, and their sexual function was determined through in person interviews. The results showed that workers who have been exposed to BPA have a higher risk of sexual dysfunction. This includes 3.9 times more chance of decreased sexual desire, 4.5 times more erectile difficulty, 7.1 times ejaculation difficulty, and a 3.9 times reduced satisfaction with sex life. Those who worked in BPA factories also reported more reduced sexual function within one year of employment in BPA factories. So, BPA may cause decreased sexual function in men (Li DK et al., Occupational exposure).
C. BPA and Babies

BPA can also affect babies in-utero. A study done in California examined 587 children from families with parents who either did or did not have occupational BPA exposure. The birth weights of their babies were recorded by interviewing the mother. The BPA exposure of the parents was determined by air sampling measurements and exposure history. It was found that maternal exposure to BPA at work during pregnancy is associated with a lower birth weight for the baby. Also, the relationship was dose-specific, being that increased BPA levels during exposure were associated with lower offspring birth weight (Miao et al.).

Additionally, it has been suggested that in-utero exposure to BPA changes the distance between the anus and genitalia (anogenital distance) in human male offspring. They compared the anogenital distance of 56 boys who were the sons of parent(s) who had occupational BPA exposure, and 97 sons of parents who were not exposed to BPA. Linear regression was used to account for the boys’ ages and weights, and after the data was analyzed, it was found that parental occupational exposure to BPA was associated with shortened anogenital distance in the male offspring. The relationship was stronger for maternal BPA exposure, with the p-value being less than 0.01. Additionally, the relationship between anogenital distance in male offspring and maternal BPA exposure during pregnancy was dose-responsive, with the anogenital distance being shorter when the mother was exposed to higher doses of BPA (p=0.008). Therefore, parental exposure to BPA can negatively affect the genital development of male offspring (Miao et al.).

It has also been found that babies who were born from mothers with higher BPA exposure were more likely to exhibit anxiety, hyperactivity, depression, and aggression. First, a study completed in 2009 in Cincinnati, Ohio, examined the effect of prenatal BPA exposure on
child behavior. 249 mothers and their children were studied. Maternal urine was collected throughout pregnancy and at birth. BPA concentration in the urine was measured using High Performance Liquid Chromatography. Additionally, their child’s behavior was analyzed at 2 years of age. It was found that there was a direct relationship between the maternal BPA concentration at week 16 of pregnancy and offspring externalizing behaviors including hyperactivity and aggression. The relationship was stronger for female children than male children (Braun, et al.).

A different study done in 2012 examined 198 African American and Dominican women and their children from pregnancy to 5 years of age. The study measured the children’s exposure to BPA and the child’s behavior. This was done by collecting urine samples from the mother during her pregnancy, and from their children between the ages 3-4 years old to measure the BPA exposure. They analyzed the urine for BPA content. They additionally surveyed the children between ages 3-5 years to analyze their behavior. The survey consisted of testing for behavioral problems, such as emotionally reactive, anxious/depressed, aggression, attention problems, sleep problems, withdrawn, and more. For male children, high BPA exposure in the womb was directly related to more behavioral problems. The highest quartile of BPA exposure for males were 1.62 times more likely to be Emotionally Reactive and 1.29 times more likely to have Aggressive behavior syndrome. For female offspring, higher prenatal BPA exposure had an indirect relationship with behavioral syndromes. The statistically significant categories included Anxious/Depressed and Aggression. Female offspring with the highest quartile of prenatal BPA exposure were 0.75 times as likely to be anxious/depressed and 0.82 times as likely to show aggressive behavior than female children with low BPA exposure. So, this study suggests that
exposure to BPA in the womb can affect child behavior in different ways depending on the sex of the child (Perera, et al.).

\[D. \text{ BPA, Heart Disease, and Type II Diabetes}\]

Some studies suggest that high bodily BPA concentrations are associated with diseases such as heart disease and Type II Diabetes. A study done at the West Virginia School of Medicine analyzed the urine of 1380 people of different races and ethnic backgrounds from the National Health and Nutritional Examination Survey from 2003-2004. It was found that the group of subjects with the highest levels of BPA in their urine (tertile 3) had a 1.5 times higher incidence of hypertension than the group of subjects with the lowest BPA urine levels (tertile 1) (Shankar, A. & Teppala, S.). This data was independent from race. The same researchers did another analysis on subjects’ urine from the National Health and Nutritional Examination Survey from 2003-2008 to determine if BPA concentration affects incidence of diabetes mellitus. The subjects were grouped into quartiles depending on the urinary concentration of BPA, with the fourth quartile being the subjects with the highest concentration. It was found that quartile 4 had a 1.68 times higher rate of diabetes than quartile 1. This data was not dependent on weight (Shankar, A. & Teppala, S. “Relationship”).

Another study done in Shanghai, China, examined the relationship between BPA, obesity, and insulin resistance. In China, 3390 adults over 40 were analyzed. Their urinary BPA levels, BMI to determine obesity, and their insulin resistance were measured. The subjects were separated into quartiles, with the fourth quartile having the highest urinary BPA concentration. It was found that the participants in the highest quartile for BPA concentration were 1.5 times as likely to obese, and 1.37 times as likely to have insulin resistance than the first quartile (Wang et al).
VII. Plastic and the Environment

Plastic additionally has many negative environmental effects. Plastic debris contain BPA as well as other chemical additives. When the plastic particles are ingested by marine animals and other wildlife, it can injure them, block their digestive systems, or poison them. These could all negatively impact the health of organisms (Knoblauch).

Floating plastic in the ocean can also have negative effects on ecosystems. Plastic is not biodegradable, meaning, it can stay intact for thousands of years without breaking down. Floating plastic in aquatic environments can act as transportation devices for invasive species. So, species from one country can migrate to another and disrupt the habitat of the new environment.

Plastic present in landfills can also leach chemicals into soil and groundwater (Knoblauch). This would spread the chemicals in plastic to the bodies of those who drink groundwater. Additionally, it would spread chemicals to plants, trees, and crops growing in the soil, some of which are consumed by wildlife and humans.

VIII: How Do We Fix This?

A. What Can We Do Instead of Recycling?

While recycling is a great start, it is unfortunately not enough to fix the plastic problem. Reducing and reusing are much more helpful than recycling. Recycling is not ideal because it takes a lot of resources and energy to recycle. To recycle, there must be certain resources available such as monitoring recycling collection sites, transportation of recyclables, and the recycling manufacturing process. Additionally, there has to be someone who wants to buy the recyclables. Also, plastic cannot be recycled back into what it originally was. Because plastic is
heat and light sensitive, it degrades when it is broken down to be recycled. Thus, most plastics that are recycled get turned into things like car bumper stickers, textiles, and plastic lumber, all of which are not recyclable (Somerville).

There are many ways to reduce plastic use. People can start with using reusable items instead of single-use plastics. For example, people can buy reusable razors instead of disposable ones, carry reusable coffee cups instead of getting a disposable one, use cloth reusable grocery bags at the supermarket, wash and reuse plastic silverware and cups, buy used items, and buy food in bulk rather than individually packaged (M. Zara). Metal straws and water bottles are effective replacements for plastic as well. Another way to reduce plastic waste is to avoid fast fashion and instead buy clothes that last longer. Buying cheap clothes can contribute to the large amount of plastic waste because they degrade faster. Many clothes are made of plastic materials, such as nylon, polyester, and acrylic. When the cheap clothes are worn out and thrown away, they will release plastic particles into the environment. Also, some sneaker companies are using repurposed plastics to make recycled shoes. While this is a good effort to recycle, when these shoes are thrown away, they will again release plastic into the environment. The best way to rectify this would be to buy clothes that last longer, so they won’t be thrown out as often (McCarthy & Sanchez).

B. Plastic Alternatives

Many scientists and companies are exploring more environmentally friendly ways of making plastics, or ways of implementing plastic alternatives. First, plant based plastics, also known as bioplastics, are being made from plant sources such as corn or potatoes. These are more biodegradable and less harmful to the earth because they can help return nutrients to the earth when they degrade. For example, researchers at Lund University in Sweden have invented
a plastic made of potato peelings and water that biodegrades in two months. It can be used to make utensils and straws. A mixture of hot water and warm potato starch is combined, poured into a mold, and cooled and set in a fridge. The potato peels are taken from fast food companies or supermarkets who deemed them unfit to sell (Back to the Future).

Additionally, Skipping Rocks lab in the UK came up with Ooho, a replacement for plastic water bottles. They have created edible water bubbles made of seaweed. The pod is inserted into one’s mouth and it bursts, releasing the water. Their manufacturing process is more efficient and cheaper than the production of plastic bottles. The packaging degrades in 6 weeks (Back To The Future).

Palm leaves are another good alternative for plastic, specifically, for plastic packaging. Instead of groceries being packaged in plastic, they can be wrapped in palm leaves from an areca palm tree. The palm leaves can be molded into a desired shape. This is an environmentally friendly alternative to plastic packaging because the leaves are natural and biodegradable.

Additionally, a Floridian Brewery called Saltwater Brewery has created plastic 6-pack rings that are biodegradable, compostable, and edible. They are made from by-products from their beer brewing process. Their ingredients include wheat and barley. The rings would not be detrimental to marine wildlife because they are edible (Back to the Future).

Mushrooms can be used as a plastic replacement in the future. Mushrooms are made up of hyphae, which are a network of filaments. They can be grown in agricultural waste and grow together within the material. The material can then be molded into any shape, thus they could take the place of many plastic items. The natural polymers adhere together like glue. The fungi are then baked at temperatures to make them inert so they cannot grow again (Bernhard).
IV: Conclusion:

BPA in plastics has been demonstrated to have a variety of detrimental human and wildlife health effects. Additionally, plastics in the environment are physically and chemically dangerous to habitats and wildlife. Because of the damage that plastic and the chemicals added to plastic are causing, there must be a reduction of plastic use and a change in the way products are made. There are many ways to easily reduce plastic usage that must be implemented to help reduce the dangers plastic can cause. Natural plastic alternatives must be implemented to replace the use of plastic with something more environmentally and health friendly.
Work Cited


Part II-Lab Report: Extraction and Quantification of BPA from Planaria

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**Abstract:**

Bisphenol A (BPA) is used to make plastics and has been found to be a xenoestrogen. The experiment described involves exposing planaria (*Girardia tigrina*), which are small regenerating flatworms, to BPA and deuterated BPA (D8-BPA). Phenotypic effects of BPA on the planaria were recorded during exposure and BPA was extracted following an incubation period to quantify the amount absorbed by the flatworms. Deuterated BPA (D8-BPA) was used in addition to BPA to distinguish added BPA from BPA already present in the organism from supply-chain contamination. A control experiment with silica microbeads and diatomaceous earth tested whether the multiple washes performed after incubation removed physisorbed BPA from planaria. Additional analysis was performed using high performance liquid chromatography (HPLC, reverse phase). Improvements in HPLC method development to analyze BPA and D8-BPA resulted in better separation between BPA and D8-BPA peaks. Gas chromatography mass spectroscopy (GCMS) is also used to quantify BPA/D8 BPA. It is concluded that planarian regeneration is negatively affected by exposure to 20 μM concentrations of BPA/D8-BPA, planaria absorb BPA and D8-BPA from solution, the washing method removed most of the physisorbed BPA, and method development involving decreasing the HPLC pump flow rate by 75% separates the D8-BPA and BPA peaks by over 20%.

**Introduction:**

Plastic is used heavily by humans in items such as “bicycle helmets, child safety seats, airbags in automobiles, cell phones, televisions, computers, other electronic equipment, roofs, walls, flooring, insulation, food packaging and utensils” (1). Bisphenol A (BPA), is a chemical commonly added to plastic to improve its durability. BPA has different known effects on planarians at different concentrations. At levels below 100 ppb, it acts as an endocrine disrupting chemical, at levels from 300-500 ppb to 1 ppm, it inhibits planaria regeneration, and above 1 ppm, it causes planarian death (2). Additionally, in previous experiments at SUNY New Paltz, it was found that planaria incubated in 20 μM BPA and D8-BPA failed to regenerate. Prior experiments to detect BPA absorbed by planaria using HPLC showed that amino acids tryptophan, tyrosine, and phenylalanine co-elute with the compound and fluoresce in the same region of the UV spectrum, and thus may interfere with BPA signal detection (3).

Overall, this experiment will help to further an understanding of the relationship between BPA absorption and the phenotype and behavior of planaria (*Girardia tigrina*), which may suggest a potential relationship between BPA exposure and the health of humans, considering their immense use of plastics. The goals of the experiments outlined were to extract and quantify absorbed BPA and D8-BPA in planaria, determine the effectiveness of worm washing
experiments at removing physisorbed BPA/D8-BPA, and increase separation of the BPA and D8-BPA peaks on HPLC. The future goal of this experiment is to correlate BPA concentration in planaria tissue with BPA exposure time.

Deuterated BPA (D8-BPA) is a deuterated form of BPA in which its carbon-bound hydrogens are replaced with deuterium atoms. By using D8-BPA, we expect to distinguish and quantify absorbed D8-BPA via experimental exposure from BPA retained via supply chain contamination in the planaria using HPLC and GCMS. Additionally, using D8-BPA helps to avoid signal disruptions of amino acids since it will have a slightly different signal in HPLC than the BPA and the amino acids.

Control experiments were performed using silica microbeads and diatomaceous earth. Because they are both made from inert inorganic compounds, they cannot absorb BPA. They also both have much larger surface area than the planaria flatworm. Silica beads are spheres, and diatomaceous earth comes from the skeletons of diatoms, which have many crevasses. Thus, silica beads have a higher surface area than the flatworm, and the diatomaceous earth has a greatly higher surface area than the flatworm. The inorganic substances are subjected to the same procedure as the worms. If the washing experiment is effective at removing most of the physisorbed BPA and D8-BPA, the extracts obtained from the control samples should show negligible BPA or D8-BPA signal in HPLC and GCMS.

**Methods:**

**I: Planaria Media**

A 47 L stock of media that mimics the planarian natural environment was made for all of the planaria to live in. Additionally, a separate 2L stock of media was made for planaria incubation experiments. For the 2 L stock, the following salts were dissolved in milliQ purified water from a Direct-Q 5UV-R filter: 0.187 g NaCl, 0.222 g CaCl$_2$, 0.2407 g MgSO$_4$, 0.0407 g MgCl$_2$, 0.0149 g KCl, and 0.2016 g NaHCO$_3$. All media stock and solutions were stored at 4°C to preserve and protect them from light, which degrades BPA and D8-BPA.

For 47 L carboy stock, deionized (DI) water was used instead of milliQ purified water. The masses used for the 2 L stock were multiplied by 23.5 to obtain the masses of reagents necessary for the 47 L media solution. To make sure the reagents dissolved, 2 L flasks were filled with DI water, and one reagent was placed in each DI water-filled flask. The dissolved contents were then poured into the 47 L carboy. DI water was then added to the same flask, swirled around, and poured into the carboy 2-3 times. Once all of the dissolved reagents were poured into the carboy, it was filled to volume with DI water by pouring 2 L flasks full of DI water into the carboy.

**II: Worm care**

Planaria were purchased from Carolina Biological Supply Company. When they were received from Carolina, they were acclimated to the laboratory. They were first put into a 50% planaria media solution (from the carboy in section I) which was diluted with the solution they were shipped in. Their solution was gradually increased to 100% planaria media from the 47 L
Planaria were fed with ground beef liver twice a week and were starved for at least 10 days prior to incubation experiments—their containers and media were also cleaned and changed on the following day after each feeding.

**III: BPA and D8-BPA stocks:**

10 mM Solutions of BPA and D8-BPA were made. For the BPA solution, 11.4 mg of BPA was added to an empty 5 mL volumetric flask and filled to volume with DMSO. A 10 mM stock solution of D8-BPA was made in a similar manner using 11.8 mg of D8-BPA in an empty 5 mL volumetric flask and filling it to volume with DMSO. The flasks were inverted to mix. Two 500 mL volumetric flasks were obtained, and were labeled as either 20 μM BPA or 20 μM D8-BPA. 1.00 mL of the respective 10 mM solution from the 5 mL flask was pipetted into the 500mL volumetric flask. The flasks were then filled to volume with the 2L planaria media stock.

**IV: Worm Incubation**

A dish of worms that had been starved for one week and transversely cut with a scalpel by other students was obtained. Three plastic Petri dishes were labeled on the cover and the bottom. One was labeled “Media”, the other labeled “20 μM BPA”, and the last “20 μM D8-BPA”. Using a disposable plastic Pasteur pipet, from the starved worm dish, 3 of the largest worms in the dish, 4 worms about 70% of that size, and 3 worms about 40% of the largest size were added to each labeled Petri dish (10 worms total in each dish). After the worms were placed in the dishes, remaining liquid was removed from the plastic Petri dishes using separate thin-tipped disposable plastic Pasteur pipets. Any remaining liquid was dried with a clean KimWipe without touching the worms. Approximately 30mL of media, BP solution (in media), or D8 solution (in media) was poured into the respective worm-containing dishes. The covers were placed on the Petri dishes and placed into an opaque plastic bin with a lid. The worms were left to incubate for two days.

While the worms were incubating, they were observed for behavioral and phenotypic changes. After two days, the amount of worms that remained were counted and recorded, along with any phenotypic differences. Using a disposable plastic Pasteur pipet, worms from the three Petri dishes were transferred to three labeled Kimble 30 mL glass centrifuge tubes. Using the thin tipped disposable plastic Pasteur pipet, any remaining liquid in the tube was removed and transferred back to the appropriate Petri dish. These solutions were later lyophilized and analyzed using GCMS by another student.

The 30 mL glass centrifuge tubes (without caps) were immersed halfway into a thermoflask containing liquid nitrogen. The tubes were placed into the liquid N₂ on an angle to prevent the glass from cracking and left until the material inside the tube had frozen. The tubes were left for a few minutes to defrost and re-capped. (See Figure 1)

**V: Washing worms**

Paper towels were laid on the lab bench to prevent contamination. A Sorvall RC5C Plus centrifuge was powered on and set to SS 34 code 05, 9500 RPM. Any labels on the 30 mL
centrifuge tube were moved to the caps of the tubes. The paper towels were labeled in three sections: Media, BPA and D8-BPA. Three corresponding disposable plastic Mohr pipets were labeled and placed in each section. A 50 mL beaker was filled with milliQ water from a milliQ stock container. 10mL of milliQ water was poured into a graduated cylinder and poured into each worm tube without touching the glass together. The tubes were capped and vortexed for several seconds using a Daigger Vortex Genie 2. The three tubes were placed in sleeves into the centrifuge rotor and another blank tube filled with 10mL of milliQ water was used to balance the instrument. The tubes were spun for 5 minutes in the Sorvall RC5C Plus centrifuge at 9500 rpm at 4°C. After 5 minutes, the water was removed using the corresponding Mohr pipets and placed into an empty glass waste container. The tubes were re-filled with 10 mL of fresh milliQ H2O with the graduated cylinder and the washing process was repeated 9x for a total of 10 washes. To keep track of the washes, a tally mark was recorded after each completion. After the 10th wash, the last of the water was removed with the designated pipets and dispensed into the waste container (Figure 1).

VI: Lyophilizing worms

Immediately after washing was completed, the tubes were labeled according to their solution and the labeled caps were removed from the tubes. The tops of the tubes were then each wrapped with Parafilm and 3 holes were poked into the top of the Parafilm using a syringe needle. The worm pellets were re-frozen using liquid nitrogen in a thermoflask. The frozen tubes were placed in a rack and lyophilized in a Millrock Technology Lyophilizer for 24 hours in the dark.

VII: Sample Preparation Prior to Analysis

After lyophilization was completed, Parafilm was removed from the tubes and 10 mL of freshly made 50:50 chloroform:methanol mixture was added to each tube. This solvent mixture was used to extract BPA or D8-BPA from the worm pellet. The samples were capped and vortexed for several seconds. The capped tubes were then Parafilmed and placed back into centrifuge sleeves. The tubes in sleeves were placed in a rack and the tubes were taped securely into the rack. The rack containing the taped 3 tubes were placed in an Innova 4330 Refrigerated Incubator shaker (New Brunswick Scientific) at 480 RPM at room temperature for about 72 hours.

After 72 hours, the rack was removed, and the tape and parafilm was removed from the tubes. The capped tubes were placed into the Sorvall centrifuge with a blank to balance the rotor. They were spun at 9800 RPM for 2 minutes. Nine 3 mL glass Kimex test tubes were labeled, 3 for each solution. Rubber rings were placed a little more than halfway up the Kimex tubes and taped onto the tubes. Approximately 3 mL of the supernatant was transferred into each of the three tubes using a micropipettor. The tubes were then placed into an Eppendorf Concentrator in the dark to evaporate the solvent. Solution from Kimex tubes containing from similar samples was combined before the evaporation was complete. Because previous HPLC experiments
provided poor separation between BPA and D8-BPA signals, GCMS was used by another student to analyze the amount of BPA and D8-BPA extracted from the planaria (Figure 1).

**Figure 1: Schematic Diagram of worm experiment.** This figure provides a visualization of the steps of the worm experiment from incubation to GCMS analysis. RPM=rotations per minute.

**VIII: Negative Control Experiment:**

The mass of 10 planaria had previously been found to be approximately 1.6 mg. To obtain the same mass of inorganic sample, 1.6 mg of diatomaceous earth (DE) was placed into a plastic 1.5 mL microcentrifuge tube. Additionally 15.36 µL of vortexed silica beads (SB) were micropipetted into a microcentrifuge tube. The silica beads contain 8.528 x 10⁹ microspheres/gram, and 8.882 x 10⁸ microspheres/mL. To obtain the equivalent of 1.6 mg, 15.36 µL of the beads were used. This volume of bead solution or mass of DE was added to six tubes total, three containing 1.6 mg DE and three containing 15.36 µL of SB. The silica beads were spun in an Eppendorf Centrifuge 5415 D and the supernatant was removed. The SB samples were then washed 3x with 1 mL of milliQ water. The DE samples were washed 10x with 1mL of milliQ water and then set off to the side. Then, 1mL of 1M NaCl was added to each of the SB tubes. The sodium ions helped to remove the ammonium groups bound to the silica beads that were present in their original solution. Sodium ions have a stronger attraction to the negatively charged silica beads surface compared to the ammonium ions. The tubes were vortexed for several seconds and placed into an Eppendorf Thermomixer R at 99º C for 24 hours at 1500 rpm. After 24 hours, the SB tubes were centrifuged and the supernatant was discarded. The SB tubes were then washed with milliQ water 10 times. All six tubes were then placed in an evaporator to dry the samples. After evaporation was complete, all tubes were incubated in 1mL of either
planaria media, 20 µM BPA, or 20 µM D8-BPA using a micropipettor and the stock solutions made previously. The incubating tubes were wrapped in aluminum foil to prevent BPA photo degradation, and placed on a spinning wheel for two days. Following incubation, the six samples were washed 10 times with 1 mL portions of milliQ water, followed by evaporation to dry the samples. A 1 mL portion of freshly made 50:50 chloroform:methanol was micropippeted into each following a similar procedure used with the worm pellets to mimic the extraction step. The samples were again vortexed, covered with aluminum foil and spun on a wheel for two days. After two days, the tubes were centrifuged, the supernatants were transferred into clean and labeled microcentrifuge tubes, and the extracts were evaporated to dryness. Samples were prepared for GCMS analysis (by another student) to detect and quantify any BPA or D8-BPA (Figure 2).

**Figure 2:** Schematic Diagram of Silica Bead Experiment. This figure provides the various steps of the control experiment using silica beads (SB) and diatomaceous earth (DE).

**IX: Analysis of control solutions containing BPA or D8-BPA**

Various BPA, D8-BPA, and combined BPA and D8-BPA solutions were made in acetonitrile in HPLC microvials. BPA or D8-BPA solution concentrations were 10, 30, and 50 nM, and the concentrations of solutions containing combined BPA and D8-BPA were all 10 nM in BPA and either 1, 15, 30, 45, 60, or 80 nM D8-BPA.

Samples were loaded into a ThermoScientific Dionex Ultimate 30000 HPLC with a 70:30 Optima Grade acetonitrile:HPLC grade H2O mobile phase. Samples were run three separate times with three different flow rates of 1.0 mL/minute, 0.5 mL/minute, and 0.25 mL/minute. The BPA and D8-BPA retention times and chromatograms were compared for each run to determine the improvement in separation between the BPA and D8-BPA peaks.
Results and Discussion:

Our results suggest that planaria behavior is impacted by BPA and D8-BPA. Behavioral observations during worm experiments showed that worms incubated in BPA or D8-BPA caused the worms to enter a dormant state where they had reduced movement and were grouped together. This is thought to be a stress response of the planaria to the endocrine-disrupting chemical. The results also indicate that planaria absorb BPA and D8-BPA from their media. Additionally, our results suggest that the washing experiment is effective at removing the majority of physisorbed BPA and D8-BPA from the inorganic samples. Therefore, signals from these compounds following analysis of the worm extracts is most likely from absorption. Lastly, the results suggest that decreasing the HPLC pump flow rate can help to separate the BPA and D8-BPA peaks.

Figures 3 and 4 show the GCMS results for the first of four separate worm experiments performed. The results show that the worms incubated in BPA and D8-BPA absorbed the compound from their solution. Intensity from mass spectra were used to quantify the amount of BPA or D8-BPA present in each extract. Normalized signal from the worm extract incubated in BPA shows a significantly higher value at the mass of BPA compared to other worm extracts from incubation in D8-BPA or just media. Additionally, this signal was significantly above the background noise level from the mass spectrometer, signifying that there was more BPA in the BPA worm extract than the other extracts, and that this amount was significant. It is most likely that these worms absorbed some of the BPA from the solution. Additionally, Figure 4 shows the GCMS results of the extracts from analysis of the D8-BPA mass signal. The media and BPA extracts show negligible signals at this mass, whereas the D8-BPA worm extract shows a much larger signal. This signal is additionally above the level of background noise. This suggests that the worms absorb D8-BPA from their solution.

![Mass spectrum intensity from BPA normalized to 2PP](image)

**Figure 3:** GCMS analysis of Worm Extracts at the mass of BPA. 2PP=2 phenyl phenol, an internal standard added to each sample. Both BPA and D8-BPA are normalized to the 2PP signal.
**Figure 4**: GCMS of Worm Extracts at the mass of D8-BPA. 2PP=2 phenyl phenol, an internal standard added to each sample. Both BPA and D8-BPA are normalized to the 2PP signal.

Both figures 5 and 6 display the GCMS results for the silica and DE control experiments. Figure 5 shows the GCMS signals at the mass of BPA, and Figure 6 shows the GCMS signals at the mass of D8-BPA. While it appears for both of these figures that the beads incubated in BPA/D8 solutions have appreciative BPA/D8 signals, they are all below the level of background noise determined for the instrument. Thus, these signals are negligible, and it can be determined that the washing experiments are successful in removing the majority of physisorbed BPA and D8-BPA from the surface of the SB/DE.

**Figure 5**: Silica Control Experiment GCMS results at the mass of BPA. 2PP=2 phenyl phenol, an internal standard added to each sample. Both BPA and D8-BPA are normalized to the 2PP signal.
Figure 6: Silica Control Experiment GCMS results at the mass of D8-BPA. 2PP=2 Phenyl Phenol. This compound is run in GCMS and all signals are normalized to its signal.

Figures 7 and 8 show results obtained through modifications made to the HPLC method during analysis of BPA or D8-BPA. An indirect relationship was observed between HPLC pump flow rate and retention time of the analyte. As the pump flow rate was decreased, the difference in retention time of BPA and D8-BPA increased. The pump flow rate for the HPLC was originally run at 1.0 mL/minute, which initially gave poor separation between the BPA and D8-BPA peaks. The flow was then decreased to 0.5 mL/min, and then 0.25 mL/min. It was found that decreasing the pump flow by 75% separated the peaks by almost 22% (Figure 7). This was determined by recording the retention times of the BPA and D8 peaks and finding the difference, and comparing it for the different pump flow rates. The original amount of separation between the two peaks at 1.0 mL/min was on average about 0.0197 minutes. When the samples were run at 0.25 mL/min, the separation increased to 0.0913 minutes. When the pump flow rate was decreased, there was a greater separation between the peaks because the mobile phase moved more slowly through the column, and the D8-BPA has a higher affinity for the AcN:H2O mobile phase compared to the BPA. So, the D8-BPA will be moving faster throughout the column since it has a higher affinity for the mobile phase, and thus it will elute first. The BPA would be moving slower throughout the column since it has a lesser affinity for the mobile phase, and thus it would be eluted later, increasing the separation between the two signals. (Figures 7,8).
Figure 7: Chromatogram Displaying Retention Time Separation. The chromatograms of the different HPLC runs were extracted from Chromeleon and graphed in Microsoft Excel. The x-axis was adjusted accordingly so the chromatograms could be overlaid and separation could be visualized.

Figure 8: HPLC Flow Rate and Difference in BPA and D8-BPA retention time. The figure displays an indirect relationship between BPA/D8-BPA difference in retention time and HPLC pump flow rate.

Conclusions and Summary:

Overall, it has been determined that regenerating planaria are negatively affected by incubation in 20 μM concentrations of BPA and D8-BPA. They absorb the endocrine disrupting chemical from their solution. Additionally, extensive washing of the worm pellet with milliQ water is effective at removing physisorbed endocrine disrupting compound. Lastly, decreasing the HPLC pump flow rate increases the separation between the BPA and D8-BPA retention times. In the future, we would like to determine the relationship between the quantity of BPA retained and phenotype. We would also like to experiment with the HPLC to obtain better
separation between the BPA and D8-BPA signals. We hope to do this by performing a gradient of AcN:H2O in which we change the ratio of the components of the mobile phase to improve separation. We additionally hope to incubate planaria in sub-nanomolar concentrations of BPA and D8-BPA. Although the GCMS isn’t capable of detecting at that low of a concentration, the fluorescence detector on HPLC is. Once we determine a HPLC method that improves the separation between the BPA and D8-BPA peaks, this experiment can be started.

References: