ToxEHS

Histopathological Effects of Bisphenol A on Soft Tissues of *Corbicula fluminea* Mull.

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Received 7 August 2018 / Received in revised form 5 January 2019
Accepted 11 January 2019
DOI 10.1007/s13530-019-0386-4

pISSN : 2005-9752 / eISSN : 2233-7784
Toxicol. Environ. Health. Sci. Vol. 11(1), 36-44, 2019

Abstract

Objective: Bisphenol A (BPA), a commonly occurring industrial chemical that is present in polycarbonate plastics and epoxy resins is mechanistically shown to affect various bodily functions of organisms. However, very limited studies have been done on the histological effects of BPA on bivalves. In this study, the toxicity of BPA was analyzed through its histological effects on the gills, digestive glands and adductor muscles of *Corbicula fluminea*, a freshwater bivalve.

Methods: Forty *C. fluminea* were exposed to set-ups with 1 μg/L, 2 μg/L and 3 μg/L of BPA for twenty-one days. Afterwhich, histolopathological analysis were done in the adductor muscles, digestive glands and gills of the clam. Histological alterations such as vacuolations, necrosis, lamellar deformation, hyperplasia, loss of epithelium, necrosis, tubular alteration, neoplasia, hemocyte infiltration, hypertrophy and pyknosis were observed and percent histological aberrations were determined per organ.

Results: Results showed that there was a significant difference in the histological alterations observed between the tissues of exposed and unexposed clams. Moreover, varying concentrations of BPA rendered differential degree of histological damage on the soft tissues of the clam. The digestive gland was the most affected tissues followed by the gill then the adductor muscles.

Conclusion: BPA were found to be toxic to *C. fluminea* as evidenced by histology. Moreover, the differential histological responses of the tissues of *C. fluminea* in different concentrations of BPA proves that they are good indicators of environmental stressors such as BPA.

Keywords: *Corbicula fluminea*, Histology, Adductor muscles, Digestive gland, Gills, Bisphenol-A

Introduction

Bisphenol A (BPA) is a chemical commonly used in the production of polycarbonate plastics and epoxy resins- both of which are utilized in applications that makes our lives easier and convenient[1]. Polycarbonate plastics are used in compact discs, medical devices as well as food and beverage containers. Epoxy resins on the other hand, are used to coat metal products such as food cans and pipes of water supply[2]. BPA enters freshwater ecosystems through effluents from sewage treatment plants, plastic factories and landfill leachate. According to Crain *et al*.[4], BPA concentration in freshwater ecosystems may vary and can reach up to 21 μg/L with the sediments containing an even greater concentration compared to the water column. BPA quickly disperse and disappear from natural water systems and this is highly attributed to adsorption by suspended materials and sediments as well as absorption of aquatic animals and plants[1,5]. BPA is a toxic compound and its effects on vertebrates and invertebrates have been thoroughly described[1,3,6,7]. Mechanistically, it has been shown to affect lipid peroxidation, endocrine function and production of reactive oxygen species in vertebrate and invertebrate organisms[1,8]. However, bibliographic data report limited information about the histological effects of BPA on mollusk in particular the bivalves, which are considered as suitable organisms for biomonitoring purposes due to their filter-feeding behavior, sessile status and ability to concentrate pollutants to several orders of magnitude above ambient levels in aquatic environmental[9]. Moreover, histological effects of this compound is only limited to the gonads of invertebrates and not on gills, digestive lands and adductor muscles which are also essential in their survival[11]. The digestive gland

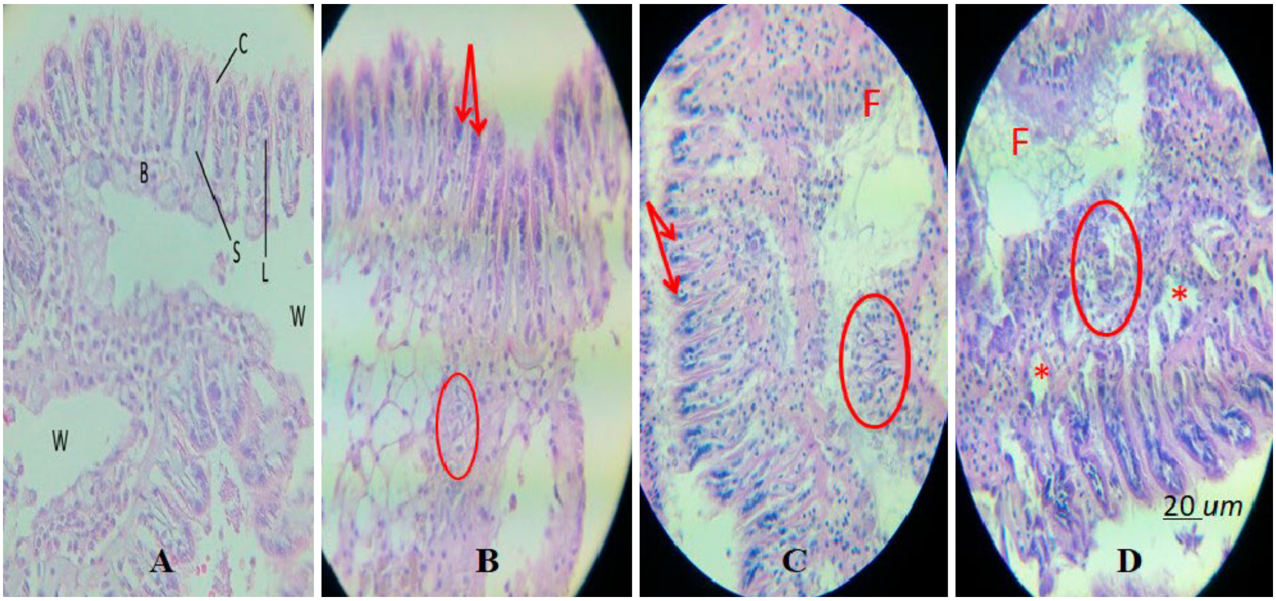


Figure 1. Photomicrographs of gills in control and BPA-exposed *C. fluminea* clams after 21 days. (A) Unexposed control clam showing regular arrangement of the gill filaments with a single layer of epithelial cells (C) supported by skeletal rods (S) forming a well-defined lamellae (L). (B): Clams exposed to 1 µg/L BPA; (C) Clams exposed to 2 µg/L; and, (D) Clams exposed to 3 µg/L BPA. Circle = hypertrophy; F = fibrosis; arrow = hyperplasia; asterisk = necrosis.

being involved in pollutant detoxification, homeostasis and bioaccumulation[11-15]; the gills are use for respiration and filtration[12-15]; and, the adductor muscles are involved in regulating the opening of the valves of clams[12-15]. This study would like to determine the histological effects of Bisphenol A on the tissues of the gills, digestive gland and adductor muscles of *Corbicula fluminea*. Specifically, it aims to to analyze the histological effects of BPA on the different organs of *Corbicula fluminea*; to compare the histological effects of BPA between the three treated Corbicula fluminea set-ups and to know which among the organ is the most and least affected.

The Asiatic clam or *Corbicula fluminea* was utilized in this study, because of their wide capacity of adaptation to extreme environment. Its wide distribution, high density, long lifespan, sedentary and filter feeding behavior, phenotypic plasticity and physiological tolerance to abiotic changes and sensitivity to various pollutants qualify them as a good sentinel organism for monitoring the status of aquatic environment[8,16-20]. Moreover, the ease in maintaining them in laboratory conditions coupled with their ability to bioaccumulate and biomagnify several contaminants make them a very convenient and highly qualified model organism for ecotoxicological studies[21-30]. The exposure of the clams to environmentally-relevant concentrations of 1 µg/L, 2 µg/L and 3 µg/L BPA[30,31] under controlled laboratory conditions aims to give data on the actual histological effects of this compound on the adductor muscles, digestive gland and gills of the test organism. This organism is a frequent food item to locals especially to lactating mothers as it contains appreciable amount of Vitamin B12 and has high calorific and glycogen content compared to any shellfish[32-34]. Thus, possible effects of the compound in the test organisms may also mirror its effects on humans.

Results

No mortality was observed both in the control and experimental groups during the 21-day exposure.

Gill Histology

Figure 1A shows the optimal histological characteristics of the clam gills. This is characterized by a well-defined lamellae made up of skeletal rods which supports a single layer of ciliated epithelial cells. Figure 1B, C on the other hand, show the alterations brought about by varying concentrations of BPA on the tissues of the gills of *C. fluminea*. Common histological aberrations that were seen in the exposed groups include vacuolation, hypertrophy, hyperplasia, lamellar deformations and necrosis. Occurrences of other alterations such as loss of epithelium, lipofuscin aggregations, fibrosis and hemocyte infiltration were also observed as concentration increases.

Figure 2 presents the mean percent aberrations of the different groups both in cellular and morphological changes and neoplasia. Under cellular and morphological changes, a dose dependent response was observed with clams exposed to 3 µg/L BPA having the highest damage of 73.29% followed by groups with 2 µg/L, 1 µg/L and unexposed group with percent aberration of 58.31%, 36.61% and 12.99% respectively. In neoplasia, clams exposed to 3 µg/L BPA registered the highest percentage of 20.02%, while a 17.99%, 16.47% and 10.21% neoplasia was seen in 1 µg/L, 2 µg/L and unexposed clams respectively. Upon subjecting the values to statistical analysis, results showed that all groups are statistically significant on cellular and morphological patterns while no significant difference were seen in neoplasia.

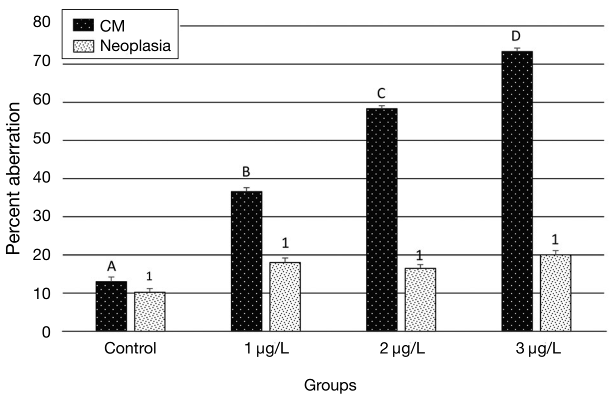


Figure 2. Mean percent aberration ±SEM of gill tissues on cellular and morphological changes (CM) and neoplastic reaction patterns. Differences in symbols would mean significant difference at $p < 0.05$ using Tukey's post hoc test.

Digestive Gland Histology

Figure 3A shows the normal digestive gland of clams from the control group. In its normal state, the digestive gland shows intact digestive tubules lined by epithelial and secretory cells that are columnar and surrounded by a well-defined basement membrane. The tubules are uniform in sizes and are regularly spaced. Moreover, intertubular spaces consist of connective tissues without significant signs of neoplasia and fibroma. Figure 3B-D illustrates the effects of a 21-day exposure of clams to different concentrations of BPA. The tubules of the digestive glands of the treated groups exhibited high instances of hyperplasia, hypertrophy and necrosis. Moreover, few incidences of granulocytoma, fibrosis, neoplasia and lipofuchsin aggregation were also observed in the digestive gland.

Figure 4 presents the mean percent aberration in tubular and intertubular areas of the digestive gland as well as the occurrence of neoplasia. Clams exposed to 3 µg/L BPA had the highest percent aberration across all areas and high incidences of neoplasia while the unexposed

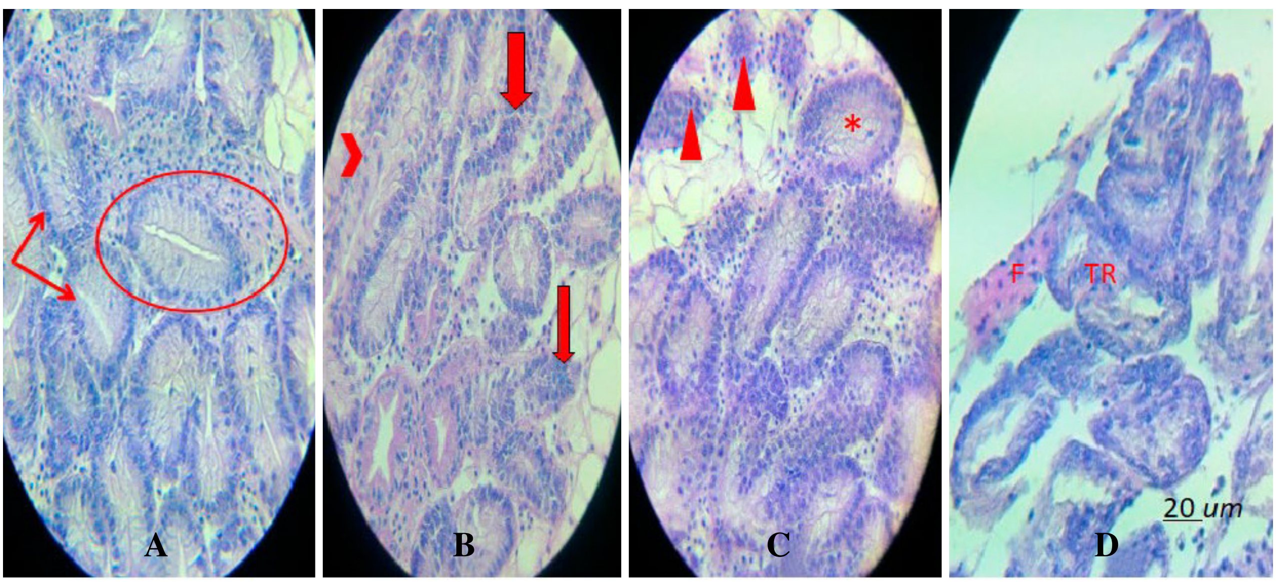


Figure 3. Photomicrographs of digestive gland in control and BPA-exposed *C. fluminea* clams after 21 days. (A) Unexposed control clam showing intact digestive tubules (encircled), surrounded by regular epithelium (thin arrow); (B): Clams exposed to 1 µg/L BPA; (C) Clams exposed to 2 µg/L; and, (D) Clams exposed to 3 µg/L BPA. Arrowhead = hypertrophy; thick arrow = hyperplasia; triangle = granulocytoma; F = fibrosis; asterisk = necrosis; TR = tubular regression.

clams had the lowest percent aberration. Clams in 3 μg/L registered a percent aberration of 78.03% in the tubular areas, 30.8% in the intertubular areas and 38.44% neoplasia. This was followed by clams exposed to 2 μg/L with a percent aberration of 74.23%, 27.47% and 33.58 % in tubular, intertubular and neoplasia. Following the 2 μg/L are the clams exposed to 1 μg/L registering a value of 60.96%, 25.86% and 27.85% respectively. The unexposed clams had the lowest aberration of 17.86% in tubular areas, 11.74% in intertubular areas and 25.68% occurrence of neoplasia. Statistical analysis were again performed to know whether values are statistically significant. Based on Figure 4, there exist a significant difference between the exposed and unexposed clams but no significant difference was seen across tissues of clams exposed to various concentrations of BPA.

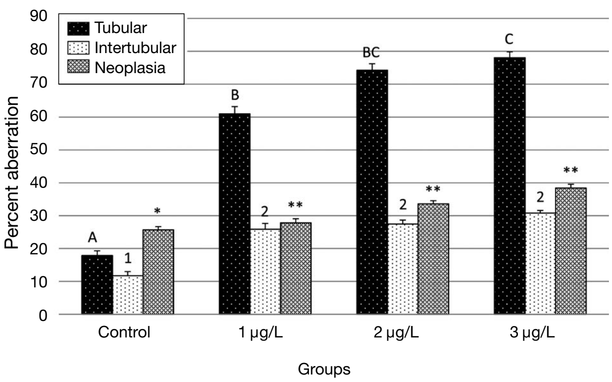


Figure 4. Mean percent aberration ±SEM of digestive gland tissues on tubular, intertubular and neoplastic reaction patterns. Differences in symbols would mean significant difference at $p < 0.05$ using Tukey's post hoc test.

Adductor Muscle Histology

Figure 5A shows the adductor muscles of clams from the control group. They displayed muscle fibers that are uniformly organized and adhering with each other. The cells are fusiform in shape with a central nucleus. Figures 5B-D illustrates the effect of varying concentration of BPA on the adductor muscles. Histological examination of the tissues revealed the occurrence of necrosis, loss of surface adherence and loss of muscle organization among the exposed group.

Figure 6 illustrates the mean percent aberration seen in adductor muscles of clams under different groups. Clams exposed to 3 μg/L BPA had the highest percent aberration of 55.32% and 14.77% followed by 1 μg/L group with a value of 50.55% and 13.3% then the 2 μg/L group with a value of 49% and 12.91. Meanwhile, the unexposed clams has the lowest percent aberration value of 25.11% and 10.63% in both cellular changes and neoplasia. Similar to the gills and digestive gland, statistical analysis was performed to determine whether there is a significant difference between groups. Results revealed that only the unexposed group is different.

Discussion

Different organs have varying responses to different

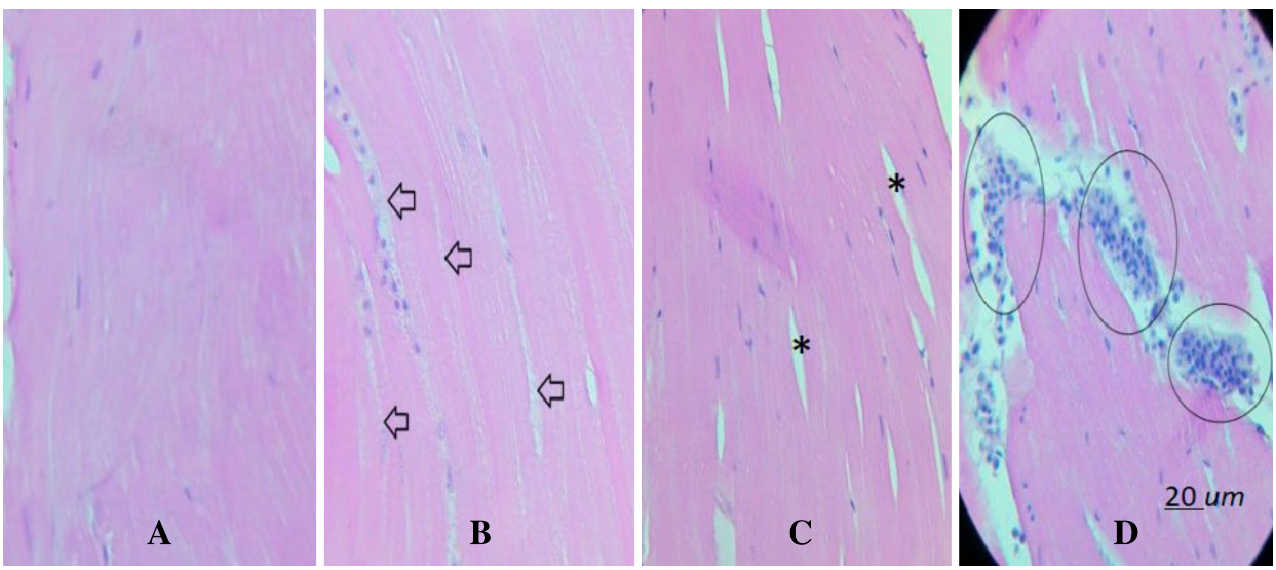
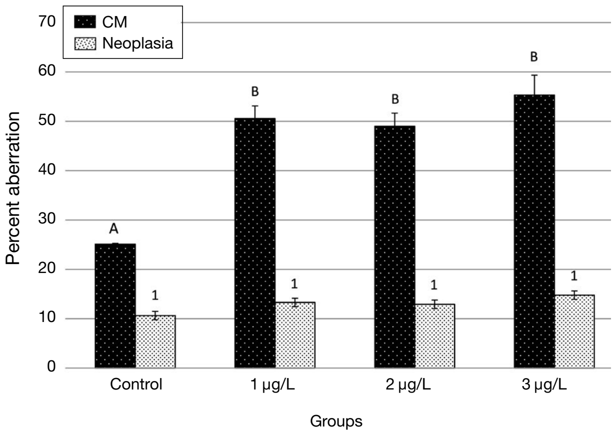


Figure 5. Photomicrograph of adductor muscles in control and BPA-exposed *C. fluminea* clams after 21 days. (A) Unexposed control clam showing organized muscle fibers; (B): Clams exposed to 1 μg/L BPA; (C) Clams exposed to 2 μg/L; and, (D) Clams exposed to 3 μg/L BPA. Asterisk = loss of surface adherence; thick arrow = necrosis; solid ring = haemocyte infiltration.



Figurea 6. Mean percent aberration ±SEM of adductor muscle tissues on cellular and moraaphological (CM) and neoplastic reaction patterns. Differences in symbols would mean significant difference at $p < 0.05$ using Tukey's post hoc test.

types of toxic compounds. In this study, the toxic effects of environmentally relevant concentrations of BPA on the histology of gills, digestive gland and adductor muscles of *C. fluminea* were established. Based on the results, the gills, digestive glands and adductor muscles showed differential responses to the presence of BPA.

Among the three organs, it was found out that the digestive gland was the most affected organ based on percent aberration values and higher incidences of neoplasia even at the lowest concentration. According to Hayashi *et al.*[35], BPA is usually highest at the digestive glands of freshwater mollusks. Based on their studies, BPA and its metabolites are often directed particularly in the lumen of the tubules of digestive gland of *C. japonica* where they are metabolized substantiating the greater histological damage seen in this organ. Similar observations were also noted by Kanapala & Arasada[36] in a study using freshwater snails. According to them, the high sensitivity of the digestive gland is directly attributed to its role in homeostasis, contaminant uptake, digestion, metabolism as well as detoxification process[35,36]. The presence of enlarged tubules and small lumen size in clams exposed to 1-2 µg/L group is a sign of hyperplasia and hypertrophy. Hyperplasia is characterized by an increase in the amount of tissue resulting from cell proliferation as a response to stimulus such as chronic inflammatory response and compensation for damage. Hypertrophy on the other hand, is an increase in the volume of tissue as a result of an increasing demand[20,38]. Hyperplasia and hypertrophy is a primary adaptive response of tissues exposed to toxic chemicals such as BPA. Cells that died due to the organism's exposure to BPA were compensated through an increase in size and production of new ones[39]. However, heightened concentrations of and continued exposure to BPA caused the necrotic pathway to progress, thus explaining the enlargement of the lumens, the presence of larger cells, and in some cases, tubular regression due the collapse of the tubules following the disappearance of the necrotized cells of those clams exposed to 3 µg/L BPA.

Necrosis triggers a proinflammatory response among the nearby cells when exposed to the toxic chemical[40]. This explains the occurrence of neoplasia in the tissues of BPA-exposed clams. In relation to this, significant hemocyte infiltration was also observed in the intertubular spaces. Since hemocytes are immune cells of *C. fluminea*, and the digestive gland is adversely and directly affected by BPA, greater immune response is needed compared to the less severely damaged tissues, i.e. gills and adductor muscles.

Next to the digestive gland, the gills of exposed clams were significantly affected as well. The gills showed a concentration-dependent response towards BPA as seen by an increasing percent aberration in its cellular and morphological attributes. Further, the statistically significant difference in histological scores seen among concentrations would show that gills have specific responses to specific amount of BPA. The increase in vacuolation and hypertrophy in clams exposed to 1 µg/L is a physiological adaptation to limit damage[1]. Vacuolation and hypertrophy are essential in confinement, isolation and sequestration of materials that could pose harm to the organism[1,37]. At higher concentrations, vacuolation and hypertrophy prove to be inefficient as a response to the slowed metabolism of the organism. According to Leonard *et al.*[41], a concentration of 2 µg/L of BPA slows the metabolic processes of the cells causing them to die, thus hyperplasia or increase in cellular number take place as an adaptation to an increasing functional demand. Further, fibrosis was also common in gill tissues exposed to 2 µg/L. According to Payan *et al.*[42], it is a degenerative response to exposure to harmful chemicals characterized by deposition of connective tissue that can obliterate the architecture of the tissues thereby leading to loss of physiological function. At the highest concentration of 3 µg/L, epithelial detachment, lamellar deformation and loss of epithelia and necrosis were seen. These mechanisms serve as barrier to prevent further entry of contaminants to damage other organs. Similar mechanism was also observed by Kumar *et al.*[43] in gills of *Lamellidens marginalis*, a freshwater bivalve. Such response has to be employed by the gills since it plays significant role in respiration, acid-base balance, ionic and osmotic regulation, food capture and maintenance of water current which greatly affects the physiological well-being of the organism[12,44-46]. The lack of significant difference in neoplastic patterns in gills of exposed and unexposed clams may be due to sequestration of the BPA made possible by the increasing incidences of vacuolations. Thus, occurrence of neoplastic damages on this tissues were halted[48].

The least affected among the organs is the adductor muscle based on its relatively low percent aberration values and alterations limited only to haemocyte infiltration, loss of surface adherence and loss of muscle organization. The infiltration of hemocytes commonly seen across in exposed samples is one of the main defense mechanisms of bivalves[46]. Hemocytes are the phagocytic cells of invertebrates that provide cell-mediated immunity in bivalves. They play a role in wound repair, internal defense, immune response, detoxification and excretion. Upon wounding or infection, damage-associated molecular patterns trigger hemocyte infiltration at the site of infection. Infiltrating hemocytes serve as a vehicle for antimicrobial peptides to phagocytose and eliminate the toxins present in the tissues[46]. Specifically, Ford *et al*.[48], concluded that the principal role of hemocyte is to plug lesions, remove debris and repair tissues. These functions aid the organism in surviving infection and injury as they repair the damages caused by its constant exposure to BPA.

Aside from haemocyte infiltration, loss of surface adherence was also observed in the adductor muscles of exposed clams. Contaminants such as BPA induces oxidative stress on lipid membranes which eventually leads to the peroxidation of lipids[49]. The peroxidation of lipids leads to an imbalance in the milieu of the muscle cell membranes, thus, causing them to lose adherence to the adjacent muscle cell membrane. On the other hand, loss of muscle bundle organization is due to the disappearance of the necrotized muscle cells as a direct result from the effect of BPA[35,51].

The very low percentage aberration in adductor muscles and the insignificant difference across exposed clams only prove that adductor muscles have a wide range of tolerance to contaminants such as BPA. This can be attributed to the reported low bioaccumulation rate of BPA metabolites in adductor muscles[35]. Additionally, adductor muscle has to develop tough defense mechanism and high endurance to contaminants as it has an essential role on the mechanical closing and opening of the organism's shell[11,12]. Failure to perform such function will lead not only to easier entrance of contaminants but eventual death of the organism.

Conclusion

In summary, the digestive gland was the most affected tissue followed by the gills then adductor muscles. The differential histological responses of the tissues of *C. fluminea* in different concentrations of BPA proves that they are good indicators of environmental stressors such as BPA. Future studies should be done on the effects of lower concentrations (<1 μg/L) on digestive gland and higher concentrations (>3 μg/L) of BPA on the adductor muscles.

Materials and Methods

Chemicals

BPA [2,2-Bis (4-hydroxyphenyl) propane; empirical formula: ($C_{15}H_{16}O_2$; MW: 228.29), ($\geq$99%; Sigma-Aldrich, USA)] was dissolved to a desired concentration with dechlorinated water via ultrasonication.

Collection and Maintenance of Test Organism

C. fluminea clam with a shell length of 20-30 mm, were obtained from a local un-impacted watershed (La Mesa, Watershed, Quezon City, Philippines). The clams were acclimatized in the laboratory for period of two weeks prior to toxicity test. During acclimatization, the clams were maintained under a 12h:12h photoperiod in a glass aquarium containing 1000L of clean aerated and dechlorinated water with sand. Further, filtration system for waste removal, constant zooplankton supply and replacement as well as quality testing of water was done every seven days.

Exposure Conditions

After acclimatization, ten clams were randomly placed in a 5-L glass container labeled as control, *S1, S2*, and *S3*- the *control* set-up containing only depurated water and sand and the experimental set-ups containing depurated water, sand and 1.0 μg/L, 2.0 μg/L, and 3.0 μg/L of BPA, respectively. Exposure period lasted for twenty-one (21) days along with proper maintenance measures being observed. Exposure period was done for 21 days as current literature and previous pilot studies indicated that this is sufficient time for uptake and effects of toxic chemicals to be seen in clams such as *C. fluminea*[8,52].

Histological Analysis

After 21 days, ten *C. fluminea* from each of the 4 set-ups were randomly selected. The specimen were prepared for histological analysis by prying open the shells and inserting a toothpick between the valves. The clams were euthanized and their adductor muscles, digestive glands and gills were excised and were separately placed in properly labeled vials containing Davidson's fixative for 24 hours. After the 24-hour period, the tissues were immediately transferred to 70% ethanol-containing vials until histological processing.

The tissues were processed, embedded in paraffin, sectioned at 5 microns, stained with Hematoxylin-Eosin at the National Kidney and Transplant Institute and examined via an Olympus light microscope. Percent aberration was determined using an index adapted from Costa *et al*.[37] with minor modifications. The categories for the gills and the adductor muscles were cellular and morphological changes (CM) and neoplasia while that of the digestive glands were tubular alterations, intertubular changes and neoplasia. Histological examination was done blindly by four researchers.

Statistical Analysis

All values were expressed as mean percent aberration ± SEM. A one-way ANOVA and Tukey's post hoc test was applied to determine whether there exist a significant difference among clams in different groups. A p value of 0.05 was considered significant. All analysis were done using the SPSS *ver* 20.

Acknowledgements

Authors are thankful to the Department of Biology, University of the Philippines Manila for providing required laboratory facilities for this work.

Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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