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Developmental and Cytogenetic Toxicity of 3D Printing Material on Sea Urchin (*Arbacia lixula*)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

In this study the ecotoxicity of 3D printing material [polylactic acid (PLA) investigated with marine echinoderm; sea urchin *Arbacia lixula.* To achieve this goal, (i) fertilization success, spermiyotoxicity and embriyotoxicity exposed to PLA concentrations (0.001, 0.005, 0.01, 0.1 and 1 g/L) were assessed for 72 h. For this purpose, our study is important to make comprehensive evaluations to ensure the safety of bioplastic formulations and to take measures to regulate the use of additives. At the same time, the additive used to increase the durability of bioplastic materials will also allow us to understand the long-term effects on ecosystems, wildlife and human health. Our aim is to minimize possible harm and ensure that the overall environmental impact of bioplastics remains positive.

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Keywords: 3D printing; polylactic acid (PLA); sea urchin.

1. INTRODUCTION

Today's studies aim to reduce overall plastic consumption and at the same time minimize its impact on ecosystems [1,2]. New methods are being developed to reduce plastic pollution, and the most striking of these is bioplastics, which are a sustainable alternative. Bio-based plastics are widely used as a replacement for traditional plastics in various applications such as packaging, automotive parts and consumer goods, thus reducing greenhouse gas emissions from fossil fuels [3]. The use of 3D printers has been increasing rapidly, which are used with raw materials in the rapid manufacturing of devices. Because of this, it has enabled the mass introduction for use at different levels. 3D printers and bioplastics offer new opportunities for applications in fields such as medicine [4]. Biopolymers has attracted great attention in the fields of sustainable packaging, energy storage, biomedicine, and textiles [2]. Polylactic acid (PLA) is considered the most prominent bioplastic due to its physicochemical properties, low price and cheapness [5-7]. PLA has been reported as an environmentally friendly compounds [4,8]. Although it is stated that it is biodegradable, Biodegratadion of PLA not occurred at normal environmental coditions on marine environment [2,9]. It is important to note that not all biodegradable plastics are suitable for all environments. Some require specific conditions such as higher temperatures facilitate their breakdown [10]. In conclusion, bio-based and biodegradable plastics offer potential benefits for environmental sustainability compared to traditional petroleum-based plastics. However, it is essential to understand their properties and limitations properly and implement appropriate waste management practices to maximize their positive impact on reducing plastic pollution. Many studies focused on microalgae [11], mollusks, and fish [12-16] but no data available on marine echinoderm are still scarce. For this reason, the ecotoxicological effects of PLA on *Arbacia lixula* were determined. Investigations of hazardous effects on early developmental stages of aquatic organisms have great importance due to the protection of the natural population's health [17,18]. The *Arbacia lixula* sea urchin is found across various European waters and plays a important role in conversation of marine ecosystems [19-21,17]. Its life cycle, including the release of mature gametes directly into seawater and pelagic larval stages, makes it an important species for understanding the impacts of contaminants on marine environments [10]. Furthermore, this work delves into investigating the potential toxicity of commercial products 3D printing filament (PLA) shortly after their introduction to markets when they are released or disposed into seawater [22-25]. Specifically focusing on PLA because of this material used by children in the school for education. This study employs *Arbacia lixula* as a model organism to examine the effects of these material.

2. MATERIALS AND METHODS

Test mediums were prepared by adding the small piece (100 µm) PLA directly to sea water; 0.001, 0.005, 0.01, 0.1 and 1 g/L test concentrations. Control group were untreated negative controls (filtered natural seawater=FSW from the same area of sea urchins). $3x10^{-4}$M $CdCl_2$ were used as a positive control. All treatments were tested six replicates. Adult *Arbacia lixula* were collected from the Aegean Sea coast (Seferihisar, Turkey). Bioassays were carried out as described previously by Arslan and Parlak [26]. For Spermyotoxicty test, 50 µl sperm cell suspensions exposed to various PLA concentrations for 30 min before insemination. Changes in the fertilization success of exposed sperm were determined by scoring the percentage of fertilized eggs (Arslan and Parlak, 2008). The embryotoxicity tests were carried out by added the 1 ml fertilized egg suspension in FSW with increasing PLA concentrations throughout development (The room temperature:19 ± 2°C). Embryotoxicity was assessed on 72-hour old pluteus larvae according to morphological criteria defined by Arslan and Parlak (2008). A sample of 100 embryos was observed under a light microscope. Developmental defects were observed on living plutei, which were slowed down their mobilization in 10^{-4} M chromium sulfate, 72 h after fertilization.

Cytogenetic tests were carried out 6 h p-f and the embryos were fixed in Carnoy's solution (ethanol, chloroform, acetic acid; 6:3:1 V: V: V). 24 h after fixation, absolute ethanol was renewed and the samples were ready to be observed under a light microscope (1000×) with oil immersion. Mitotic activity (numbers of metaphase and anaphase) and chromosome aberrations (chromosome bridges, lagging chromosomes, multipolar spindles, free chromosome sets, fragmented chromosomes) were scored in each embryo, thus allowing to assess both quantitative endpoints and mitotic anomalies.

EPA Probit Analysis Program used for calculating LC/EC Values Version 1.5. Dunnets tests were used to compare the differences in the frequency distribution of the evaluated parameters (N: normal plutei, R: retarded plutei, P1: skeletal malformations, P2: blocked gastrula or blastula and D: dead) between the negative control (FSW) and the treatment groups by applying the logarithmic transformation to normalize distributions.

3. RESULTS AND DISCUSSION

It was observed that sperms were exposed to PLA for 30 minutes, resulting in significant changes in their fertilization capacity.The fertilization rate was observed 100% in the control group. At the first concentration of 0.001g-PLA/L no change was observed. It was determined that fertilization did not have a negative effect on this amount of PLA. The fertilized egg rate decreased to 92% at 0.005 g-PLA/L. This ratio decreased to 86.33% at 0.01 g-PLA/L in parallel with the increase in the amount of PLA, and to 59% at the final concentration of 1g-PLA, with a decrease of approximately 43% (Fig. 1). The impact of PLA on fertilization was determined as EC50 0. 69 g/L PLA by probit analyses Table 1. The scores of developmental defects of larvae showed that offspring quality was significantly decreased (Fig. 2) at all concentrations tested (p<0.0001). EC50 value of PLA was estimated as 0.341 g/L for spermyotoxicity as shown in Table 1. This results bring us a conclusion that the PLA has less effects on fertilization success of sperms but extreamly decreased offspring quality of exposed sperms became more important from the ecotoxicological point of view.

Results of embryotoxicty tests, significant effects were observed at concentrations ranging from 0.001 to 1 g/L-PLA. The embryotoxicity tests shows the classic dose-response curve indicating a decreased percentage of normal larvae development with increasing PLA concentrations (Fig. 3). The impact of PLA on exposed embryos was estimated as EC50 0.155 g/L PLA concentration by probit analyses. According to the toxicity criteria of Woelke (1965) at 0.01 g-PLA/L, the normal pluteus frequency decreased by approximately 20% to 80%.

Table 1. EC50/LC50 Levels of PLA on Sea urchin *A. lixula*

Results of Biotest	EC50/LC50 Levels (g-PLA/L)
Fertilization Success	0. 69
Offspring Quality	0.341
Embryotoxicty	0.155

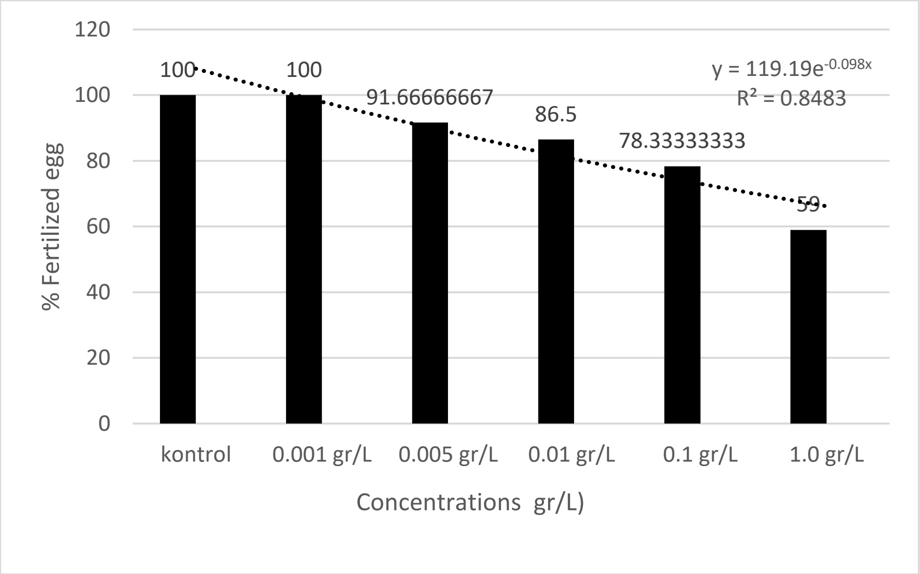

Fig. 1. Effects of PLA on fertilization success

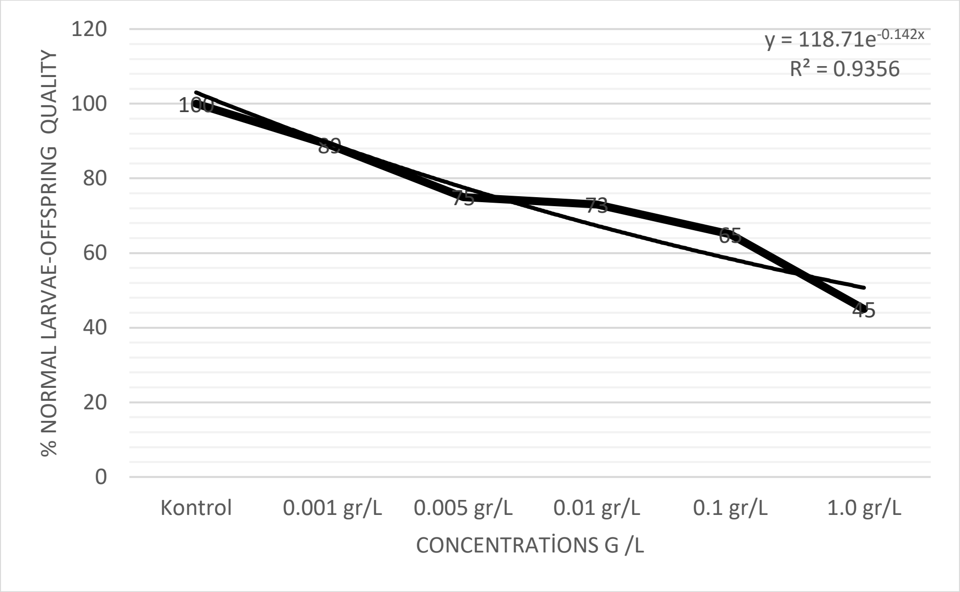

Fig. 2. Spermyotoxicity after PLA exposure in *A. lixula* sea urchin sperm. Offspring quality percentage of *A. lixula* embriyos

In parallel with this decrease, the frequency of individuals with deformation in the skeletal system increased by 23%. It has been determined that this concentration is toxic according to the frequency of pluteus with developmental disorders (Woelke 1965).

The cytogenetic results for PLA are shown in Fig. 4 as shown in Fig. 4 ratio of metaphase and anaphase were significantly decrease. Furthermore, mitotic activity in the embryos were inhibited at 0.01 g/L ($p < 0.05$) and 1 g/L-PLA ($p < 0.001$). Fig. 5 showed that the number of Interphase Embryos (IE) differed at 0.01 to 1g-PLA/L.Its increased at high PLA concentrations. As shown in Fig. 4, a significant difference was observed in average total mitotic aberrations in embryos exposed to 0.001 to 1 g-PLA/ L compared to controls.

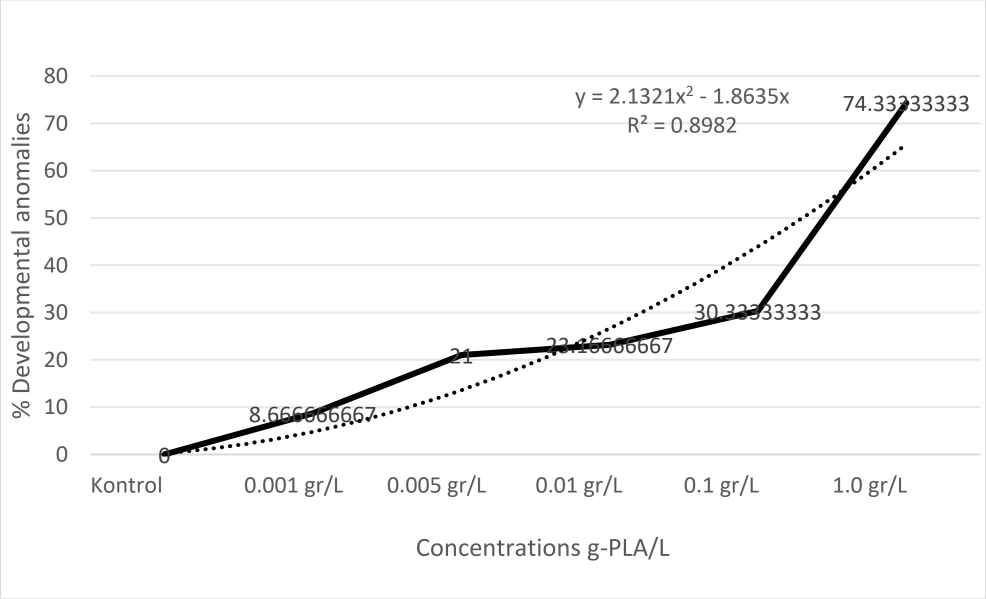

Fig. 3. Embyotoxic effects of PLA on *A. lixula*

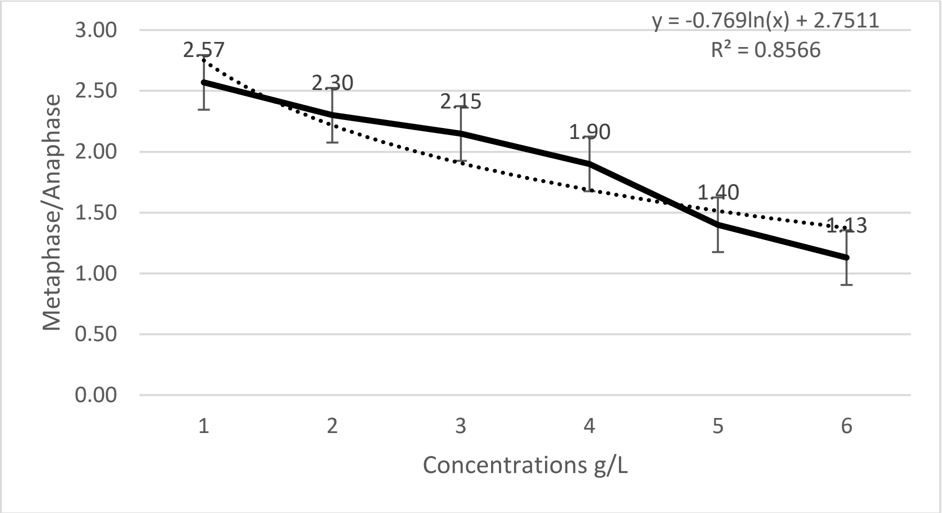

Fig. 4. Cytogenetic toxicity of PLA on embryos. Metaphase/Anaphase ratio

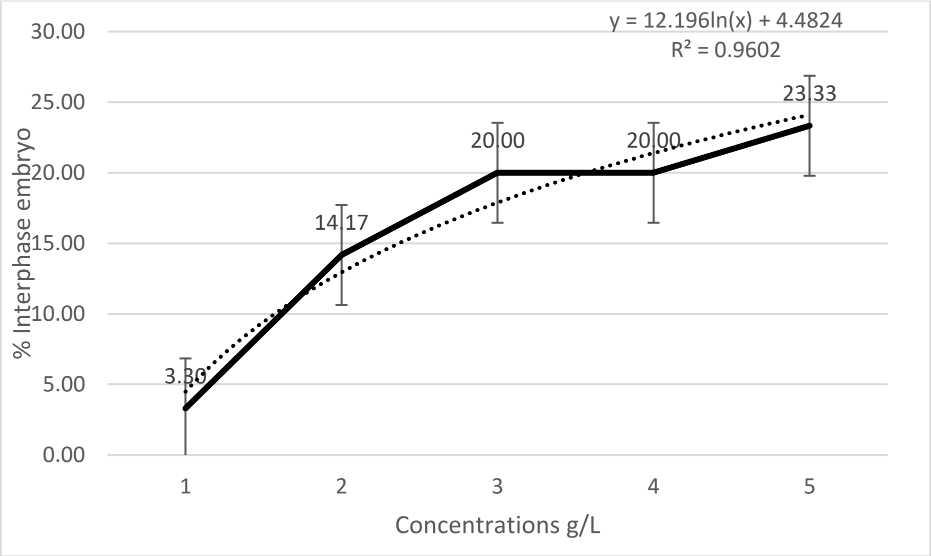

Fig. 5. Cytogenetic toxicity after PLA exposure. Percentages of interphase embryos

3D Polylactic acid (PLA) based printers are increasing their use and popularity worldwide However, this technology also causes environmental pollution, especially microplastic pollution in the aquatic environment [27]. Reported by Rodríguez-Hernandez et al., [28] the formation of nanoplastic pollution as a result of the cleaning process of the products taken from the 3D printer and their physicochemical characterization were reported. As a result of the study, they reported that nano-sized plastic particles easily enter the aquatic environment and that these residues aggregate around 1 mm on average in seawater. At the same time, researchers have stated that the resulting and clumping nanoplastics interact with pH and other positively charged pollutants, becoming an unexpected environmental problem and public health risk. Previous studies have reported that biodegradable microplastics (PLA), which are used extensively to reduce microplastic pollution, cause a toxicity similar to microplastics. In the study conducted by Green [29] PLA potentially negatively affects the oyster *Ostrea edulis* as much as traditional microplastics. In addition, PLA hazardous effects on the life and health of *Danio rerio*, *Mytilus edulis*, *Microcosmus exasperates* and *Daphnia magna*. And also causes oxidative stress, reproductive problems, intestinal damage, immunosuppression. have been reported [29,30,31].

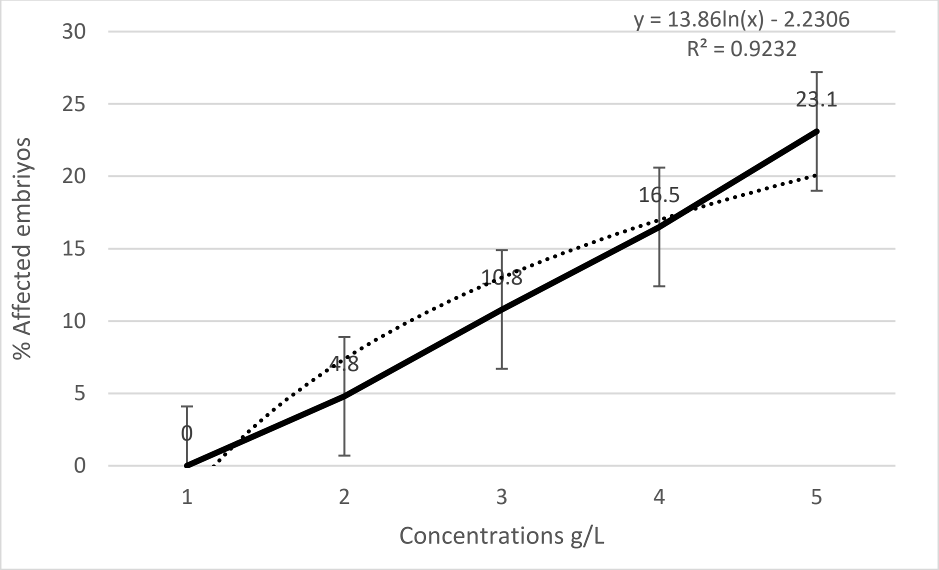

Fig. 6. Cytogenetic toxicity after PLA exposure in *A. lixula* sea urchin embryos. Percentage of affected embryos (percent embryos having ≥1 mitotic aberrations)

It has been stated by many researchers that the PLA used in 3D printers are potentially toxic [32]. Many toxicity studies have shown that print parts and leachates of 3D printers are contaminated with *Daphnia magna* [33,34]. The aim of our study is to investigate the toxic effects of PLA on sea urchin *A. lixula* in both acute and chronic periods. Montalvão [9] reported in their study that although PLA is considered biodegradable due to its microbial origin, it almost does not decompose in aquatic environments. For this reason, ecotoxicity studies conducted in recent years have focused on the damages and risks that 3D printer raw materials may cause as a result of unconscious and incorrect use. The study by An et al., [35] reported that the ecotoxicity of PLA on *Daphnia magna*. According to result of An et al., [35] study, the survival rate for *D. magna* declined to 52.4%, and end of Chronic exposure at 1 and 5 mgL^{-1} PLA caused a decrease of offspring. This study contributes that biodegradable microplastics (PLA) toxic effects on *D. magna* which could be similar to conventional Microplastics effects on aquatic organism. When our results compare with An et al., [35] research similar results were observed. In our results PLA exhibited the fertilization and normal development and also cause a genetic hazards at sea urchin. In conclusion, Previous studies and our study showed that the importance of PLA contaminations. Balentine et al., [36] investigated the acute and chronic toxicity of 3D printer resin against *Ceriodaphnia dubia* and as a result, it was reported that the LC50 value varied between 2.6 and 33 mg/L as a result of 48-hour acute toxicity tests. Researchers have also determined that 3D printing resin inhibits growth with IC25 values of 0.33 to 16 mg/L. Uribe-Echeverría and Beiras were tested the effects of a polyvinyl chloride (PVC) toy polylactic acid containers (PLA), and polylactic acid/polyhydroxyalkanoate 3D printing filament (PLA/PHA) using Para*centrotus lividus* sea urchin larvae. As a result of their study, they reported that the PVC toy was very toxic, whereas PHB showed mild toxicity, even though it was considered a non-toxic polymer. Uribe-Echeverría and Beiras exposed sea urchin embryos to the 3D printing material PLA and stated that, unlike our study, PLA containers and PLA/PHA filament were harmless to the larvae. The reason for this result is probably that the researchers used the materials diluted, whereas in our study we carried out the tests by adding them directly to the medium. It has been reported by several researchers that PLA is acutely toxic to algae. Li et al., [11] reported that PLA caused a inhibition of growth on *Skeletonoma costatum* and also they concluded that the exposure of *S. costatum* to 0.1, 0.2, 0.3 and 0.5 mg/L PLA induced significant reduction of Chl *a* content. A lack of information about the toxicity of PLA to the developmental stages of the sea urchin *Arbacia lixula* was observed.

4. CONCLUSION

It can be concluded that PLA affect the *A. lixula* during reproduction and embryonic developmental stages. As a result of biotests conducted with the PLA printing filament tested in this study, it was revealed that it negatively affected fertilization, sperm, embryos and mitotic stages, and revealed the need for the use of already commercialized, safe biobased and biodegradable products and attention in waste management.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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