

Research Article



Pathological effects of *Aspergillus* toxicity on gill structure of *Litopenaeus vannamei* in Iran by two different toxicological investigations

Jamshidizadeh S.¹; Amrollahi Biuki N.^{1,2*}

Received: July 2021

Accepted: September 2023

Abstract

This histological study was conducted to discover the response of *Litopenaeus vannamei* to various levels of aflatoxigenic fungi (*Aspergillus*) toxicity. For this purpose, 400 specimens of live shrimp were gathered from a shrimp culture site in Iran. According to the results of the high-pressure liquid chromatography (HPLC) analysis, *Aspergillus parasiticus* had a higher production capability of total aflatoxin (TAF) (1073.804 ng g⁻¹) compared to *Aspergillus flavus* (292.349 ng g⁻¹). Two experiments with different toxicities of *Aspergillus* were assayed. In both experiments, the shrimps in 6 experimental groups were exposed to 0, 1, 5, 10, 15, and 20 ml of fungal spore suspension (FSS) in the feed (E1) and culture medium water (E2) in triplicate in each group for 4 weeks. The histopathology results of the gill tissue in the control group were normal. However, the inflammation, hemocytic infiltration, melanization, edema, and necrosis (as the main histopathological changes in the gill tissue) were observed after 28 days of the experiment when the toxicity of both experiments reached 18 µg kg⁻¹ total aflatoxin and 1 ml FSS in E1 and E2, respectively. Furthermore, it was recognized that the histological alterations index (HAI) of gill was higher in E2 (0-130) than in E1 (0-74).

Keywords: *Aspergillus parasiticus*, *Aspergillus flavus*, Aflatoxin, Gill, *Litopenaeus vannamei*

1-Department of Marine Biology, Faculty of Marine Science and Technology, University of Hormozgan, Bandar Abbas, Iran.

2-Department of Modern Technologies, Mangrove Forest Research Center, University of Hormozgan, Bandar Abbas, Iran.

*Corresponding author's Email: amrollahi@hormozgan.ac.ir

Introduction

In recent decades, with the expansion of the shrimp farming trade, there has been an urge to observe the physiological conditions of farm-raised penaeids regarding the incidence of many issues through this trade (Deng *et al.*, 2020; Fang *et al.*, 2020; Jannathulla and Dayal., 2023). Many researchers have reported the fungal contamination in the shrimp propagation process that caused economic losses for the global aquaculture industry (Zeng *et al.*, 2016; Deng *et al.*, 2020; Kracizy *et al.*, 2021; Su *et al.*, 2023). Also, various studies have shown that mycotoxins as a secondary metabolite of *Aspergillus* fungi have become a serious issue in the aquafeed and have also affected the health of aquatic animals especially aquatic invertebrates such as *Fenneropenaeus indicus* (Ghaednia *et al.*, 2013), Common carp (*Cyprinus carpio*) (Al-Rubaiy *et al.*, 2018) and *Litopenaeus vannamei* (Zhao *et al.*, 2017; Jamshidizadeh *et al.*, 2019; Wang *et al.*, 2019; Kracizy *et al.*, 2021).

Aflatoxin is an aromatic hydrocarbon that belongs to an oversized cluster of mycotoxins created by fungal species such as the *Aspergillus* genus, notably *A. flavus* and *A. parasiticus*, and species of the *Penicillium* and *Rhizopus* genera (Albero *et al.*, 2022; Mirza Alizadeh *et al.*, 2022; Smaoui *et al.*, 2023). These *Aspergillus* spp. create four aflatoxin varieties such as aflatoxins B1, B2, G1 and G2, which the symbols B and G represent abbreviations for Blue and Green fluorescent colors produced under UV irradiation (Al-Ghouti *et al.*, 2022;

Pisoschi *et al.*, 2023). Among these varieties, AFB1 is known as the most potent teratogenic and is considered a carcinogen, hepatotoxic, and immunosuppressant with the greatest toxicity for aquatic animals and humans (Alam *et al.*, 2022; Cao *et al.*, 2022; Gao *et al.*, 2023; Mohamed *et al.*, 2023).

In marine shrimp, the toxicity of AF can lead to abnormalities, such as physiological disorders, digestive function problems, poor growth, and histological changes, which decrease aquatic animal production (Zeng *et al.*, 2016; Jamshidizadeh *et al.*, 2019; Deng *et al.*, 2020; Kracizy *et al.*, 2021; Su *et al.*, 2023). To ensure the health of farm-raised shrimps and increase the efficiency of the aquaculture industry, the monitoring of aquatic feed and culture ponds for *Aspergillus* fungal contamination is an essential factor that is commonly used in most countries. Since fungi invasion is related to environmental conditions such as temperature and humidity and improper feed storage methods to the growth of toxigenic fungi, the probability of aflatoxicosis in an area with a humid tropical climate like the Hormozgan region in Iran is common. In this regard, preventing aflatoxicosis and minimizing the economic failure caused by aquatic diseases, depends on the monitoring of pathogenic microorganisms in aquaculture ponds.

In recent years, *Litopenaeus vannamei* (Boone, 1931) has become the most widespread crustacean species cultured worldwide, especially in Asian countries (Abdirad *et al.*, 2022;

Hembrom *et al.*, 2023; Mondal *et al.*, 2023). Although many researchers have studied the effects of various toxic substances on *Litopenaeus vannamei*, the effect of total aflatoxin (TAF) toxicity as a result of fungal contamination in shrimp farms has not been considered yet. Since the gills are closely related to both the hemolymph or blood system and external environment of aquatic animals which are generally multifunctional for acid-base balance, osmoregulation, respiration, and excretion of nitrogenous waste in shrimp, the tissue of this organ acts as a host of a secondary metabolite of toxigenic fungus (Callaghan *et al.*, 2016; Allen and Weihrauch, 2021). However, there is rare information about the effect of aflatoxins on the structures of the gill.

Therefore, in the present research, it was tried to determine the pathogenicity of *Aspergillus* spp. For this purpose, histopathology of the gill of *Litopenaeus vannamei* was assayed as a biomarker for monitoring the physiological conditions or controlling these economically important pathogens in shrimp farms.

Materials and methods

Research design

In this research, toxic substances were tested for the histopathological study of the gill by employing two following experiments for 4 weeks (Fig. 1): 1) E1 (experimental shrimps exposed to aflatoxin in feed) and 2) E2 (experimental shrimps exposed to aflatoxin in culture medium water).

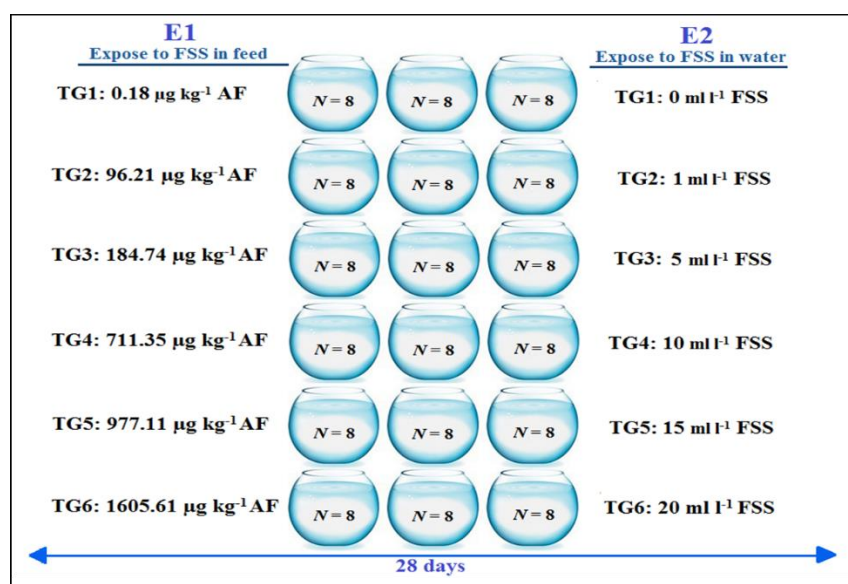


Figure 1: Design of experiments (E1 and E2). FSS: fungal spore suspension, AF: total aflatoxin detected in experimental diets.

In these experiments, several groups of 8 shrimps were introduced into glass containers with 20 l-adaptation culture medium water. In the first experiment (E1), shrimps in six testable groups were

fed with aflatoxin-contaminated diets for 28 days as follows: diet 1 (control) without FSS and diets 2-6 (with 1, 5, 10, 15, and 20 ml of FSS per kg). Aflatoxin concentrations of the experimental diets

were found to be 0.18, 96.21, 184.74, 711.35, 977.11, and 1605.61 $\mu\text{g kg}^{-1}$, respectively. In the second experiment (E2), various volumes of fungal spore suspension (FSS) were transferred to 6 groups of 20 l-culture medium water as follows: group 1 (control) without FSS and treatment groups 2-6 with 1, 5, 10, 15, and 20 (ml) of FSS per l, respectively.

Toxic substances

Fungal strains, growth medium, and growth conditions

In this study, two species of aflatoxigenic *Aspergillus* including *A. flavus* (PTCC 5018), and *A. parasiticus* (NRRL 5286) were used to compare the TAF production. The growth method of mentioned strains was modified as follows: Briefly, fungi were cultured on sabouraud dextrose agar (SDA) in Petri dishes containing medium and were allowed to grow in the dark and sterile environment in an incubator at 27°C for 7 days for the analysis of TAF production.

Quantification of total aflatoxin (TAF)

After 7 days of incubation in the dark at 27°C on SDA medium, samples were prepared and extracted for aflatoxin quantitation by HPLC according to a previous study (Abbas *et al.*, 2004). TAF (ng g^{-1}) in the samples was quantified to introduce a suitable species of *Aspergillus* with a high capacity of aflatoxin production for further process.

Experimental toxic substances

In the current study, the experimental protocol was modified and designed according to the previous studies (Casado *et al.*, 2001; Lim *et al.*, 2001; Bintvihok and Kositcharoenkul, 2006; Deng *et al.*, 2010). After comparing the TAF production capacity between experimental fungi, *A. parasiticus* was cultured on SDA as explained in the previous section. To track of research process, the conidiospore from the culture was collected in 1 ml of sterile water. The spore concentration of the suspension samples was determined using a hemocytometer as described in (Nesci *et al.*, 2003).

In the first experiment (E1), shrimps in six testable groups were fed with aflatoxin-contaminated diets for 28 days. The aflatoxin-contaminated shrimp meal was prepared using six group of commercial shrimp meal (100 gr) with the same basal combination (Table 1). Next, different volumes of FSS (with a concentration of 10^6 spore mL^{-1}) were placed on the diets with a moisture ratio of 20% separately, as follows: diet 1 without FSS (control) and diets 2-6 with 1, 5, 10, 15, and 20 (ml) of FSS per kg, respectively. To allow aflatoxin production, the mix is placed in an orbital shaker at 150 rpm and 27°C for 7 days. Then, the meal contaminated with aflatoxin was oven-dried at 90°C such that its moisture content was decreased to 10%. Finally, the TAF was subjected to feed analysis by the HPLC technique. The meal contaminated with aflatoxin was kept at -20°C until use in the feeding experiment. In the second

experiment (E2), various volumes of fungal spore suspension (FSS, with a concentration of 10^6 spore mL^{-1}) were transferred to 6 groups of 20 l-culture medium water as follows: group 1

(control) without FSS and treatment groups 2-6 with 1, 5, 10, 15, and 20 (mL) of FSS per l, respectively.

Table 1: Basal components of the shrimp feed (g kg^{-1} diet).

Ingredients	Content
Fish meal	200
Soybean meal	300
Wheat flour	245
Soy protein concentrate	70
Beer yeast	40
Chicken meal	30
Shrimp meal	30
Soy oil	20
Fish oil	10
Soy lecithin	10
Vitamin premix1	10
Mineral premix2	10
Monocalcium phosphate	20
Choline chloride	5
Total	1000

1 Vitamin premix (kg^{-1} diet): vitamin A, 250 000 IU; riboflavin, 750 mg; pyridoxine HCl, 400 mg; cyanocobalamin 1 mg; thiamine, 250 mg; menadione, 250 mg; folic acid, 125 mg; biotin, 10 mg; a-tocopherol, 2.5 g; myo-inositol, 8000 mg; calcium pantothenate, 1250 mg; nicotinic acid, 2000 mg; vitamin D3, 45 000 IU; vitamin C, 7000 mg.

2 Mineral premix (kg^{-1} diet): $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g; CaCO_3 , 37.9 g; KCl, 5.3 g; KI, 0.04 g; NaCl, 2.6 g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.02 g; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.9 g; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.03 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.5 g; $\text{Ca}(\text{HPO}_4)_2 \cdot 2\text{H}_2\text{O}$, 9.8 g.

Shrimp and experimental conditions

In the present study, live and healthy samples (Juvenile Pacific white shrimps, *Litopenaeus vannamei*), with an average weight of 9.74 ± 1.52 (g), were caught from the Tiab located in the east of Bandar Abbas, Hormozgan province ($56^\circ 51' \text{E}$ and $27^\circ 06' \text{N}$) in Iran and were transferred to the laboratory of Hormozgan University. The location of the sampling site is shown in Figure 2. Then, for the aim of adaptation, 8

shrimps were put into 18 fiberglass containers ($\text{DO } 7.71 \pm 0.74 \text{ mg/L}$, $30.09 \pm 2.84 \text{ ppt}$, $\text{pH } 8.23 \pm 0.32$, and $26.06 \pm 2.80^\circ\text{C}$) for two weeks. Shrimp feed pellets were used for feeding shrimps four times a day during the adaptation period. The water was replaced every 24 h and the experiment was carried out for 28 days. Dead samples were eliminated and recorded during the experiment.

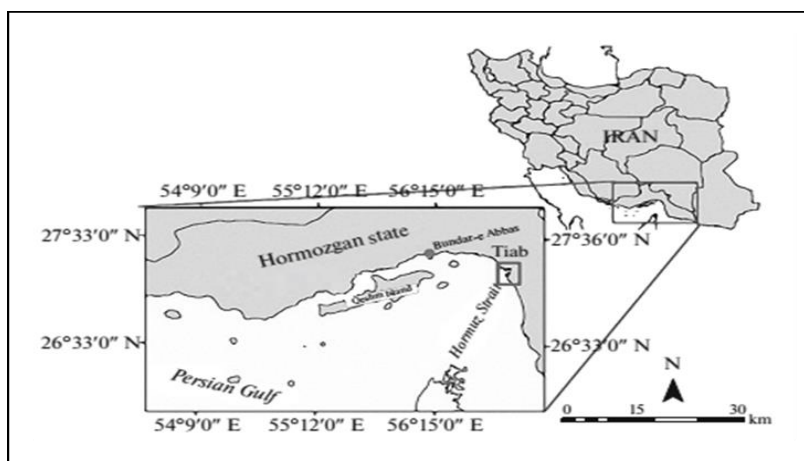


Figure 2: Location of sample collection.

Collection of sample

Shrimps were deprived of feed for 24 h before the sample collection process. Next, three shrimps from each aquarium were caught and their gill was separated for histological studies.

Histopathological process

Preparation of tissue slides

After 4 weeks of exposing the experiment, the gill of shrimps was evaluated using a light microscope (OLYMPUS) through routine histological methods. The gills were immersed in the Bouin's solution fixative for 24 to 72 h to prevent autolysis of this organ before further processing and were exposed to ethyl alcohol 70% for long-term maintenance. Next, following the routine H&E (Harri's hematoxylin and eosin phloxine) stain protocol, tissues were prepared for microscopic evaluation. Briefly, gills were dehydrated via a series of enhancing ethanol concentrations cleared in xylene and infiltrated with liquid paraffin at 57°C using an automatic processor of tissue (DS 2080/H). Afterward, they were

placed in paraffin as a tissue paraffin block. After trimming of tissue blocks, they were cut into 5-µm thick slides using a semiautomatic-rotary microtome. Finally, the samples were dyed with H&E stain protocol. The tissue slides were examined through an optical microscope and photomicrographs were performed by a digital microscope camera (Dino Eye AM423X, Taiwan).

Histological alterations index (HAI)

After investigating the photomicrographs of tissue slides, the histological damage intensity of the gill in experimental shrimps was measured as an HAI. Tissue injuries were categorized into three progressive stages during this method. Tissue damage levels are denoted as stage I, stage II, and stage III (Table 2) with "no effect on organ operation", "more intense injury and impaired organ operation", and "very intense and irretrievable changes to operation and structure", respectively (da Silva Montes *et al.*, 2011).

Table 2: Classification of the histological alterations found in gill of *Litopenaeus vannamei* examined during experiments (da Silva Montes *et al.*, 2011).

Stage I	Stage II	Stage III
Curling and clubbing of the lamellae	Inflammation of lamella	Necrosis
Lamellar epithelial hyperplasia	Hemocytic infiltration	
Atrophy of lamellae	Melanization	
Oedema	Pillar cell disruption	
Desquamation of epithelial lamellae		

Values of HAI were determined according to the following formula

(Poleksić and Mitrović-Tutundžić, 1994):

$$\text{HAI} = [1 \times \Sigma (\text{stage I})] + [10 \times \Sigma (\text{stage II})] + [100 \times \Sigma (\text{stage III})]$$

In this histologic analysis, the average HAI value calculated using this method was classified into five categories: 0-10 (normal tissue operation), 11-20 (mild to moderate change), 21-50 (moderate to severe change), 51-100 (severe change), and >100 (irreparable change).

Statistical analysis

Statistical analysis of this research and plotting the graphs were carried out using SPSS 22 and Excel software packages, respectively. HAI variables were compared utilizing an independent Student's t-test ($p < 0.05$). Other experimental parameters were compared by a One-way and Two-way ANOVA test ($p < 0.05$). Eventually, the comparison between experimental groups was made by Duncan's multiple range test ($p < 0.05$).

Results

Aflatoxin levels produced by experimental fungi

After incubation of *A. flavus* and *A. parasiticus* in the dark at 27°C for 7 days, TAF was extracted from each sample. Table 3 shows levels of aflatoxins (ng g^{-1}) as mean \pm standard deviation of three independent plates for each fungal strain. Compared with *A. flavus*, aflatoxin production by *A. parasiticus* was found to be significant ($p < 0.05$) after 7 days. A higher level of aflatoxin with significantly different compared with *A. flavus* ($p < 0.05$) associated with AFB1 by 86.2% TAF ($925.895 \text{ ng g}^{-1}$) in the *A. parasiticus* strain. In contrast, AFG1 and AFG2 production by *A. flavus* was not detected. Estimating the aflatoxin by HPLC method revealed the significant production of TAF in *A. parasiticus* by $1073.84 \text{ ng g}^{-1}$, indicating a 27.2% increase from $292.349 \text{ ng g}^{-1}$ by *A. flavus*.

Table 3: Production of aflatoxins by experimental *Aspergillus* spp.

<i>Aspergillus</i> spp.	B1	Aflatoxin type			Total aflatoxin
		B2	G1	G2	
<i>A. flavus</i> (ng g ⁻¹)	5.528±0.603 ^b	286.821±23.690 ^a	NO	NO	292.349
<i>A. parasiticus</i> (ng g ⁻¹)	6.415±1.127 ^c	925.895±24.241 ^a	4.477±1.205 ^c	137.017±39.012 ^b	1073.804

Note: Data represent mean±SD of three replicates. Values with different superscripts in the same column indicate statistical difference at $p<0.05$

Aflatoxin levels in the experimental diets
In current study, aflatoxin contamination (0.18 µg kg⁻¹) was detected in diet without FSS (control group). However, aflatoxin concentrations of diets 2-6

(with 1, 5, 10, 15, and 20 ml of FSS per kg) were found to be 96.21, 184.74, 711.35, 977.11, and 1605.61 µg kg⁻¹, respectively (Table 4).

Table 4: Aflatoxin levels of the experimental diets.

Experiment al diets	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Fungi spore suspension (mL kg ⁻¹)	0	1	5	10	15	20
Detected Aflatoxin (µg kg ⁻¹)	0.18±0.02 ^f	96.21±3.80 ^e	184.74±25.33 ^d	711.35±30.12 ^c	977.11±35.07 ^b	1605.61±44.18 ^a

Note: Data represent mean±SD of three replicates. Values with different superscripts in the same column indicate statistical difference at $p<0.05$.

Gill histopathology and histological alterations index
In the present research, histological evaluation of the gill of shrimps indicated that the gill tissue of the

control group was normal; like the typical histologic structure previously introduced for Penaeidae shrimps (Fig. 3).

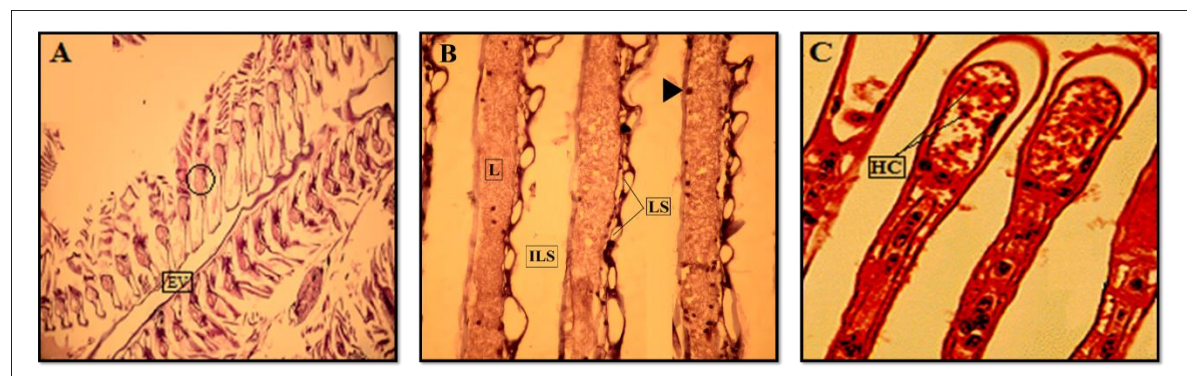


Figure 3: Normal structure of gill of *Litopenaeus vannamei*. Circle: each tip of filament is occupied by a hemolymphatic lacuna, EV: efferent vessel (a); L: normal lamellae structure, ILS: uniform interlamellar spaces, LS: lamellar sinus, Triangle: pillar cells (b); HC: hemocytes (c). Scale bars: a and b: 20µm; c: 10µm.

However, according to the study of tissue slides photomicrographs, when the feed and culture medium water was contaminated for experimental shrimps, notable histological damage was observed in shrimps from both experiments (Figs. 4 and 5). In E1, the gills indicated hemocytic infiltration and epithelial inflammation at a concentration of $711.35 \mu\text{g kg}^{-1}$ AF. Pathology of melanization was presented when the concentration of AF exceeded $977.11 \mu\text{g kg}^{-1}$ AF. Furthermore, a maximum HAI value of 48.3 and 74.3 was observed in the treatment groups of 5 and 6, suggesting moderate to severe and severe changes to the gills (Fig. 6). The fusion of lamella

and hyperplasia of the basal epithelial cells was observed at a concentration of 711.35 until $1605.61 \mu\text{g kg}^{-1}$ AF (with different severities). (In E2, similar pathologies were detected coupled with necrosis, atrophied hemolymphatic lacuna, curling, and clubbing of the lamellae. This result is associated with the level of aflatoxin on the branchial lamellae (Fig. 5). These observations were reflected in the enhanced HAI values in treatments 4, 5, and 6 with means of 85.3, 117.6, and 130.3, respectively, indicating damage shift from severe to irreparable alteration in gill structure.

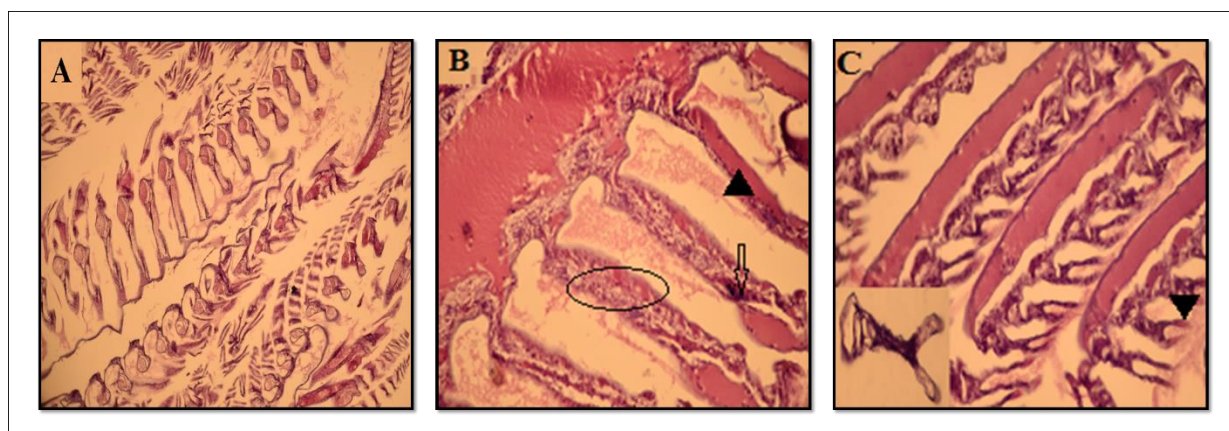


Figure 4: Gill structure of *Litopenaeus vannamei* exposed to aflatoxin in E1. Abnormal lamella (a); Arrow: melanisation, Circle: oedema, Triangle: haemocytic infiltration (b); Triangle: clubbing of the lamellae (c). Scale bars: a: 20µm; b and c: 10µm.

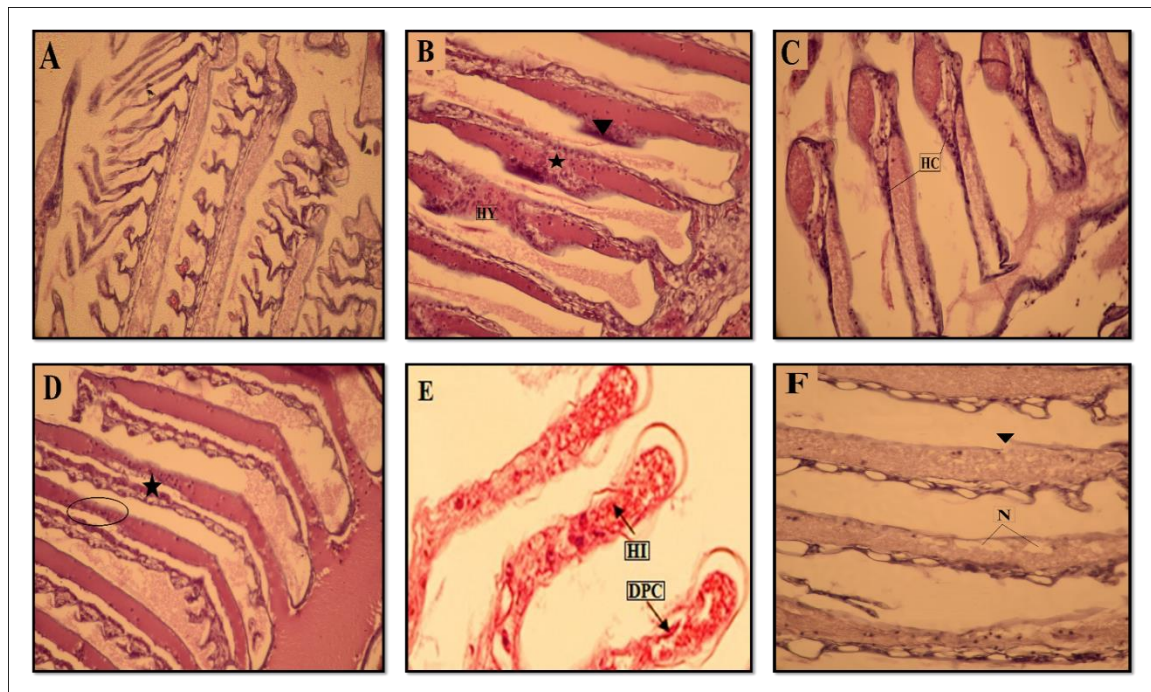


Figure 5: Gill structure of *Litopenaeus vannamei* exposed to aflatoxin in E2. Dissemination of gill lamellae coupled with curling and clubbing of the lamellae (a). HY: hyperplasia, Star: inflammation with accumulation of hemocytes, Triangle: melanization (b); HC: accumulation of hemocytes (c); Circle: atrophy of lamellae, Star: accumulation of hemocytes (d); DPC: disrupted pillar cells, HI: haemocytic infiltration (e); N: Necrosis in gill lamellae, Triangle: desquamation of the cuticle (f). Staining: H&E. Scale bars: a: 20µm; b-f: 10µm.

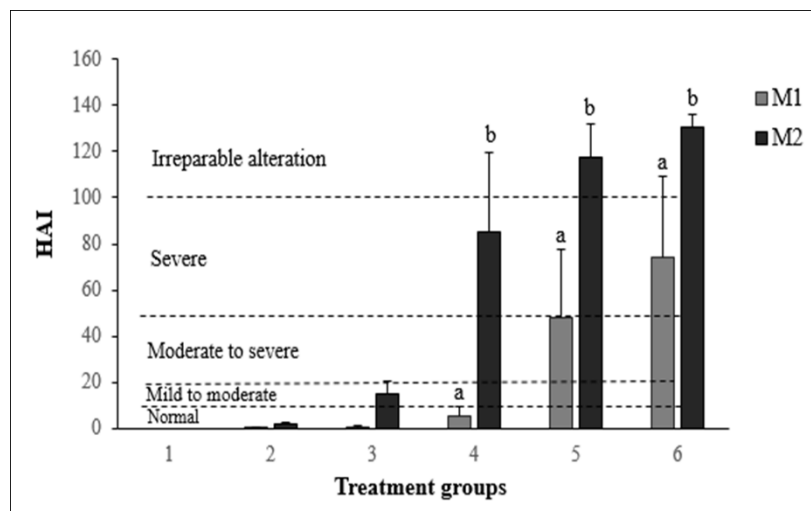


Figure 6: Histological alteration index (HAI±SD) values of gills in E1 (gray bars) and E2 (black bars). Different letters indicate significant differences ($p < 0.05$) between experiments in the same treatment groups.

Figure 6 presents the values of HAI in both experiments in the experimental shrimps, which are significantly different between experiments in treatment groups of 4-6 ($p < 0.05$).

According to HAI values, the observed alterations in the gill of treatment shrimps were relevant to the concentration of dietary aflatoxin. Therefore, by increasing the content of

aflatoxin in the diet, the HAI value was also increased significantly ($p < 0.05$). Evaluating the tissue photomicrographs indicated a maximum of HAI with a mean of 74.3 and 130.3 in a sixth treatment group in E1 and E2, respectively, indicating severe and irreparable alteration. Small values of HAI were detected to be equal to 0, 0.3, 0.6, 5 and 48 in E1 and 2, 15, 85, 117 and 130 in E2.

Discussion

All infective microorganisms possess some attributes that permit them to invade and cause harm to organisms that are exposed to their toxic substances. Toxicity is a term that refers to the flexibility of a being to provide toxins capable of reworking the conventional operation of cells or tissues and destroy them. Since some toxins are secreted outside the producer microorganism and cause severe harm after they penetrate the host organism (Atlas, 1995), the toxicant substances of *Aspergillus* spp toward shrimp were very high, showing their important histopathological effects.

Many researchers have reported that some strains of fungi are isolated from aquaculture ponds, aquafeed, and even the body of shrimp. A study of Ochoa *et al.* (2015) recognized that strains of *Geotrichum* and *Fusarium* isolated from the walls and bottoms of the culture ponds of *L. vannamei*. Also, aquaculture center in Asian countries like southern provinces of Iran is thought to be potential locations for fungal contamination (Gonçalves *et al.*, 2017; Gonçalves *et al.*, 2018a, b;

Jamshidizadeh *et al.*, 2019). This fungal contaminant might have occurred due to factors such as the low quality of aquafeed and pond water caused by inappropriate management of shrimp farms in a wet tropical climate. Therefore, such places provide a suitable spot for mold growth, followed by contamination of shrimp feed and water with fungal substances. Lahouar *et al.* (2016) have shown that temperature and water activity affect *Aspergillus* spp growth, leading to aflatoxin production. Among the isolated fungal strains such as *Penicillium*, *Fusarium*, *Cladosporium*, *Candida*, and different genera, *Aspergillus* spp is known to be the most important and powerful fungal species to produce aflatoxins, which are mycotoxins known as toxigenic potential (Guo *et al.*, 2018; Yu *et al.*, 2020; Navale *et al.*, 2021; Wei *et al.*, 2023).

Production of aflatoxin is reported to occur by 3 major species of *Aspergillus*; *A. flavus*, *A. parasiticus*, and less frequently by another *Aspergillus* species (Ahmed *et al.*, 2020; Navale *et al.*, 2021). Many researchers have recognized variations within the substance production of *A. flavus* and *A. parasiticus*, although they are closely connected species (D'Mello and Macdonald, 1998; Klich and Pitt, 1988; Do and Choi, 2007; Nikolic *et al.*, 2018; Rushing and Selim, 2019; Ráduly *et al.*, 2020). In the current experiment, the aflatoxin production capability of important *Aspergillus* spp was analyzed with HPLC. As shown in Table 3, *A. parasiticus* synthesizes AFB and AFG

types, whereas *A. flavus* creates only AFB. Therefore, *A. parasiticus* produces a high concentration of TAF (1073.804), suggesting a better production capability of TAF than *A. flavus*. Many researchers have reported that over 90% of all *A. parasiticus* produces high concentrations of aflatoxin, as well as B and G types. On the opposite side, it has been reported that no more than 50% of *A. flavus* can synthesize toxins and if they produce simply B aflatoxins (Rushing and Selim, 2019; Priesterjahn *et al.*, 2020). In another study (Priesterjahn *et al.*, 2020), *A. flavus* produced aflatoxins B1 and B2, whereas *A. parasiticus* generated aflatoxins B1, B2, G1, and G2. According to these results, in the current study, *A. parasiticus* was chosen for analysis of the toxicity of Aflatoxigenic *Aspergillus* and utilized for the preparation of experimental diets, water, and tracking the further process.

Nowadays, increasing amounts of plant proteins such as soybean, peanut, corn, cottonseed, or wheat are being combined into aquatic animal feeds to avoid the heavy prices of animal-based resources (Hua *et al.*, 2019; Dawood and Koshio, 2020; Salehpour *et al.*, 2022). A high amount of these plant components are polluted with mycotoxins over time (Dawood and Koshio, 2020; Jia *et al.*, 2022). The most mycotoxins typically detected in aquaculture feed are different types of aflatoxin (AFB1, AFB2, AFG1, and AFG2) (Ottinger and Kaattari, 1998; Huang *et al.*, 2011). In current research, it was discovered that although FSS was not added to the control shrimp diet, 0.18 $\mu\text{g kg}^{-1}$ of TAF was detected. This result

seems to be due to a suitable climate for fungus growth, the low quality of shrimp feed components, and improper strategies of shrimp processing and aquafeed storage in Hormozgan, Iran. Many studies have shown the prevalence of aflatoxin in industrial aqua feeds (Gonçalves *et al.*, 2017; Gonçalves *et al.*, 2018a; Gonçalves *et al.*, 2018b; Hassaan *et al.*, 2020). The analysis of the mycotoxin prevalence in Asian countries (Gonçalves *et al.*, 2018b) indicates the common aflatoxin contamination of commercial aquafeed in this region, which is due to the climate conditions of these countries. This researcher has reported that aflatoxin contamination in 69% of the aqua feed samples caused a high level of aflatoxin with a quantity of 51.82 $\mu\text{g kg}^{-1}$. Bautista *et al.* (1994) examined industrial shrimp feeds in the Philippines and reported different levels of AFB1 contamination from non-found to 120 $\mu\text{g kg}^{-1}$. Deng *et al.* (2010) reported AFB1 contamination of 62.6, 13.3, and 1.6 $\mu\text{g kg}^{-1}$ in peanut meal, soybean meal, and fish meal, respectively. Bintvihok *et al.* (2003) indicated the presence of aflatoxin in shrimp feeds utilized in aquaculture industry. Zeng *et al.* (2016) found 16.9 $\mu\text{g kg}^{-1}$ of aflatoxin B1 within the control shrimp diet with no additional aflatoxin. Other studies also detected aflatoxin in the control aquatic diet (Han *et al.*, 2010; Rajeev Raghavan *et al.*, 2011; Huang *et al.*, 2014).

In aquatic animals, the gills are multi-functional organs that operate between the animal and the ambient environment. Gills are referred to as the foremost

susceptible organ because they are subjected to all or any toxicant pathogens within the environment (Salehpour *et al.*, 2021; Lavanya and Dayakar, 2022). Also, Randall *et al.* (1998) incontestable that the gills are often a significant route of uptake even for chemical contamination. Histopathological changes in respiratory organ structure by any annoyance materials like poison dissolved or suspended within the water directly affect the mechanisms of respiration and osmoregulation. They eventually might lead to hypoxia, impaired gas exchange, metabolism failure, and issues with the ionic and acid-base balance of aquatic animals. (Callaghan *et al.*, 2016; Allen and Weihrauch, 2021). Hence, aflatoxicosis may have an economically important impact on the production level of aquaculture farms. According to these issues, in the current study, histopathological damages of this substance in the gill of *L. vannamei* exposed to *A. parasiticus* metabolite were evaluated.

The histologic survey of the control gills within the present research and other scientists (de Araujo and Valenti, 2018), demonstrates that the gill structures of shrimps are shaped primarily by normal lamellae structure, efferent vessel, pillar cells, lamellar epithelium, hemocytes, and hemolymphatic lacuna. The role of hemocytes as a part of the cellular psychoanalytic process of crustaceans has been proved. Also, their larger presence is regarded as a symptom of inflammation (Frischer *et al.*, 2022).

Despite, increased level of hemocytes in the gill layer of *L. vannamei* can be a sign of blood circulation damage caused by inflammation. Shahafve *et al.* (2017) evaluated histopathology of gills in the fish treated with sublethal levels of aflatoxin (0.5, 0.7, and 1.4 mg kg⁻¹ feed) for 21 days. Necrosis of gills, curling, and clubbing of the lamellae, inflammation of cells, and gill epithelium necrosis are the main histopathological alterations reported by this scientist within the gill of experimental fish. Necrosis is a lesion in which the cell cytoplasm is uniformly stained in all parts of the cell. In fact, necrosis is a sign of a decrease in cell activity and cell death. Eventually, these cells are cytolysis or phagocytes by lymphocytes (Gente *et al.*, 1999; Xu *et al.*, 2022).

Necrosis in the gill indicates aflatoxin effects on the endothelial cells of the vascular system and most pathologic damages are determined in these cells due to their sensitivity to aflatoxin (Zeng *et al.*, 2019). Similar changes were determined in rohu fish (*Labeo rohita*) exposed to AFB1 (Sahoo *et al.*, 2001). This researcher reported epithelium hyperplasia and cell necrosis in gill lamellae of rohu fed with aflatoxin-contaminated. Similar changes are determined in grass carp (*Ctenopharyngodon idella*), rainbow trout (*Oncorhynchus mykiss*), Nile tilapia (*Oreochromis niloticus*), juvenile maroon clownfish (*Premnas biaculeatus*), and crab (*Portunus pelagicus*) (Romano and Zeng, 2007; Rodrigues *et al.*, 2014; Abdel-Daim *et*

al., 2020; Imani *et al.*, 2020; He *et al.*, 2023). Al-Azri *et al.* (2015) found the hyperplasia of the primary lamellae's epithelial layer, desquamation of epithelial cells, and cellular degeneration at aflatoxin concentrations more than 100 µg, which led to necrosis of epithelial tissues of gill by day thirty of the test. Hyperplasia of gill filaments is a long-term response of squamous cells that often occurs in response to low levels of harmful agents. Epithelial cell hyperplasia is a chronic response to microbial and parasitic infections or chemical agents. The destruction of pillar cells is due to the intense flow of blood into the gill blades or even the direct effect of pollutants on these cells. Swelling of the lamellae is the reason for chronic changes in the gill structure. By microscopic examination, it is clear that the epithelial cells accumulate at the upper layer of lamella, so they are club-shaped. (Sharifpour *et al.*, 2011).

According to the literature, several studies report that toxic substances caused changes in the structure of the gill and the histological damage was enhanced by raising the concentration of toxic substances in gills. Nevertheless, the effects of fungal toxic metabolite in medium water of aquatic animals have received less attention. Soegianto *et al.* (2013) discovered that copper caused changes in the structure of the gill, where the histologic injury deteriorated with rising copper concentration, leading to gill hyperplasia and necrosis. Usman *et al.* (2013) stated that the gill structure of *L. vannamei* juveniles considerably altered with a sublethal exposure of lead

for 15 days. Such exposure affects its organs including gill hyperplasia, hemocytic accumulation in lamellae, and inflammation. Moreover, these serious structural injuries suggest that copper may prevent the physiological functions of the gill. This researcher also showed that histologic injury will increase with increasing lead concentration. Similar microscopic anatomy alterations are discovered in different crustaceans subjected to heavy metals (Frías-Espéricueta *et al.*, 2008; Shukla *et al.*, 2019; de Jesus *et al.*, 2021) According to this study, once crustaceans are subjected to various levels of metals, they show changes including a blackened look of the gills, necrosis, and hyperplasia of gill cells, abnormal structure of the epithelium, and desquamation of cuticle. Furtado *et al.* (2015) found hyperplasia in the gills of *L. vannamei* subjected to concentrations of nitrate for a long period of the experiment. Lamela *et al.* (2005) identified edema within the gills after they cultured *L. schmitti* below low salinity and attributed it to a discount in osmoregulatory capability that influenced vascular permeableness.

According to the results of the present study, water and feed quality in deterioration aquaculture centers triggered changes to the structure of the gill and also the microscopic anatomy injury with an increasing quantity of FSS in water. Similarly, TAF in feed resulted in abnormal lamella (clubbing of the lamellae), edema, inflammation, hemocytic infiltration, and melanization (Figs. 4 and 5). In the current study, to

match the intensity of lesions in shrimps subjected to various experiments of aflatoxin contamination, histopathological study results of every experimental group were quantified semi quantitatively as histological alternation index (HAI) of gill (Table 2).

Pathologies that were maintained at stage I and were thought to be normal structures, after 4 weeks of both experiment, have turned into stage II tissue damage, actually changed from moderate to irreparable alteration. Within the treatment groups, HAI value in E1 grows at a lower rate than E2 reaching a severe level. However, injuries in E2 converted significantly to irreparable alteration that caused by water pollution with aflatoxin. In treatment group 4, once a normal structure of the gill was maintained in E1, injury in E2 accrued to severe alteration significantly. Also, when toxicity increased in treatment groups 5 and 6, the tissue injury accrued from moderate and severe in E1 severally to irreparable alteration in E2. Moreover, analysis of HAI indicated that the type and magnitude of histopathological alterations in gill tissue vary based on aflatoxin level in both experiments. So, once the largest amounts of toxicity were detected in treatment groups 5 and 6, the injury to the gills became irreparable and could have induced the high toxicity in experiment E2 (Fig. 6). Moreover, concerning the values of HAI (>100) in treatment groups exposed to $FSS \geq 15$ mL, we discovered cellular degeneration caused by changes from stage-3 lesions, like necrosis of the gill epithelial tissues

of these experimental groups with a completely various severity in E2.

A Histological study by Ferguson (1989) recognized that the hyperplasia of gill lamellae could also be elicited by the impact of the poisonous substance that changes glycoprotein within the mucous secretion covering the cells. Therefore, it influences the negative bars of the epithelium and inflicts interpolation to the adjacent lamellae. It seems that exposure to aflatoxin also enhanced the secretion of mucus by the gills, probably resulting in disabled gas exchange. Thus, they enhance the thickness of the epithelial cells that are in contact with the external environment. These alterations within the epithelial cells are examples of protection mechanisms against solid or microbial agents in suspension. Accordingly, they enhance the gap between the blood and the external environment. Eventually, they serve as an obstacle to the importation of contaminants (Hadi *et al.*, 2009), due to the impaired oxygen uptake (Figueiredo-Fernandes *et al.*, 2007).

The mentioned damage due to the presence of *Aspergillus* spp in the feed and water used in the aquaculture industry indicates the disability of gill function. In this regard, its potential to affect the respiratory/osmoregulatory capacity of *L. vannamei* can cause an economically significant effect on the production of farm-raised shrimp. Large amounts of toxicants, which contaminate shrimp farms through feed and water, are accumulated in gill filaments, covering their surfaces and

blocking the chambers of gill. Metabolic acidosis and tissue hypoxia and damage to immune system, as well as the flexibility of shrimp to infection are caused and as a result the adverse effects of poisons (Bauer, 1999; Scholnick *et al.*, 2006; Martin and Hose, 2010). Shrimp subjected to contaminant stress have low inviolability and reduced resistance against numerous toxicant substances (Liao *et al.*, 2012; Li *et al.*, 2017). In our experiment, contaminated feed and water confirm the histologic response in *L. vannamei*.

In the present study, more damage within the gills was determined in shrimp that were exposed to greater toxicity by the rise within the *A. parasiticus* spore suspension (up to 15 and 20 mL L⁻¹) in water (E2). The main damages related to the gills were hemocytic infiltration, lamellae inflammation, melanization, and necrosis. In this research, three phases of histological changes were found in gills under enhancing toxicity in feed and water: (i) inflammation in gill lamellae and edema; (ii) hemocytic infiltration by toxic detection; and (iii) melanization and necrosis. These pathologies can cause moderate to irreversible changes resulting in respiratory failure and a high risk of gathering the cultured shrimp. Besides, this study discovered that once toxicity increased in both experiments, a greater histological alteration index (HAI) is recorded in gills. This response reveals the sensibility of the gills to the existence of aflatoxigenic *Aspergillus* in feed and water. Impairment of gill function can cause respiratory loss of *L.*

vannamei, leading to an economically significant effect on aquaculture. Since these fungi are capable of producing mycotoxins, further studies are necessary to regulate and interfere with these economically important pathogens in aquaculture. Thus, this study suggests monitoring the physiological conditions as a biomarker to control and prevent these economically important pathogens. Further research works should also be conducted to assess the impact of the toxic secondary metabolite of fungus at the histological levels in other organs of shrimp.

Acknowledgments

We would like to acknowledge the Faculty of Marine Science and Technology, University of Hormozgan, Bandar Abbas, Iran.

References

- Abbas, H.K., Zablotowicz, R., Weaver, M., Horn, B., Xie, W. and Shier, W., 2004.** Comparison of cultural and analytical methods for determination of aflatoxin production by Mississippi Delta *Aspergillus* isolates. *Canadian Journal of Microbiology*, 50(3), 193-199. DOI:10.1139/w04-006
- Abdel-Daim, M.M., Dawood, M.A., Aleya, L. and Alkahtani, S., 2020.** Effects of fucoidan on the hematic indicators and antioxidative responses of Nile tilapia (*Oreochromis niloticus*) fed diets contaminated with aflatoxin B1. *Environmental Science and Pollution*

- Research*, 27(11), 12579-12586. DOI:10.1007/s11356-020-07854-w
- Abdirad Z, Ghaednia B, Kakoolaki S, Mirbakhsh M. and Ghorbani Vaghei R., 2022.** The effects of rearing Pacific white-leg shrimp (*Litopenaeus vannamei* Boone, 1931) in biofloc system on the immune responses and survival rate in challenge with *Vibrio harveyi*. *Iranian Journal of Fisheries Sciences*, 21(3), 705-725.
- Ahmed, A. E., AL-Kahtani, M. M., El-Diasty, E. M., Ahmed, A. S., Saber, H., Abbas, A. M. and Hussein, M. A., 2020.** Diversity of Toxigenic Molds and Mycotoxins Isolated from Dairy Products: Antifungal Activity of Egyptian Marine Algae on *Aspergillus* and *Candida* Species. *Journal of Pure & Applied Microbiology*, 14(1). DOI:10.22207/JPAM.14.1.23
- Alam, S., Nisa, S. and Daud, S., 2022.** Mycotoxins in environment and its health implications. *Hazardous Environmental Micro-pollutants, Health Impacts and Allied Treatment Technologies*, 28(5), 289-318. DOI:10.1007/978-3-030-96523-5-12
- Al-Azri, H.H., Ba-Omar, T., Elshafie, A. and Barry, M.J., 2015.** Histopathological and Ultrastructural Changes in the Liver and Gills of the Killifish *Aphanius dispar* (Cyprinodontidae) Exposed to Aflatoxin B1. *Sultan Qaboos University Journal for Science*, 20(1), 1-10. DOI:10.24200/squjs.vol20iss1pp1-10
- Albero, B., Fernández-Cruz, M. L. and Pérez, R. A., 2022.** Simultaneous Determination of 15 Mycotoxins in Aquaculture Feed by Liquid Chromatography–Tandem Mass Spectrometry. *Toxins*, 14(5), 316. DOI: 10.3390/toxins14050316
- Al-Ghouthi, M.A., AlHusaini, A., Abu-Dieyeh, M.H., Abd Elkhabeer, M. and Alam, M.M., 2022.** Determination of aflatoxins in coffee by means of ultra-high performance liquid chromatography-fluorescence detector and fungi isolation. *International Journal of Environmental Analytical Chemistry*, 102(18), 6999-7014. DOI:10.1080/03067319.2020.1819993
- Allen, G.J.P. and Weihrauch, D., 2021.** Exploring the versatility of the perfused crustacean gill as a model for transbranchial transport processes. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 254, 110572. DOI:10.1016/j.cbpb.2021.110572
- Al-Rubaiy, A.G., AL, I.A.A.A.J. and Rudainy, I.B., 2018.** Toxicity effects of aflatoxin B1 on growth indices and histopathological alteration in *Cyprinus carpio*. *Iraqi journal of biotechnology*, 17(3).
- Atlas, R.M, 1995.** Microorganisms in our World. Mosby Year Book, Inc, USA. pp5-13.
- Frischer, M. E., Landers, S. C., Walker, A. N., Powell, S. A. and Lee, R. F., 2022.** Black gill in marine decapod crustaceans: A review. *Reviews in Fisheries Science &*

- Aquaculture*, 30(4), 498-519.
DOI:10.1080/23308249.2022.2047153
- Bauer, R.T., 1999.** Gill-cleaning mechanisms of a dendrobranchiate shrimp, *Rimapenaeus similis* (Decapoda, Penaeidae): Description and experimental testing of function. *Journal of Morphology*, 242(2), 125-139. DOI:10.1002/(SICI)1097-4687(199911)242
- Bautista, M.N., Lavilla-Pitogo, C.R., Subosa, P.F. and Begino, E.T., 1994.** Aflatoxin B1 contamination of shrimp feeds and its effect on growth and hepatopancreas of pre-adult *Penaeus monodon*. *Journal of the Science of Food and Agriculture*, 65(1), 5-11. DOI:10.1002/jsfa.2740650103
- Bintvihok, A., Ponpornpisit, A., Tangtrongpiros, J., Panichkriangkrai, W., Rattanapanee, R.K. and Kumagai, S., 2003.** Aflatoxin contamination in shrimp feed and effects of aflatoxin addition to feed on shrimp production. *Journal of Food Protection*, 66(5), 882-885. DOI:10.4315/0362-028X-66.5.882
- Bintvihok, A. and Kositcharoenkul, S., 2006.** Effect of dietary calcium propionate on performance, hepatic enzyme activities and aflatoxin residues in broilers fed a diet containing low levels of aflatoxin B1. *Toxicon*, 47(1), 41-46. DOI:10.1016/j.toxicon.2005.09.009
- Callaghan, N.I., Allen, G.J.P., Robart, T.E., Dieni, C.A. and MacCormack, T.J., 2016.** Zinc oxide nanoparticles trigger cardiorespiratory stress and reduce aerobic scope in the white sucker, *Catostomus commersonii*. *NanoImpact*, 2, 29-37. DOI:10.1016/j.impact.2016.06.004
- Cao, W., Yu, P., Yang, K. and Cao, D., 2022.** Aflatoxin B1: Metabolism, toxicology, and its involvement in oxidative stress and cancer development. *Toxicology Mechanisms and Methods*, 32(6), 395-419. DOI:10.1080/15376516.2021.2021339
- Casado, J., Theumer, M., Masih, D., Chulze, S. and Rubinstein, H., 2001.** Experimental subchronic mycotoxicoses in mice: individual and combined effects of dietary exposure to fumonisins and aflatoxin B1. *Food and Chemical Toxicology*, 39(6), 579-586. DOI:10.1016/S0278-6915(00)00174-5
- D'Mello, J. and Macdonald, A., 1998.** Fungal toxins as disease elicitors. *Environmental Toxicology*, CRC Press Science, USA. 414 P.
- da Silva Montes, C., Rosa Filho, J.S. and Rocha, R.M., 2011.** Histological biomarker as diagnostic tool for evaluating the environmental quality of Guajará bay, Brazil. *Environmental Monitoring*, IntechOpen, Brazil. 540 P.
- Dawood, M.A. and Koshio, S., 2020.** Application of fermentation strategy in aquafeed for sustainable aquaculture. *Reviews in Aquaculture*,

- 12(2), 987-1002.
DOI:10.1111/raq.12368
- de Araujo, M.C. and Valenti, W.C., 2018.** Efeito da intensidade luminosa no desenvolvimento larval do camarão-da-amazônia, *Macrobrachium amazonicum*. *Boletim do Instituto de Pesca*, 37(2), 155-164.
- de Jesus, W.B., de Oliveira Mota, T.D.S., Soares, S.H., Pinheiro-Sousa, D.B., de Oliveira, S.R.S., Torres, H.S. and Neta, R.N.F.C., 2021.** Biomarkers and occurrences of heavy metals in sediment and the bioaccumulation of metals in crabs (*Ucides cordatus*) in impacted mangroves on the Amazon coast, Brazil. *Chemosphere*, 271, 129444. DOI:10.1016/j.chemosphere.2020.129444
- Deng, S.X., Tian, L.X., Liu, F.J., Jin, S.J., Liang, G.Y., Yang, H.J. and Liu, Y.J., 2010.** Toxic effects and residue of aflatoxin B1 in tilapia (*Oreochromis niloticus* × *O. aureus*) during long-term dietary exposure. *Aquaculture*, 307(3-4), 233-240. DOI:10.1016/j.aquaculture.2010.07.029
- Deng, Y., Deng, Q., Wang, Y., Sun, L., Wang, R., Ye, L. and Gooneratne, R., 2020.** Tolerance and bioaccumulation of aflatoxin B1 in invertebrate *Litopenaeus vannamei* and vertebrate *Oreochromis niloticus*. *Aquaculture*, 524, 735055. DOI:10.1016/j.aquaculture.2020.735055
- Do, J.H. and Choi, D.K., 2007.** Aflatoxins: detection, toxicity, and biosynthesis. *Biotechnology and Bioprocess Engineering*, 12(6), 585-593. DOI:10.1007/BF02931073
- Fang, H., Wang, B., Jiang, K., Liu, M. and Wang, L., 2020.** Effects of *Lactobacillus pentosus* HC-2 on the growth performance, intestinal morphology, immune-related genes and intestinal microbiota of *Penaeus vannamei* affected by aflatoxin B1. *Aquaculture*, 525, 735289. DOI:10.1016/j.aquaculture.2020.735289
- Figueiredo-Fernandes, A., Ferreira-Cardoso, J.V., Garcia-Santos, S., Monteiro, S.M., Carrola, J., Matos, P. and Fontáinhas-Fernandes, A., 2007.** Histopathological changes in liver and gill epithelium of Nile tilapia, *Oreochromis niloticus*, exposed to waterborne copper. *Pesquisa Veterinária Brasileira*, 27(3), 103-109. DOI:10.1590/S0100-736X2007000300004
- Frías-Espéricueta, M., Castro-Longoria, R., Barrón-Gallardo, G., Osuna-López, J., Abad-Rosales, S., Páez-Osuna, F. and Voltolina, D., 2008.** Histological changes and survival of *Litopenaeus vannamei* juveniles with different copper concentrations. *Aquaculture*, 278(1-4), 97-100. DOI:10.1016/j.aquaculture.2008.03.008
- Furtado, P.S., Campos, B.R., Serra, F.P., Klosterhoff, M., Romano, L.A. and Wasielesky, W., 2015.** Effects of nitrate toxicity in the Pacific white shrimp, *Litopenaeus vannamei*, reared with biofloc

- technology (BFT). *Aquaculture International*, 23(1), 315-327. DOI:10.1007/s10499-014-9817-z
- Gao, Z., Luo, K., Peng, J., Zhu, Q., Liu, C., Wang, X. and Zhang, H., 2023.** The natural occurrence, toxicity mechanisms and management strategies of Fumonisin B1: A review. *Environmental Pollution*, 121065. DOI:10.1016/j.envpol.2023.121065
- Gente, S., Poussereau, N. and Fèvre, M., 1999.** Isolation and expression of a nitrogen regulatory gene, *nmc*, of *Penicillium roqueforti*. *FEMS Microbiology Letters*, 175, 291-297. DOI:10.1111/j.1574-6968.1999.tb13633.x
- Ghaednia, B., Bayat, M., Sohrabi Haghdooost, I., Motallebi, A.A., Sepahdari, A., Mirbakhsh, M. and Mehrabi, M.R., 2013.** Effects of aflatoxin B1 on growth performance, health indices, phagocytic activity and histopathological alteration in *Fenneropenaeus indicus*. *Iranian Journal of Fisheries Science*, 12(4), 813-826.
- Gonçalves, R., Schatzmayr, D., Hofstetter, U. and Santos, G., 2017.** Occurrence of mycotoxins in aquaculture: preliminary overview of Asian and European plant ingredients and finished feeds. *World Mycotoxin Journal*, 10(2), 183-194. DOI:10.3920/WMJ2016.2111
- Gonçalves, R., Hofstetter, U., Schatzmayr, D. and Jenkins, T., 2018a.** Mycotoxins in Southeast Asian aquaculture: plant-based meals and finished feeds. *World Mycotoxin Journal*, 11(2), 265-275. DOI:10.3920/WMJ2017.2239
- Gonçalves, R.A., Naehrer, K. and Santos, G.A., 2018b.** Occurrence of mycotoxins in commercial aquafeeds in Asia and Europe: a real risk to aquaculture? *Reviews in Aquaculture*, 10(2), 263-280. DOI:10.1111/raq.12159
- Guo, M., Jiang, W., Luo, J., Yang, M. and Pang, X., 2018.** Analysis of the fungal community in ziziphi spinosae semen through high-throughput sequencing. *Toxins*, 10(12), 494. DOI: 10.3390/toxins10120494
- Hadi, A., Shokr, A. and Alwan, S., 2009.** Effects of aluminum on the biochemical parameters of fresh waterfish *Tilapia zillii*. *Journal of Science and Its Applications*, 3(1), 33-41.
- Han, D., Xie, S., Zhu, X., Yang, Y. and Guo, Z., 2010.** Growth and hepatopancreas performances of gibel carp fed diets containing low levels of aflatoxin B1. *Aquaculture Nutrition*, 16(4), 335-342. DOI:10.1111/j.1365-2095.2009.00669.x
- Hassaan, M.S., Nssar, K.M., Mohammady, E.Y., Amin, A., Tayel, S. I. and El-Haroun, E.R., 2020.** Nano-zeolite efficiency to mitigate the aflatoxin B1 (AFB1) toxicity: Effects on growth, digestive enzymes, antioxidant, DNA damage and bioaccumulation of AFB1 residues in Nile tilapia (*Oreochromis niloticus*). *Aquaculture*, 523, 735-752. DOI:10.1016/j.aquaculture.2020.735123

- He, X.N., Zeng, Z.Z., Feng, L., Wu, P., Jiang, W.D., Liu, Y. and Zhou, X.Q., 2023. Aflatoxin B1 damaged structural barrier through Keap1a/Nrf2/MLCK signaling pathways and immune barrier through NF- κ B/TOR signaling pathways in gill of grass carp (*Ctenopharyngodon idella*). *Aquatic Toxicology*, 257, 106424. DOI:10.1016/j.aquatox.2023.106424
- Hembrom, P.S., Barik, S., Deepthi, M., Kanno, S. and Grace, T., 2023. Influence of gut microbiome on health and development of penaeid shrimps. *Aquatic Sciences*, 86(1), 4. DOI:10.1007/s00027-023-01018-x
- Hua, K., Cobcroft, J.M., Cole, A., Condon, K., Jerry, D.R., Mangott, A. and Strugnell, J.M., 2019. The future of aquatic protein: implications for protein sources in aquaculture diets. *One Earth*, 1(3), 316-329. DOI:10.1016/j.oneear.2019.10.018
- Huang, Y., Han, D., Zhu, X., Yang, Y., Jin, J., Chen, Y. and Xie, S., 2011. Response and recovery of gibel carp from subchronic oral administration of aflatoxin B1. *Aquaculture*, 319(1-2), 89-97. DOI:10.1016/j.aquaculture.2011.06.024
- Huang, Y., Han, D., Xiao, X., Zhu, X., Yang, Y., Jin, J. and Xie, S., 2014. Effect of dietary aflatoxin B1 on growth, fecundity and tissue accumulation in gibel carp during the stage of gonad development. *Aquaculture*, 428, 236-242. DOI:10.1016/j.aquaculture.2014.03.010
- Imani, A., Sarvi Moghanlou, K., Ghafari Farsani, H., Mahmoudi, S.S., Noori, F. and Farzaneh, M., 2020. Histopathological effect of aflatoxin B1 on some internal tissues of rainbow trout (*Oncorhynchus mykiss*). *Journal of Fisheries*, 73(2), 149-161. DOI:10.22059/jfisheries.2020.300305.1157
- Jamshidizadeh, S., Amrollahi Biuki, N., Yousefzadi, M. and Aramideh, A., 2019. Response of Pacific white leg shrimp (*Litopenaeus vannamei*) on exposure to aflatoxin in feed. *Aquaculture Research*, 50(7), 1973-1984. DOI:10.1111/are.14086
- Jannathulla, R. and Dayal, J.S., 2023. Beneficial effects, challenges and opportunities of the filamentous fungus, *Aspergillus niger* with special reference to the shrimp feed industry—A review. *Reviews in Aquaculture*, 15(4), 1311-1334. DOI:10.1111/raq.12775
- Jia, S., Li, X., He, W. and Wu, G., 2022. Protein-sourced feedstuffs for aquatic animals in nutrition research and aquaculture. *Recent Advances in Animal Nutrition and Metabolism*, 237-261. DOI:10.1007/978-3-030-85686-1_12
- Klich, M. and Pitt, J., 1988. Differentiation of *Aspergillus flavus* from *A. parasiticus* and other closely related species. *Transactions of the British Mycological Society*, 91(1), 99-108. DOI:10.1016/S0007-1536(88)80010-X
- Kracizy, R.O., Brazão, C.C., de Marco Viott, A., Ribeiro, K.,

- Koppenol, A., dos Santos, A.M.V. and Ballester, E.L.C., 2021.** Evaluation of aflatoxin and fumonisin in the diet of pacific white shrimp (*Litopenaeus vannamei*) on their performance and health. *Aquaculture*, 544, 737051. DOI:10.1016/j.aquaculture.2021.737051
- Lahouar, A., Marin, S., Crespo-Sempere, A., Saïd, S. and Sanchis, V., 2016.** Effects of temperature, water activity and incubation time on fungal growth and aflatoxin B1 production by toxinogenic *Aspergillus flavus* isolates on sorghum seeds. *Revista Argentina de Microbiologia*, 48(1), 78-85. DOI:10.1016/j.ram.2015.10.001
- Lamela, R.E.L., Silveira Coffigny, R., Quintana, Y.C. and Martínez, M., 2005.** Phenoloxidase and peroxidase activity in the shrimp *Litopenaeus schmitti*, Pérez-Farfante and Kensley (1997) exposed to low salinity. *Aquaculture Research*, 36(13), 1293-1297. DOI:10.1111/j.1365-2109.2005.01344
- Lavanya, K. and Dayakar, Y., 2022.** Histopathological alterations in the white leg shrimp *L. Vennamei* supplemented with farm isolated probiotic (LAB) upon challenge with White Spot Syndrome Virus (WSSV). *International Journal of Science and Research Archive*, 7(2), 479-486. DOI:10.30574/ijrsra.2022.7.2.0317
- Li, E., Wang, X., Chen, K., Xu, C., Qin, J. G. and Chen, L., 2017.** Physiological change and nutritional requirement of Pacific white shrimp *Litopenaeus vannamei* at low salinity. *Reviews in Aquaculture*, 9(1), 57-75. DOI:10.1111/raq.12104
- Liao, S., Li, Q., Wang, A., Xian, J., Chen, X., Gou, N. and Xu, X., 2012.** Effect of nitrite on immunity of the white shrimp *Litopenaeus vannamei* at low temperture and low salinity. *Ecotoxicology*, 21(6), 1603-1608. DOI:10.1007/s10646-012-0947-7
- Lim, H.A., Ng, W.K., Lim, S.L. and Ibrahim, C., 2001.** Contamination of palm kernel meal with *Aspergillus flavus* affects its nutritive value in pelleted feed for tilapia, *Oreochromis mossambicus*. *Aquaculture Research*, 32(11), 895-905. DOI:10.1046/j.1365-2109.2001.00625.x
- Martin, G. and Hose, J., 2010.** The shrimp book: Functional anatomy of penaeid shrimp. Nottingham University Press, England, 920 P.
- Mirza Alizadeh, A., Mousavi Khaneghah, A. and Hosseini, H., 2022.** Mycotoxins and mycotoxigenic fungi in aquaculture and seafood: a review and new perspective. *Toxin Reviews*, 41(3), 1058-1065. DOI:10.1080/15569543.2021.2010759
- Mohamed, S. I., Shehata, S. A., Bassiony, S. M., Mahgoub, S.A. and Abd El-Hack, M. E., 2023.** Does the use of different types of probiotics possess detoxification properties against aflatoxins contamination in rabbit diets?. *Probiotics and Antimicrobial*

- Proteins*, 15(5), 1382-1392. DOI:10.1007/s12602-022-09990-w
- Mondal, S., Deepika, A., Hundare, S., Poojary, N., Abraham, T. J., Sreedharan, K. and Rajendran, K.V., 2023.** A study on the natural host range of Enterocytozoon hepatopenaei in different species of shrimp and co-habiting aquatic fauna. *Indian Journal of Agricultural Research*, 70(3), 98-106. DOI:10.21077/ijf.2023.70.3.129795-13
- Navale, V., Vamkudoth, K.R., Ajmera, S. and Dhuri, V., 2021.** *Aspergillus* derived mycotoxins in food and the environment: Prevalence, detection, and toxicity. *Toxicology Reports*, 8, 1008-1030. DOI:10.1016/j.toxrep.2021.04.013
- Nesci, A., Rodriguez, M. and Etcheverry, M., 2003.** Control of *Aspergillus* growth and aflatoxin production using antioxidants at different conditions of water activity and pH. *Journal of Applied Microbiology*, 95(2), 279-287. DOI:10.1046/j.1365-2672.2003.01973.x
- Nikolic, M., Nikolic, A., Jaukovic, M., Savic, I., Petrovic, T., Bagi, F. and Stankovic, S., 2018.** Differentiation between *Aspergillus flavus* and *Aspergillus parasiticus* isolates originated from wheat. *Genetika-Belgrade*, 50(1), 143-152. DOI:10.2298/GENSR1801143N
- Ochoa, J.L., Ochoa-Alvarez, N., Guzmán-Murillo, M.A., Hernandez, S. and Ascencio, F., 2015.** Isolation and risk assessment of *Geotrichum spp.* in the white shrimp (*Litopenaeus vannamei* Boone, 1931) from culture ponds. *Latin American Journal of Aquatic Research*, 43(4), 755-765. DOI:10.3856/vol43-issue4-fulltext-14
- Ottinger, C. and Kaattari, S., 1998.** Sensitivity of rainbow trout leucocytes to aflatoxin B1. *Fish and Shellfish Immunology*, 8(7), 515-530. DOI:10.1006/fsim.1998.0154
- Pisoschi, A.M., Iordache, F., Stanca, L., Petcu, A. I., Pur DOI:u, L., Geicu, O.I. and Serban, A.I., 2023.** Comprehensive overview and critical perspective on the analytical techniques applied to aflatoxin determination—a review paper. *Microchemical Journal*, 108770. DOI:10.1016/j.microc.2023.108770
- Priesterjahn, E.M., Geisen, R. and Schmidt-Heydt, M., 2020.** Influence of light and water activity on growth and mycotoxin formation of selected isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. *Microorganisms*, 8(12), 2000. DOI:10.3390/microorganisms8122000
- Ráduly, Z., Szabó, L., Madar, A., Pócsi, I. and Csernoch, L., 2020.** Toxicological and medical aspects of *Aspergillus*-derived mycotoxins entering the feed and food chain. *Frontiers in microbiology*, 10, 2908. DOI:10.3389/fmicb.2019.02908
- Rajeev Raghavan, P., Zhu, X., Lei, W., Han, D., Yang, Y. and Xie, S., 2011.** Low levels of Aflatoxin B1 could cause mortalities in juvenile hybrid sturgeon, *Acipenser*

- ruthenus* ♂ × *A. baeri* ♀. *Aquaculture Nutrition*, 17(2). DOI:10.1111/j.1365-2095.2009.00725.x
- Randall, D., Connell, D., Yang, R. and Wu, S., 1998.** Concentrations of persistent lipophilic compounds in fish are determined by exchange across the gills, not through the food chain. *Chemosphere*, 37(7), 1263-1270. DOI:10.1016/S0045-6535(98)00124-6
- Rodrigues, R.V., Romano, L.A., Schwarz, M.H., Delbos, B. and Sampaio, L.A., 2014.** Acute tolerance and histopathological effects of ammonia on juvenile maroon clownfish *Premnas biaculeatus* (Block 1790). *Aquaculture Research*, 45(7), 1133-1139. DOI:10.1111/are.12054
- Romano, N. and Zeng, C., 2007.** Ontogenetic changes in tolerance to acute ammonia exposure and associated gill histological alterations during early juvenile development of the blue swimmer crab, *Portunus pelagicus*. *Aquaculture*, 266(1-4), 246-254. DOI:10.1016/j.aquaculture.2007.01.035
- Rushing, B.R. and Selim, M.I., 2019.** Aflatoxin B1: a review on metabolism, toxicity, occurrence in food, occupational exposure, and detoxification methods. *Food Chem. Toxicol.* 124, 81-100. DOI:10.1016/j.fct.2018.11.047
- Sahoo, P., Mukherjee, S., Nayak, S. and Dey, S., 2001.** Acute and subchronic toxicity of aflatoxin B 1 to rohu, *Labeo rohita* (Hamilton). NISCAIR-CSIR, India, pp. 453-458.
- Salehpour, R., Amrollahi Biuki, N., Mohammadi, M., Dashtiannasab, A. and Ebrahimnejad, P., 2021.** The dietary effect of fucoidan extracted from brown seaweed, *Cystoseira trinodis* (C. Agardh) on growth and disease resistance to WSSV in shrimp *Litopenaeus vannamei*. *Fish & Shellfish Immunology*, 119, 84-95. DOI: 10.1016/j.fsi.2021.09.005
- Salehpour, R., Amrollahi Biuki, N., Mohammadi, M., Dashtiannasab, A., Ebrahimnejad, P. and Ghasemi, A., 2022.** Evaluation of growth performance and biochemical parameters of western white leg shrimp, *Litopenaus vanname* (Boone, 1931) fed with fucoidan enriched diet. *Journal of Marine Science and Technology*, 21(4), 41-54. DOI:10.22113/jmst.2021.268814.2412
- Scholnick, D.A., Burnett, K.G. and Burnett, L.E., 2006.** Impact of exposure to bacteria on metabolism in the penaeid shrimp *Litopenaeus vannamei*. *The Biological Bulletin*, 211(1), 44-49. DOI: 10.2307/4134576
- Shahafve, S., Banaee, M., Haghi, B.N. and Mohiseni, M., 2017.** Histopathological study of common carp (*Cyprinus carpio*) fed aflatoxin-contaminated diets. *International Journal of Aquatic Biology*, 5(2), 63-70. DOI:10.22034/ijab.v5i2.264
- Sharifpour, I., Abtahi, B., Heidary Jamebozorgi, F., Seyfabadi, S. and Taghizadeh, R., 2011.** Experimental

- assessment of the histopathological effects of water-soluble fraction of crude oil on gill tissue of juvenile *Rutilus frisii kutum*. *Iranian Scientific Fisheries Journal*. 20(1), 89-100.
- Shukla, S., Tiwari, K.J., Lodhi, H.S., Shukla, S., Mishra, A. and Sharma, U.D., 2019.** Histopathological alterations in gills of freshwater prawn, *Macrobrachium dayanum* (Crustacea-Decapoda) after acute and sub-acute exposure of lead nitrate. *Journal of Applied and Natural Science*, 11(2), 568-574. DOI:10.31018/jans.v11i2.2118
- Smaoui, S., D'Amore, T., Agriopoulou, S. and Mousavi Khaneghah, A., 2023.** Mycotoxins in Seafood: Occurrence, Recent Development of Analytical Techniques and Future Challenges. *Separations*, 10(3), 217. DOI:10.3390/separations10030217
- Soegianto, A., Irawan, B. and Usman, N., 2013.** Effects of sublethal copper concentrations on gills of white shrimp (*Litopenaeus vannamei*, Boone 1931). *Bulletin of Environmental Contamination and Toxicology*, 91(6), 630-634. DOI:10.1007/s00128-013-1113-5
- Su, C., Li, J., Pan, L., Zhang, M., Chen, Z. and Lu, M., 2023.** Immunotoxicity and the mechanisms of aflatoxin B1-induced growth retardation in shrimp and alleviating effects of bile acids. *Journal of Hazardous Materials*, 459, 132266. DOI: 10.1016/j.jhazmat.2023.132266
- Usman, N., Irawan, B. and Soegianto, A., 2013.** Effect of lead on survival, osmoregulation, and histological changes of the gills of the white shrimp, *Litopenaeus vannamei*, Boone, 1931. *Journal of the World Aquaculture Society*, 44(4), 547-556. DOI:10.1111/jwas.12054
- Wang, Y., Wang, B., Liu, M., Jiang, K., Wang, M. and Wang, L., 2019.** Comparative transcriptome analysis reveals the different roles between hepatopancreas and intestine of *Litopenaeus vannamei* in immune response to aflatoxin B1 (AFB1) challenge. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*, 222, 1-10. DOI:10.1016/j.cbpc.2019.04.006
- Wei, G., Guo, X., Liang, Y., Liu, C., Zhang, G., Liang, C. and Dong, L., 2023.** Occurrence of fungi and mycotoxins in herbal medicines and rapid detection of toxin-producing fungi. *Environmental Pollution*, 122082. DOI:10.1016/j.envpol.2023.122082
- Xu, T., Liu, Q., Chen, D. and Liu, Y., 2022.** Atrazine exposure induces necroptosis through the P450/ROS pathway and causes inflammation in the gill of common carp (*Cyprinus carpio* L.). *Fish and shellfish immunology*, 131, 809-816. DOI:10.1016/j.fsi.2022.10.022
- Yu, J., Guo, M., Jiang, W., Yang, M. and Pang, X., 2020.** Assessment of the microbiome and potential aflatoxin associated with the medicinal herb *Platycladus orientalis*. *Frontiers in microbiology*, 11, 582679. DOI:10.3389/fmicb.2020.582679

- Zeng, S., Long, W., Tian, L., Xie, S., Chen, Y., Yang, H. and Liu, Y., 2016.** Effects of dietary aflatoxin B1 on growth performance, body composition, haematological parameters and histopathology of juvenile Pacific white shrimp (*Litopenaeus vannamei*). *Aquaculture Nutrition*, 22(5), 1152-1159. DOI:10.1111/anu.12331
- Zeng, Z.Z., Jiang, W.D., Wu, P., Liu, Y., Zeng, Y.Y., Jiang, J. and Feng, L., 2019.** Dietary aflatoxin B1 decreases growth performance and damages the structural integrity of immune organs in juvenile grass carp (*Ctenopharyngodon idella*). *Aquaculture*, 500, 1-17. DOI: 10.1016/j.aquaculture.2018.09.064
- Zhao, W., Wang, L., Liu, M., Jiang, K., Wang, M., Yang, G. and Wang, B., 2017.** Transcriptome, antioxidant enzyme activity and histopathology analysis of hepatopancreas from the white shrimp *Litopenaeus vannamei* fed with aflatoxin B1 (AFB1). *Developmental and Comparative Immunology*, 74, 69-81. DOI:10.1016/j.dci.2017.03.031