Dose- and Time-Dependent Histopathological and Immunotoxic Effects of DEHP on the Spleen of *Sparidentex hasta*: Implications for Fish Health and Veterinary Toxicology

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Abstract

This study comprehensively evaluated the dose- and time-dependent effects of di-(2-ethylhexyl) phthalate (DEHP), a widely used environmental contaminant, on the spleen of *Sparidentex hasta* over a 14-day exposure period. Fish were exposed to five concentrations of DEHP (0, 5, 10, 50, and 100 µg/g), and splenic responses were assessed through quantitative morphometric indices, including spleen weight (SW), Spleen Somatic Index (SSI), and Spleen-to-Length Ratio (SLR), as well as qualitative histopathological analysis. Statistical comparisons were performed using SPSS 26, and graphical representations were generated in Excel. Results demonstrated significant dose- and time-related increases in SW (0.10 g in controls vs. 0.27 g at 100 µg/g, $p < 0.05$), SSI (0.089% vs. 0.276%, $p < 0.05$), and SLR (0.563% vs. 1.698%, $p < 0.05$). Histopathological examinations revealed progressive structural alterations with increasing DEHP concentrations, including sinusoidal congestion, lymphocytic hyperplasia, melanomacrophage center (MMC) accumulation, and focal necrosis. Lower doses induced mild adaptive responses, whereas higher exposures resulted in pronounced tissue disorganization and immune activation. Strong correlations between morphometric indices and microscopic lesions suggested that splenic enlargement reflects both oxidative stress and immunotoxic effects. These findings underscore the sensitivity of the spleen as a reliable biomarker for detecting sub-lethal immunotoxicity in fish exposed to environmental pollutants. Overall, this study highlights the importance of integrating quantitative indices with histopathological evaluation to assess the ecotoxicological impacts of DEHP, providing valuable insights for environmental monitoring, fish health management, and aquaculture sustainability. The results further emphasize the potential ecological and economic consequences of DEHP contamination in aquatic ecosystems.

Keywords: Diethylhexyl Phthalate; Fish diseases; Histology; Immunotoxicity; Spleen pathology; Toxicology

1. Introduction

Phthalate esters, particularly di-(2-ethylhexyl) phthalate (DEHP), are widely used plasticizers in polymer industries. Due to their weak chemical binding to polymer matrices, DEHP can readily leach into aquatic environments, accumulating in sediments, water, and aquatic organisms [1]. Industrial effluents, urban wastewater, and oil-related activities further increase the concentration of these contaminants in coastal waters, making the semi-enclosed Persian Gulf particularly vulnerable to pollution [2].

Global studies have demonstrated that DEHP exerts multiple adverse effects on aquatic organisms, including endocrine disruption, oxidative stress, genotoxicity, and immunotoxicity [3,4].

The spleen, as a primary immune organ in fish, plays a central role in hematopoiesis and immune defense. Histopathological alterations, including lymphocyte depletion, sinus congestion, and changes in melanomacrophage centers (MMCs), serve as sensitive biomarkers of environmental stress and contaminant exposure. International studies have reported spleen alterations, MMC accumulation, and lymphocyte reduction in fish exposed to DEHP and similar pollutants [5,6].

The fish species *Sparidentex hasta*, widely distributed in the Persian Gulf and southern Iranian coasts, is of significant economic and ecological importance. As a carnivorous species prone to bioaccumulation of pollutants and dependent on shallow coastal habitats, it represents a sensitive model for evaluating environmental contamination. Recent studies have shown that S. hasta exhibits tissue alterations when exposed to petroleum hydrocarbons and heavy metals [7].

Despite global evidence of DEHP toxicity, no studies have investigated its histopathological effects on fish from the Persian Gulf. The present study represents the first assessment of DEHP-induced spleen alterations in S. hasta. In addition to histopathological evaluation, key physiological parameters, including body weight, total length, and spleen indices (SSL, SRL), were measured to provide a comprehensive assessment of DEHP effects.

This investigation offers novel insights into the immunotoxicological impacts of phthalates in fish and provides valuable information for pollution monitoring and sustainable management of marine resources in the Persian Gulf.

2. Materials and Methods

2.1. Fish Acquisition and Acclimation

A total of 105 male *Sparidentex hasta*, averaging 100 ± 8.5 g in weight and 16.3 ± 0.2 cm in total length, were obtained from a commercial hatchery in Bandar Imam Khomeini, Khuzestan Province, Iran. Fish were acclimated for 10 days in 6000-L fiberglass tanks filled with filtered and UV-sterilized seawater to ensure adaptation to laboratory conditions. During this period, fish were fed a commercial diet at 3% of their body weight twice daily (morning and evening). Water quality parameters including temperature, salinity, and pH were monitored daily, and 30% of the tank water was renewed daily to maintain optimal conditions.

2.2. Physiological and Organ Indices

To evaluate the health status and physiological responses of the fish, organosomatic indices and condition factors were calculated. Given the focus on the spleen, the splenosomatic index (SSI) and spleen-to-length ratio (SLR) were determined in addition to the general condition factor (CF). Sampling was performed on Day 1, Day 7, and Day 14.

The condition factor was calculated according to Equation (1), the splenosomatic index was calculated using Equation (2), and the spleen-to-length ratio was obtained from Equation (3)

- **Condition Factor (CF)** = (Body weight / Total length^3) × 100 (1)
- **Splenosomatic Index (SSI)** = (Spleen weight / Total weight) × 100 (2)
- **Spleen-to-Length Ratio (SLR)** = (Spleen weight / Total length) × 100 (3)

All measurements were obtained using a digital scale (±0.01 g) and a measuring board (±0.1 cm). These indices allowed tracking of physiological changes induced by DEHP-associated microplastic exposure.

2.3. Experimental Setup

Following acclimation, fish were randomly assigned to seven experimental groups (15 fish per group). DEHP (Di-(2-ethylhexyl) phthalate, CAS No. 117-81-7, purity 99.7%) was obtained from Sigma-Aldrich and dissolved in olive oil to prepare stock solutions. Concentrations were calculated based on mean fish body weight and prepared fresh weekly.

Four DEHP exposure groups received intraperitoneal injections at 5, 10, 50, and 100 µg/g body weight. Two control groups were included: a negative control receiving olive oil only and an untreated sham control. Fish were anesthetized with 2-phenoxyethanol prior to injections, gently handled with sterile equipment, and returned to their respective tanks immediately after dosing.

2.4. Spleen Tissue Sampling

Spleen tissues were collected on Days 1, 7 and 14 post-exposures. Fish were anesthetized, and a midline incision was made to carefully excise the spleen. Tissues were immediately fixed in 10% neutral-buffered formalin for subsequent histological analysis [5, 7,8].

2.5. Histological Procedures

Excised spleens were processed using an automated tissue processor for dehydration, clearing, and paraffin embedding. Paraffin blocks were sectioned at 5 µm thickness with a rotary microtome, mounted on glass slides, and stained with hematoxylin and eosin (H&E) following standard protocols:

1. Two xylene baths (20 min each) for clearing.
2. Descending ethanol series (100%, 90%, 80%, 70%) for rehydration.
3. Hematoxylin staining for 10 min, followed by eosin for 20 min.
4. Ascending ethanol series for dehydration and final clearing in xylene.

For each fish, three non-overlapping sections were prepared and examined under a light microscope. Five randomly selected microscopic fields per section were evaluated for histopathological scoring to ensure accurate representation of splenic alterations. These procedures ensured optimal preservation and visualization of spleen histoarchitecture [9].

2.6. Microscopy and Histopathological Assessment

Slides were examined under a light microscope at multiple magnifications. Representative images were captured to document histopathological changes such as lymphocyte depletion, congestion, hemorrhage, and increased melano-macrophage centers. All analyses were performed in accordance with standard aquatic histology protocols.

2.7. Statistical methods

All data are presented as mean ± standard deviation (SD). The normality of datasets was examined using the Shapiro–Wilk test, confirming that physiological indices were approximately normally distributed. Two-way analysis of variance (ANOVA) was employed to evaluate the effects of DEHP dose (5, 10, 50, and 100 µg/g) and exposure duration (Day 7 and Day 14) as independent factors, as well as their interaction (Dose × Time). When significant main effects or interactions were detected, Tukey's post hoc test was applied to identify pairwise differences among treatment groups and between sampling days within the same dose.

For parameters measured only once (e.g., baseline values before exposure), one-way ANOVA followed by Tukey's post hoc test was used. Differences were considered statistically significant at $P < 0.05$. All statistical analyses were performed using SPSS software (version 26.0)**,** and the graphs were prepared in Microsoft Excel (Microsoft Office 365).

3. Results

3.1. DEHP-Induced Modifications in Fish Spleen Indices and General Health

On day 1, prior to DEHP exposure, the general condition and health status of the fish were evaluated using spleen indices and the condition factor (CF). The spleen somatic index (SSI), representing the relative spleen weight to total body weight, was 0.10 ± 0.005. The spleen-to-body length ratio (SLR), calculated as spleen weight divided by total body length, was 0.613 ± 0.031. The condition factor (CF), indicative of overall health and nutritional status, was 2.31 ± 0.12. These baseline values were used to assess subsequent changes in spleen indices on days 7 and 14 (Table 1).

Table 1. Spleen indices and general health parameters of fish at day 1.

Index	Mean ± SD	Formula	Description
(CF)	2.31 ± 0.12	CF = (Total weight / Total length³) × 100	Indicator of general health and nutritional status
(SSl)	0.10 ± 0.005	SSI = (Spleen weight / Total body weight) × 100	Indicates relative spleen size compared to body weight
(SLR)	0.613 ± 0.031	SLR = (Spleen weight / Total body length)	Reflects spleen size relative to body length

Note: Values are presented as mean ± SD. CF = Condition Factor, SSI = Spleen Somatic Index, SLR = Spleen-to-Length Ratio

Exposure of *S. hasta* to increasing concentrations of DEHP (5, 10, 50, and 100 µg/g) led to a notable rise in spleen weight (SW) compared to both control and negative control (oil solvent, SC) groups. As illustrated in Figure 1, this enhancement was observed as early as day 7 and persisted through day 14. The magnitude of the effect was concentration-dependent, with the heaviest spleens recorded in fish exposed to 100 µg/g DEHP. Additionally, SW values were higher on day 14 than on day 7 at all tested concentrations, indicating a cumulative and time-dependent impact of DEHP on spleen mass.

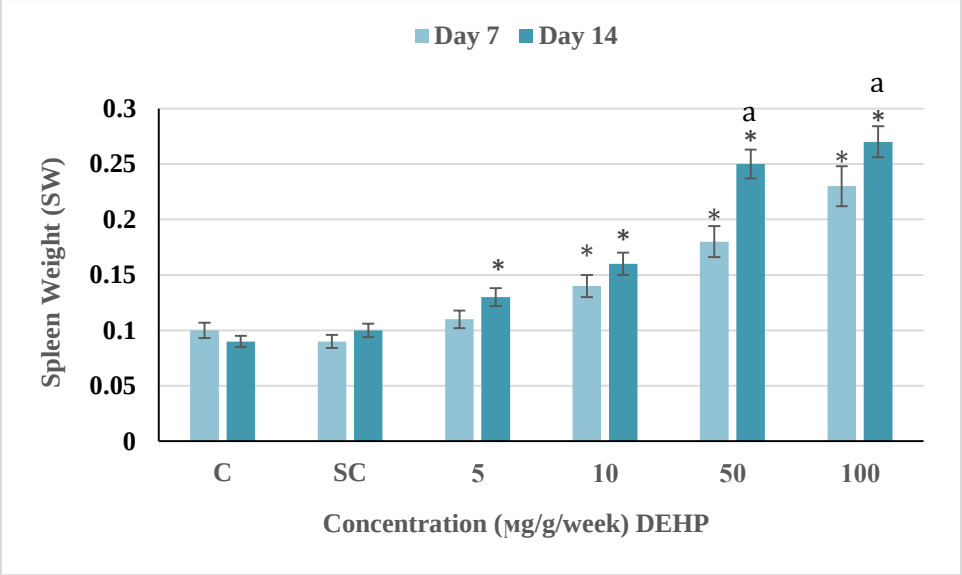


Figure 1. Spleen weight (SW) of *S. hasta* following exposure to DEHP at 5, 10, 50, and 100 µg/g, along with control and negative control groups (oil solvent, SC), for 7 and 14 days. Data are presented as mean ± SEM (n = 15). * Indicates a significant difference compared to the control and negative control groups ($p < 0.05$), and a indicates a significant difference between day 7 and day 14 ($p < 0.05$).

The spleen somatic index (SSI) in *S. hasta* indicated that exposure to varying concentrations of DEHP induced significant alterations in this parameter. As shown in Figure 2, on day 7, SSI values in the 10, 50, and 100 µg/g DEHP treatments were significantly higher compared to the control and negative control groups. The increase was concentration-dependent, with the highest value observed in the 100 µg/g DEHP treatment. By day 14, the concentration-dependent trend persisted, and SSI values in all DEHP-exposed groups remained significantly elevated relative to the control and negative control. The more pronounced increase in SSI on day 14, particularly at higher concentrations, suggests a cumulative and time-dependent effect of DEHP on spleen tissue.

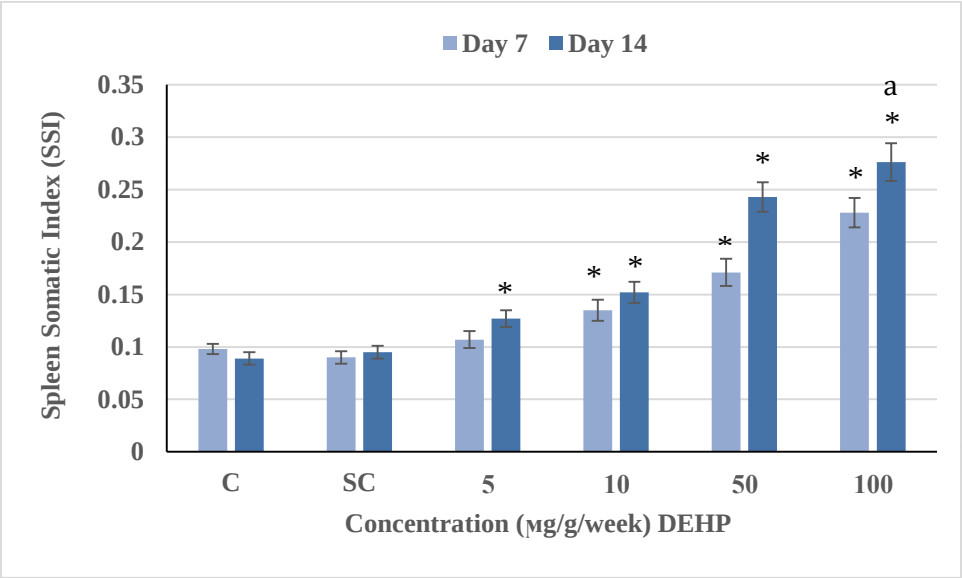


Figure 2. Spleen somatic index (SSI) of *S. hasta* following exposure to DEHP at 5, 10, 50, and 100 µg/g, along with control and negative control groups (oil solvent, SC), for 7 and 14 days. Data are presented as mean ± SEM (n = 15). * Indicates a significant difference compared to the control and negative control groups ($p < 0.05$), and a indicates a significant difference between day 7 and day 14 ($p < 0.05$).

The spleen-to-total body length ratio (SLR) of *S. hasta* exhibited a pronounced and progressive increase following exposure to DEHP at 5, 10, 50, and 100 µg/g. As illustrated in Figure 3, all DEHP-treated groups showed elevated SLR values as early as day 7, which further intensified by day 14, indicating a clear time-dependent effect. The magnitude of change was dose-dependent, with the highest SLR observed in fish exposed to 100 µg/g DEHP. These results suggest that DEHP not only increases spleen mass but also alters its proportional relationship to body length, reflecting cumulative physiological and potentially immunotoxic effects over the exposure period.

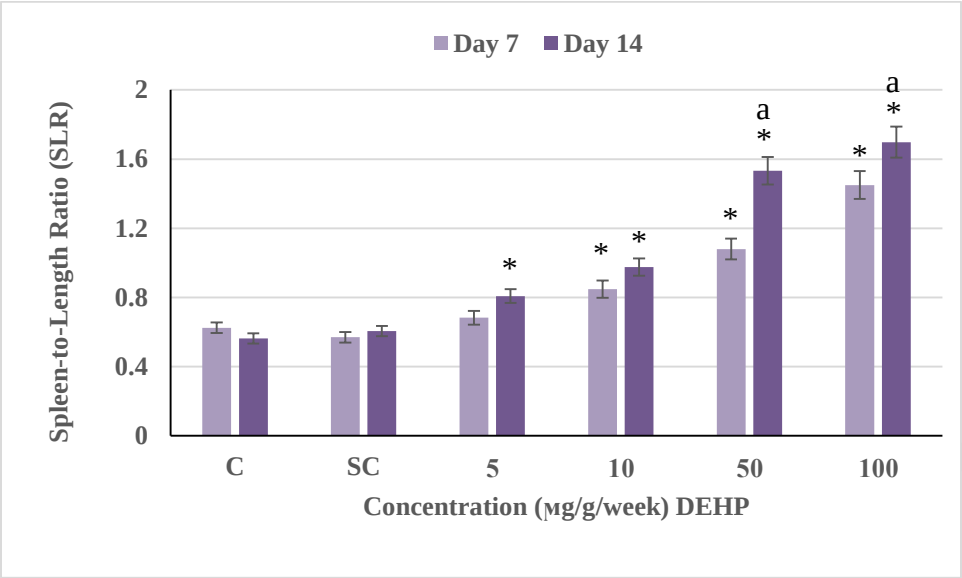


Figure 3. Spleen-to-body length ratio (SLR) of *S. hasta* following exposure to DEHP at 5, 10, 50, and 100 µg/g, along with control and negative control groups (oil solvent, SC), at days 7 and 14. Data are presented as mean ± SEM (n = 15). * Indicates a significant difference compared to the control and negative control groups ($p < 0.05$), and a indicates a significant difference between day 7 and day 14 ($p < 0.05$).

3.2. Comparative Results (Day 1 → Day 7 → Day 14)

The heatmap illustrates semi-quantitative changes in spleen indices, including spleen weight (SW), spleen somatic index (SSI), and spleen-to-body length ratio (SLR), from day 1 to days 7 and 14 in control and DEHP-exposed groups. Scores range from 0 to 3, where 0 indicates no change, 1 represents minor changes, 2 corresponds to moderate changes, and 3 indicates strong changes relative to baseline. In the control group, all indices remained stable (score 0), while the Oil Control group exhibited only minor increases (score 1). In contrast, DEHP-treated groups showed clear dose- and time-dependent elevations: low concentrations (5 and 10 µg/g) reached moderate scores (1–2), and higher concentrations (50 and 100 µg/g) reached strong scores (3) by day 14. The heatmap provides a clear visual representation of the progressive splenic responses across groups and time points, highlighting both the magnitude and temporal pattern of DEHP-induced changes (Table 2).

Table 2. Semi-quantitative (0–3) changes in spleen indices (SW, SSI, SLR) from day 1 to days 7 and 14 in control and DEHP-exposed groups

	SW Day 1	SW Day 7	SW Day 14	SSI Day 1	SSI Day 7	SSI Day 14	SLR Day 1	SLR Day 7	SLR Day 14
Control	0	0	0	0	0	0	0	0	0
Oil Control	0	0	0	0	0	0	0	0	0
5 µg/g	0	1	2	0	1	2	0	1	2
10 µg/g	0	1	2	0	1	2	0	1	2
50 µg/g	0	2	3	0	2	3	0	2	3
100 µg/g	0	2	3	0	2	3	0	2	3

SW = spleen weight; **SSI** = spleen somatic index (%); **SLR** = spleen-to-body length ratio.
Scores: 0 = no change, 1 = minor change, 2 = moderate change, 3 = strong change relative to day 1.
Heatmap colors correspond to the scores: 0 = green, 1 = yellow, 2 = orange, 3 = red.

3.3. Histopathological Analysis of the Spleen on Day 7: Dose-Dependent Effects of DEHP

Histological examination of the spleen on day seven revealed well-preserved architecture in the control groups (Figures 4-A and 4-B). In these groups, the parenchyma exhibited normal organization with evenly distributed cells and uniformly basophilic nuclei. No signs of necrosis, degeneration, congestion, or abnormal melanomacrophage centers (MMCs) were observed, confirming that the control tissues could serve as a baseline reference for comparison with DEHP-treated groups.

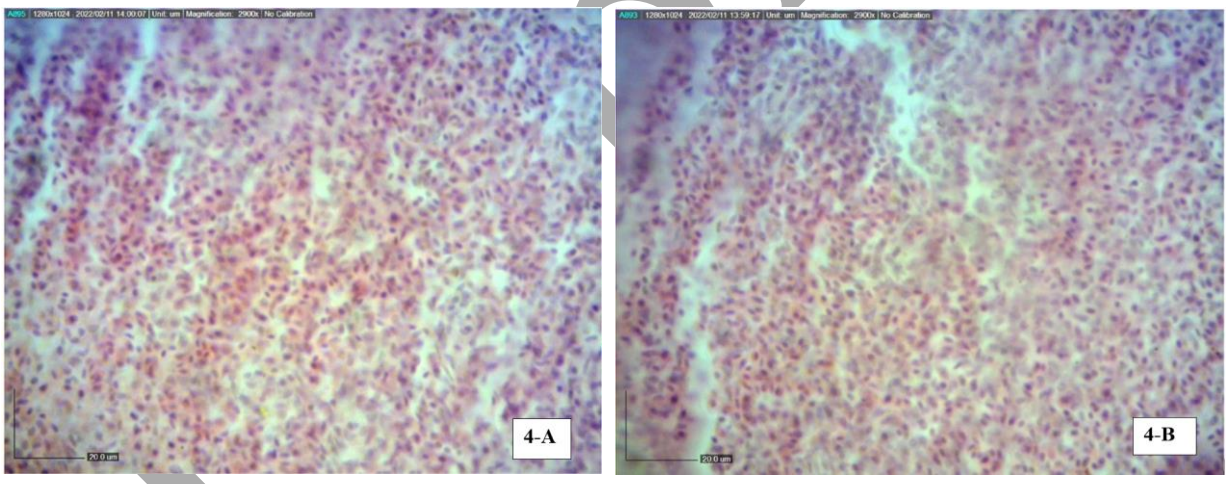
Exposure to 5 µg/g DEHP (Figure 4-C) induced mild histopathological alterations, including congestion and dilated sinusoids with partial loss of normal architecture, slight lymphocytic hyperplasia (★), scattered necrotic or degenerative regions (○), and basophilic nuclei indicative of cellular stress (→). Numerous MMCs with pigment deposition (melanin, hemosiderin, lipofuscin; ◌) were also observed, suggesting early immune activation and oxidative stress.

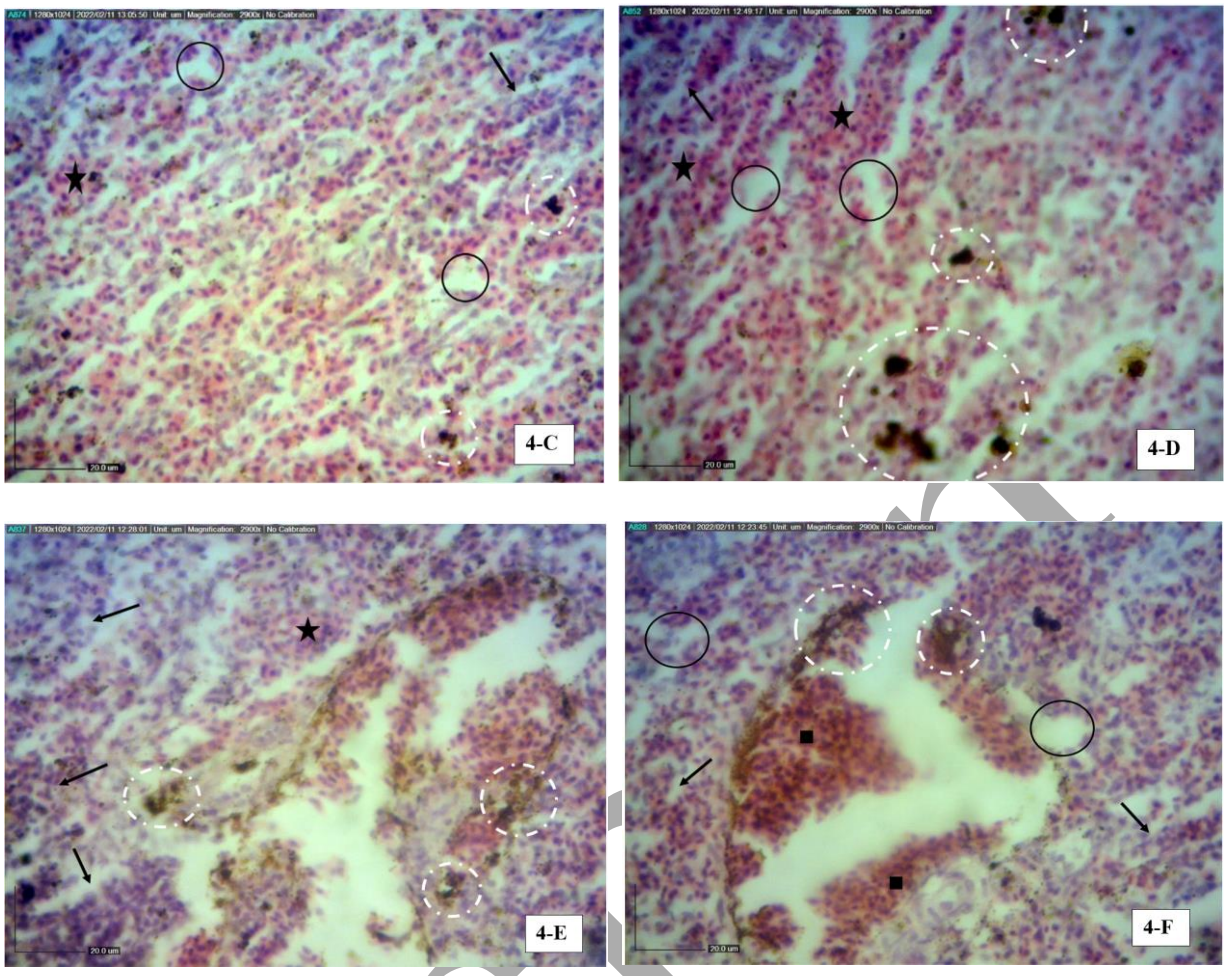
At 10 µg/g DEHP (Figure 4-D), these alterations were slightly more pronounced. Sinusoidal spaces were more evident (○), and MMCs were scattered throughout the parenchyma (◌), reflecting early immune activation and oxidative stress. Lymphocyte presence remained mild (★), and there was no evidence of widespread necrosis or acute inflammation, indicating a controlled early stress response.

In the 50 µg/g DEHP group (Figure 4-E), moderate histopathological changes were observed. Basophilic nuclei were more prominent, MMCs exhibited greater pigment accumulation (◌), and lymphocytic hyperplasia was pronounced (★), reflecting both cellular stress and activation of the immune system.

The 100 µg/g DEHP group (Figure 4-F) showed marked histopathological alterations. The splenic architecture exhibited extensive congestion, abundant MMCs and immune cell aggregates (★, ◌), mild necrotic areas (○), and increased inflammatory cell infiltration around blood vessels and ellipsoids (■). These findings indicate a strong dose-dependent response to DEHP, characterized by severe cellular stress, immune activation, and structural impairment of the spleen.

Overall, the splenic histopathological responses on day seven exhibited a clear dose-dependent pattern, ranging from mild changes at low DEHP concentrations to marked alterations at higher doses, highlighting the immunotoxic and cytotoxic potential of DEHP in *Sparidentex hasta*.





Figures 4-A to 4-F: Comparative histopathology of spleen tissue in control and DEHP-treated groups on day 7. Figure 4-A, 4-B (Control group): Normal splenic architecture with evenly distributed cells and basophilic nuclei; no necrosis, degeneration, congestion, or abnormal MMCs observed**.** Figure 4-C (5 µg/g DEHP): slight lymphocytic hyperplasia (★), scattered necrotic areas (○), basophilic nuclei (→), and increased MMCs with pigment deposition (◌), indicating early immune activation. Figure 4-D (10 µg/g DEHP): scattered necrotic areas (○), scattered MMCs (◌), and mild lymphocytic hyperplasia (★), reflecting controlled early immune response**.** Figure 4-E (50 µg/g DEHP): Moderate alterations, with prominent basophilic nuclei (→), higher pigment accumulation in MMCs (◌), and more pronounced lymphocytic hyperplasia (★). Figure 4-F (100 µg/g DEHP): Severe histopathological changes, including extensive congestion, abundant MMCs (◌), lymphocyte aggregation (★), necrotic areas (○), and marked inflammatory cell infiltration around vessels (■), indicating strong dose-dependent immune activation and cellular stress. Scale bar: ×400

Table 3 summarizes the dose-dependent histopathological changes in the spleen on day 7. Increasing DEHP concentrations progressively disrupted splenic architecture, enhanced lymphocytic activity, and promoted melanomacrophage center (MMC) formation, whereas control groups maintained normal histology. This overview highlights the graded cytotoxic and immunomodulatory effects of DEHP exposure.

Table 3. Dose-dependent histopathological alterations in the spleen of *Sparidentex hasta* on day 7 following DEHP exposure

Alterations / Groups	Control	5 µg/g	10 µg/g	50 µg/g	100 µg/g
Lymphocytic hyperplasia	0	1	1	2	3
Necrosis	0	1	1	2	3
MMC and pigment deposition	0	1	1	2	3
Basophilic nuclei	0	1	1	2	3
Congestion	0	0	0	1	3
Perivascular inflammatory infiltration	0	0	0	0	3

Histopathological alterations were graded using a semi-quantitative scoring system as follows: **0 = absent; 1 = mild; 2 = moderate; 3 = severe lesion**.

3.4. Histopathological Analysis of the Spleen on Day 14: Dose-Dependent Effects of DEHP

On day 14, histological analysis of the spleen in the control groups (Figures 5-A and 5-B) revealed well-preserved architecture, with no significant pathological changes. The tissue exhibited normal parenchymal organization, with evenly distributed cells and uniformly basophilic nuclei. No signs of necrosis, degeneration, or congestion were observed, and melanomacrophage centers (MMCs) appeared normal. These findings confirm that the control tissues remained stable throughout the study, serving as a reliable baseline reference.

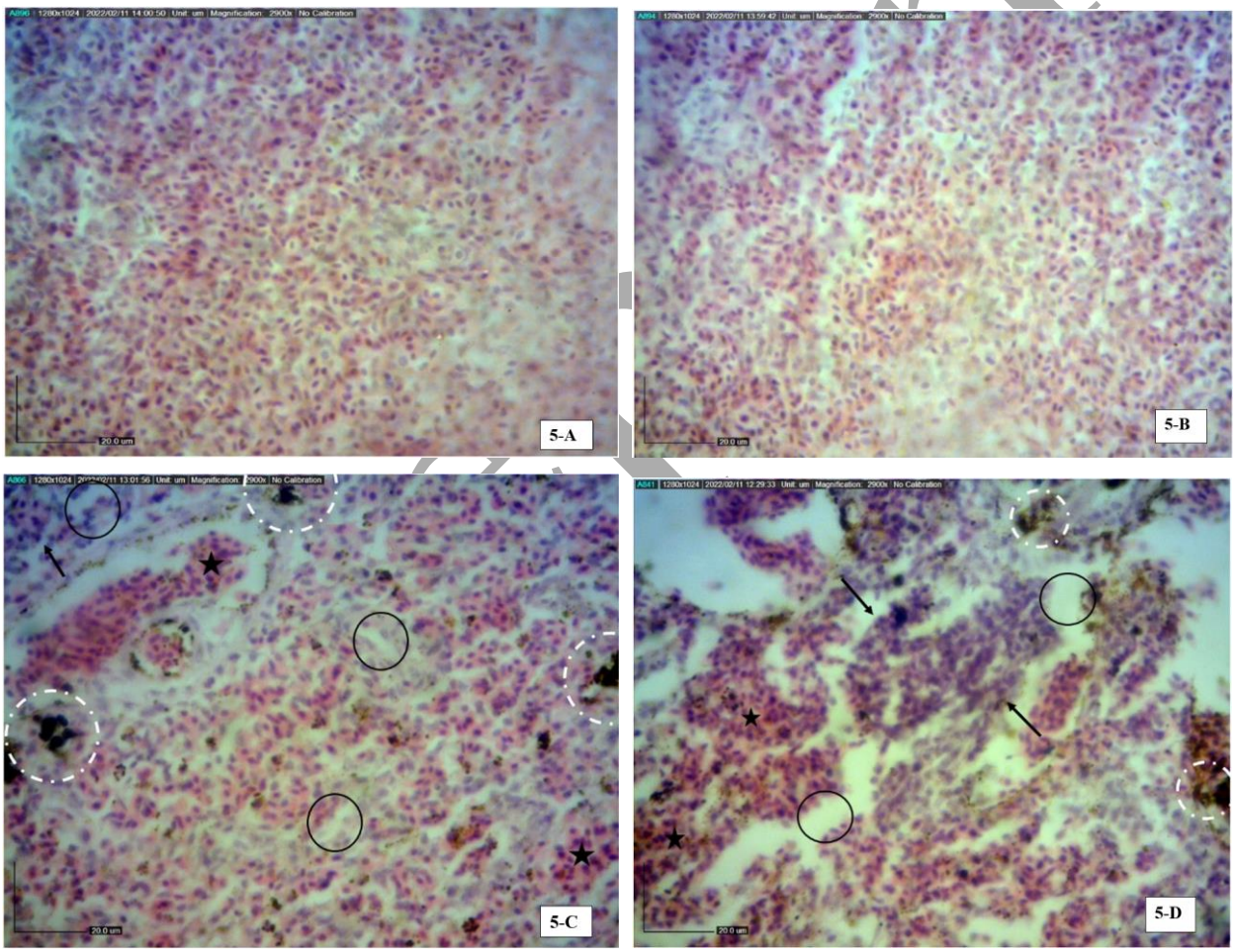
In the 5 µg/g DEHP group (Figure 5-C), mild histopathological alterations were observed, slightly more pronounced compared to day 7. Congestion and dilation of sinusoids were more evident, and lymphocytic hyperplasia was slightly increased (★), indicating enhanced immune cell activity. Scattered areas of degeneration and necrosis were noted (○), and cells with basophilic nuclei were more prominent (→), reflecting cellular stress. Additionally, there was an increase in MMCs with pigment accumulation (melanin, hemosiderin, lipofuscin; ◌) throughout the parenchyma, suggesting further immune activation and oxidative stress.

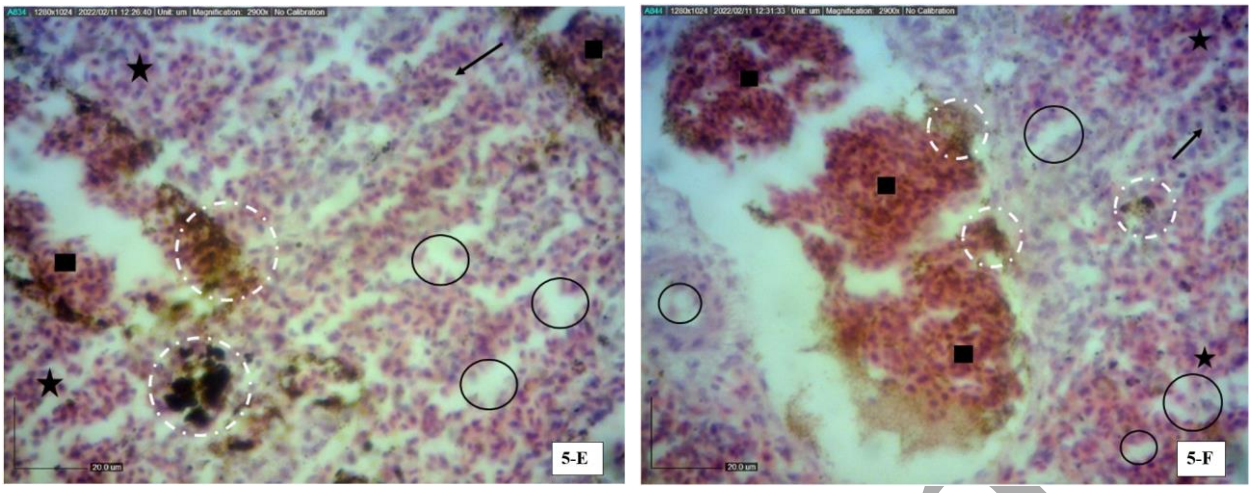
At 10 µg/g DEHP (Figure 5-D), the histopathological alterations became more evident. Sinusoids appeared more dilated (○), and MMCs with pigment deposition (◌) were more abundant across the parenchyma. Lymphocytic hyperplasia remained marked (★), and basophilic nuclei were more frequent (→), indicating heightened cellular stress. These findings reflect a stronger immune activation and a controlled response to DEHP-induced stress.

In the 50 µg/g DEHP group (Figure 5-E), more significant changes were detected. The presence of basophilic nuclei was notably increased (→), lymphocytic hyperplasia was

prominent (★), and MMCs exhibited greater pigment accumulation (◌). Scattered necrotic and degenerative areas (○) were also present, pointing to the growing severity of splenic cellular and immune responses to DEHP exposure.

The 100 µg/g DEHP group (Figure 5-F) exhibited the most severe histopathological alterations. Extensive congestion was evident, along with abundant MMCs (◌) and marked lymphocytic aggregation (★). Mild to moderate necrotic regions (○) were observed, accompanied by inflammatory cell infiltration around blood vessels and ellipsoids (■). These findings highlight a severe dose-dependent response to DEHP, characterized by profound cellular stress, widespread immune activation, and structural impairment of the spleen.





Figures 5-A to 5-F: Comparative histopathology of spleen tissue in control and DEHP-treated groups on day 14. Figure 5-A, 5-B (Control group): Normal splenic architecture with evenly distributed cells and basophilic nuclei; no necrosis, degeneration, congestion, or abnormal MMCs observed. Figure 5-C (5 µg/g DEHP): Mild alterations including congestion, slight lymphocytic hyperplasia (★), scattered necrotic areas (○), basophilic nuclei (→), and increased MMCs with pigment deposition (melanin, hemosiderin, lipofuscin; ◌), indicating early immune activation and cellular stress**.** Figure 5-D (10 µg/g DEHP): More evident sinusoidal spaces (○), scattered MMCs (◌), mild to moderate lymphocytic hyperplasia (★), and more frequent basophilic nuclei (→), reflecting heightened immune activation and cellular stress. Figure 5-E (50 µg/g DEHP): Moderate alterations with prominent basophilic nuclei (→), pronounced lymphocytic hyperplasia (★), MMCs with higher pigment accumulation (◌), and scattered necrotic/degenerative areas (○), indicating increased immune and cellular response**.** Figure 5-F (100 µg/g DEHP): Severe histopathological changes, including extensive congestion, abundant MMCs (◌), marked lymphocyte aggregation (★), necrotic areas (○), and widespread inflammatory cell infiltration (■), reflecting strong dose-dependent immune activation and cellular stress. Scale bar: ×400

Table 4 summarizes the dose-dependent histopathological changes in the spleen on day 14. Higher DEHP concentrations led to increasing disruption of splenic architecture, lymphocytic hyperplasia, and enhanced MMC formation. Necrosis and degeneration were more prominent at elevated doses, reflecting a dose-dependent cytotoxic and immunotoxic response. Control groups showed normal histology with no significant changes.

Table 4. Dose-dependent histopathological alterations in the spleen of *Sparidentex hasta* on day 14 following DEHP

Alterations / Groups	**Control**	**5 µg/g**	**10 µg/g**	**50 µg/g**	**100 µg/g**
Lymphocytic hyperplasia	0	1	2	2	3
Necrosis	0	1	1	2	3
MMC and pigment deposition	0	1	2	2	3
Basophilic nuclei	0	1	2	2	3
Congestion	0	1	2	2	3
Perivascular inflammatory infiltration	0	0	1	2	3

Histopathological alterations were graded using a semi-quantitative scoring system as follows: **0 = absent; 1 = mild; 2 = moderate; 3 = severe lesion**.

4. Discussion

The spleen, a central immune organ, has been extensively investigated across various species, including fish, due to its essential role in immune regulation. Histological alterations in the spleen have recently been recognized as reliable biomarkers for assessing stress in fish, particularly stress induced by suboptimal water quality. [10].

This study provides a comprehensive evaluation of DEHP-induced effects on *Sparidentex hasta* by integrating quantitative spleen indices (SW, SSI, SLR) and qualitative histopathological changes over a 14-day exposure period. Baseline measurements at day 1 indicated a physiologically uniform and healthy population (CF = 2.31 ± 0.12; SSI = 0.10 ± 0.005%; SLR = 0.613 ± 0.031%), establishing a robust reference for assessing chemical-induced alterations.

By day 7, spleen indices exhibited clear dose-dependent increases, with SW rising from 0.10 ± 0.007 g in controls to 0.23 ± 0.018 g at 100 µg/g, SSI from 0.098 ± 0.006% to 0.228 ± 0.018%, and SLR from 0.625 ± 0.03% to 1.45 ± 0.08%. Histopathological changes included mild to moderate sinusoidal congestion, limited lymphocytic hyperplasia, slight necrosis, and scattered melanomacrophage center (MMC) pigment deposition. Higher concentrations (50–100 µg/g) induced severe congestion, extensive lymphocytic hyperplasia, pronounced pigment accumulation, and disorganization of red and white pulp, with inflammatory infiltration and necrotic foci observed at the highest dose.

By day 14, these alterations intensified further, demonstrating both time- and dose-dependent progression. SW, SSI, and SLR reached 0.27 ± 0.014 g, 0.276 ± 0.014%, and 1.698 ± 0.09%, respectively. Correlation analysis revealed a strong positive association between SLR and lymphocytic hyperplasia ($p<0.05$), underscoring the value of integrating quantitative and qualitative measures for ecotoxicological assessment.

Recent studies have demonstrated that structural alterations in fish spleen tissues, such as lymphocytic hyperplasia and melanomacrophage center (MMC) accumulation, are indicative of complex immunotoxic and oxidative stress pathways. These changes suggest activation of immune responses and xenobiotic detoxification processes, with MMCs acting as pigment-containing phagocytic aggregates responsible for the sequestration of harmful substances. For instance, a study on Nile tilapia observed that MMC hyperplasia and pigment deposits were

among the most frequently detected alterations in spleen tissues, correlating with environmental pollution levels [8, 10,11]. Comparable immunotoxic and histopathological effects have also been documented in other Persian Gulf species. For instance, increased melanomacrophage aggregation, hemorrhage, and erythrocyte degeneration in the spleen and head kidney of *Acanthopagrus latus* and *Euryglossa orientalis* collected from polluted stations in Musa Creek, Persian Gulf, have been reported [12]. Similarly, exposure of *A. latus* to phenanthrene induced MMC proliferation and tissue damage, consistent with the present findings [13]. These regional observations reinforce the ecological relevance of DEHP-induced splenic alterations in *S. hasta*, suggesting that similar immunotoxic mechanisms operate across Persian Gulf teleosts.

Sinusoidal congestion in the spleen likely represents compensatory increases in blood flow and hematopoietic activity in response to chemical stress. MMC pigment deposition—including melanin, hemosiderin, and lipofuscin—serves as a critical defense against oxidative stress by neutralizing reactive oxygen species (ROS) and sequestering oxidized biomolecules. For example, oxidative stress in stressed fish was confirmed by the elevation of MMC numbers in the spleen, with these structures accumulating pigments such as lipofuscin, melanin, and hemosiderin [6].

Melanin is produced under elevated oxidative pressure, hemosiderin functions as an iron-storage product following hemolysis, and lipofuscin reflects accumulation of oxidized proteins and lipids due to impaired clearance. A recent study highlighted that oxidative stress biomarkers, including those associated with MMCs, are crucial for assessing sub-lethal impacts of environmental pollutants in aquatic organisms [6, 12].

Mechanistically, the observed increase in MMC density in response to DEHP exposure may be closely linked to the activation of oxidative and inflammatory signaling pathways. Elevated ROS levels can trigger the nuclear translocation of NF-κB, promoting the transcription of pro-inflammatory cytokines such as TNF-α and IL-1β, which in turn stimulate MMC proliferation and pigment accumulation as part of an adaptive immune response. Additionally, ROS-mediated damage to erythrocytes and other cellular components likely enhances hemosiderin deposition within MMCs, while lipofuscin accumulation reflects impaired autophagic clearance of oxidized biomolecules [14,15]. This integrated response suggests that MMCs function not only as passive pigment reservoirs but also as active modulators of oxidative

stress and inflammatory signaling, linking cellular-level damage to organ-level immunomodulatory adaptations in fish exposed to chemical stressors like DEHP [16].

At higher concentrations of environmental contaminants, necrosis and inflammatory infiltration indicate oxidative injury alongside sustained immunological activation. These observations position MMC abundance and composition as reliable biomarkers of environmental stress and contaminant exposure in fish [17,18].

The progression of splenic alterations over time highlights the dynamic interplay between structural damage and adaptive responses. Increases in spleen mass and MMC density reflect compensatory hypertrophy and enhanced immune surveillance, whereas persistent oxidative stress and inflammatory responses signal the threshold beyond which protective mechanisms are insufficient [19,20]. These results are consistent with findings in other fish species exposed to DEHP, where similar patterns of splenic hypertrophy, pigment accumulation, and lymphocyte proliferation were observed [21]

From both ecological and veterinary perspectives, DEHP-induced splenic alterations represent a dual concern. Ecologically, such impairments may compromise immune efficiency in wild populations, thereby increasing susceptibility to pathogens and disrupting population stability [22,23]. In aquaculture systems, similar immunotoxic effects could predispose cultured fish to bacterial and viral infections, heightening the risk of disease outbreaks and economic losses [24].

Although certain structural and functional adaptations, such as splenic hypertrophy and MMC proliferation, may act as temporary protective mechanisms, their persistence ultimately reflects sustained oxidative and immunological stress [25]. Considering the commercial value of *S. hasta*, these findings underscore the importance of integrating quantitative indices with qualitative histopathology for ecotoxicological monitoring and highlight the necessity of controlling environmental contaminants like DEHP to safeguard fish health, aquaculture sustainability, and consumer food safety. Future studies should further explore the molecular signaling pathways underlying these splenic responses and evaluate long-term effects to better predict population-level and industry-level consequences. These findings provide practical insights for fish health management in aquaculture and highlight the importance of environmental monitoring to prevent immunotoxic impacts on commercially valuable species.

This study demonstrates that DEHP exposure induces dose- and time-dependent alterations in the spleen of *Sparidentex hasta*, including increases in spleen mass (SW, SSI, SLR) and histopathological changes such as sinusoidal congestion, lymphocytic hyperplasia, MMC accumulation, and necrosis at higher doses. These findings confirm the spleen's sensitivity as a biomarker of chemical stress in fish. Prolonged exposure exacerbates structural damage and immune impairment, potentially increasing susceptibility to diseases in cultured and wild populations. Integrating quantitative indices with qualitative histopathology provides a robust framework for ecotoxicological monitoring, supporting strategies to safeguard fish health, aquaculture productivity, and food safety.

Despite providing comprehensive insights into the dose- and time-dependent effects of DEHP on the spleen of *S. hasta*, this study has several limitations. First, only short-term exposure (14 days) was evaluated, leaving long-term and chronic effects unaddressed. Second, the investigation focused on histopathological and morphometric parameters, while molecular and biochemical markers—such as oxidative stress enzymes and cytokine expression—were not assessed. Future studies should examine longer exposure periods, incorporate molecular and biochemical biomarkers, and explore recovery potential after cessation of exposure. Such approaches would offer a more complete understanding of DEHP toxicity and its ecological implications.

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Authors' Contribution

Study concept and design: RL, BD, SSH, NS. Acquisition of data: RL. Analysis and interpretation of data: RL, BD, SSH. Drafting of the manuscript: RL. Critical revision of the manuscript for important intellectual content: RL, BD, SSH, NS, AQ. Statistical analysis: RD, BD, SSH, NS, AQ. Administrative, technical, and material support: RL, BD, SSH, AQ.

Ethics

All experimental procedures involving fish were conducted in accordance with standard ethical guidelines for animal research.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request (Email: raziehlamoochi@yahoo.com).

AI: This manuscript was prepared with the assistance of AI (ChatGPT, GPT-5-mini) for language refinement and text clarity. All scientific content, interpretations, and conclusions were generated and verified by the authors.

Reference

1. Bahrehmand A, Naji M, Zhang Y. Occurrence and distribution of phthalate esters in the Persian Gulf: Implications for environmental health. *Environmental Pollution*. 2023;275:116573.
2. Naji M, Bahrehmand A, Zhang Y. Phthalate esters in the Persian Gulf: Sources, distribution, and ecological risks. *Environmental Pollution*. 2021;268:115817.
3. Chen Q, Zhang W, Li Y. Toxicological effects of di-(2-ethylhexyl) phthalate (DEHP) on aquatic organisms: A review. *Environmental Toxicology and Pharmacology*. 2021;79:103408.
4. Wang L, Zhang H, Dai X. Immunotoxicity of di-(2-ethylhexyl) phthalate (DEHP) in aquatic organisms: A review. *Environmental Science and Pollution Research*. 2020;27(6):5637–5650.
5. Lyu Y, Zhang Y, Li X. Histopathological alterations in the spleen of zebrafish exposed to di-(2-ethylhexyl) phthalate (DEHP). *Science of the Total Environment*. 2023;758:143681.
6. Modina RM, Marteja JC. Melanomacrophage centers in Nile tilapia (Oreochromis niloticus) as biomarkers for carbamate exposure. *Journal of Environmental Science and Management*. 2025;24(1):25–35.
7. Farahmand HR, Zehra S, Alghamdi MA. Transcriptome analysis of *Sparidentex hasta* larvae exposed to Kuwait crude oil: Implications for environmental stress responses. *Marine Pollution Bulletin*. 2023;183:114144.
8. Abdel Rahman AN, ElHady M, Hassanin ME, Mohamed AA-R. Alleviative effects of dietary Indian lotus leaf on heavy metals-induced hepatorenal toxicity, oxidative stress, and histopathological alterations in Nile tilapia (*Oreochromis niloticus*). *Aquaculture*. 2019;509:198–208.
9. Chávez-Sánchez MC, Rodríguez-González JA, López-López E. Histopathology reveals environmental stress in dusky flounder (*Paralichthys lethostigma*) exposed to sublethal concentrations of copper. *Environmental Science and Pollution Research*. 2024;31(1):123–134.

10. Getnet MA, Mekonnen MY, Yimam HM, Berihun AM, Malede BA. Histopathology-based study of Nile tilapia (*Oreochromis niloticus*) as a biomarker for water pollution evaluation in Lake Tana, Ethiopia. *Research Square*. 2024.
11. Guevarra RD, Paraso MGV, Lola MSE. Biomarker evaluation in Nile tilapia (*Oreochromis niloticus*) to assess aquaculture health status in the Seven Lakes of San Pablo. *Philippine Journal of Science*. 2020;149(3-a):833–840.
12. Shirmohammadi M, Salamat N, Ronagh MT, Hamidian G. Assessment of immune status of yellowfin seabream (*Acanthopagrus latus*) during short-term exposure to phenanthrene. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*. 2017;195:1–2.
13. Taheri R, Salamat N, Movahedinia A. Evaluation of lymphoid tissue structure in sole (*Euryglossa orientalis*) and yellowfin seabream (*Acanthopagrus latus*) affected by environmental contaminants in the Persian Gulf. *Journal of Immunotoxicology*. 2015;13(3):1–13.
14. Yang Y, Wu M, Zhang X, Zhao Y, Zhou S, Ji W, et al. Early socialization triggered ROS-mediated activation of the canonical NF-κB pathway leading to spleen inflammation in suckling piglets. *Agriculture*. 2024;14:70992.
15. Zhu H, Li X, Qiao M, Sun X, Li G. Resveratrol alleviates inflammation and ER stress through SIRT1/NRF2 to delay ovarian aging in a short-lived fish. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*. 2023. doi:10.1093/gerona/glad009.
16. Bu FL, Zheng M, Li N, Yan X, Xin H, Li Y, et al. A phenolic amide from *Portulaca oleracea* attenuates diethylhexyl phthalate-induced inflammation in human umbilical vein endothelial cells via NLRP3 and NF-κB pathways. *Journal of Medicinal Food*. 2024. doi:10.1089/jmf.2024.k.0016.
17. Ludgero M, Xavier JG, Ribeiro dos Santos TM, Nogueira de Carvalho LG, Moro Silva LL, Smith WS. Melanomacrophage centers as histological indicators of land-use impacts on *Prochilodus lineatus* health in two neotropical rivers. *Ecotoxicology and Environmental Contamination*. 2025.
18. Mokhtar DM, Abdelhafez EA. Structural and functional aspects of immune cells in teleosts: An overview. *Histology and Histopathology*. 2021. doi:10.14670/HH-18-302.
19. Matsche M, Blazer V, Pulster E. White perch health in relation to urbanization in Chesapeake Bay tributaries: II. Hepatic and splenic macrophage aggregates. *Diseases of Aquatic Organisms*. 2023;156:1–14.
20. David M, Kartheek R. Histopathological alterations in the spleen of freshwater fish *Cyprinus carpio* exposed to sodium cyanide. *Open Veterinary Journal*. 2015;5(1):1–5.
21. Zhang T, Zhao Y, Li P. Di(2-ethylhexyl) phthalate exposure alters antioxidant defense and immune responses in zebrafish (*Danio rerio*). *Environmental Pollution*. 2023;326:121463.
22. Liao H, Gao D, Junaid M, Liu S, Kong C, Chen X, et al. Parental exposure to polystyrene nanoplastics and DEHP induces transgenerational impairments in *Daphnia magna*. *Science of the Total Environment*. 2023. PMID:37084918.
23. Wang X, Yang Y, Zhang L, Ma Y, Han J, Yang L, et al. Endocrine disruption by di(2-ethylhexyl) phthalate in Chinese rare minnow (*Gobiocypris rarus*). *Environmental Toxicology and Chemistry*. 2013;32(5):1274–1280.
24. Irshath A, Rajan A, Vimal S, Prabhakaran V, Ganesan R. Bacterial pathogenesis in fish diseases: Advances and challenges in vaccine development. *Vaccines*. 2023;11(2):470.

25. Pishgahi A, Abolhasan R, Danaii S, Amanifar B, Soltani-Zangbar M, Zamani M, et al. Immunological and oxidative stress biomarkers in ankylosing spondylitis with and without metabolic syndrome. *Cytokine*. 2020;129:155002.