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Impact of Azolla on the histopathology of the liver and intestine of the fingerling carp *Cyprinus carpio*

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ABSTRACT

This study aims to evaluate the histopathological effects of *Cyprinus carpio* treated with Azolla growing on the pond with sheep manure fertilizer. Heavy metals contamination in aquatic ecosystems is known to be recognized by aquatic macrophytes. This study aims to estimate the accumulative of lead(Pb) and Zinc (Zn) in Azolla and to detect if these heavy metals cause damage to fish tissues. Azolla cultured in ponds fertilized with sheep manure for eight days, the Pb and Zn were estimated in the sheep manure which were (28.5 and 139.3) mg /kg respectively the percentage of these heavy metals in the macrophytes was 50% for Pb and 19% for Zn. *Cyprinus carpio* were divided into five groups depending on the rate of Azolla concentrated with Pb and Zn added to fish feed as the T1 (0%), T2(4.5%), T3(9%), T4(13.5%) and T5(18%). Histopathological changes and lesions vary in severity according to the level of Azolla in microscopic examination of the intestine and liver from these groups, which range in the abnormal most villi shape, with adherence to condensed heavy metals, goblet cells and juncture hyperplasia, its necrosis, and villi damage. Also, liver lesions are characterized by vacuolar degeneration and proliferation of sinusoids in fish of T2; The lesions are more severe in other groups, including isolated infiltration of inflammatory cells, vasculitis, vasogenic oedema with thrombus formation, the concentration of the bile duct, atrophy of the pancreas with the necrotic portal area. In the conclusion of this study that Azolla macrophytes are good bioaccumulation of Pb and Zn that can be transmitted into fish and cause histopathological changes and tissue damage, other studies should be conducted to determine the correlation between concentrated heavy metals in Azolla plants and histopathological and physiological changes in fish.

INTRODUCTION

Aquaculture is the quickest-growing food-producing industry perceived as having the best chance of meeting the rising demand for aquatic food; fish is one of the most important sources of protein from aquaculture areas, giving significantly to food safety and economic growth (**Kumari *et al*., 2017; Yakubu *et al*., 2022**). However, bad management, toxicity, and increased concentrations of heavy metals cause damage to the aquatic system and lead to bioaccumulative in fish tissue, cause immune function and fish health disturbances, and expose fish to many disease outbreaks. **(Shelke and Wani, 2016 ;Jaber *et al*., 2021)**. Marine pollution is one of the most dangerous domestic, agricultural, industrial and human problems **(Athalye *et al*., 2001)**. Heavy metals' toxic, pathological effects are characterized by inhibition of antioxidant enzymes, D.N.A. damage, loss of cell permeability balance and release of reactive oxygen species **(Gill and Tuteja, 2010 ; Lushchak, 2011) .**

Water plants such as water hyacinth, algae, bivalves, Azolla and fungi have recently been the focus of research on bioabsorption and bioavailability by living organisms **(Jamuna and Noorjahan , 2009)**. Azolla inter to aquatic culture because it is classified as **Smith (2003)** categorized as activated sludge and has suiTable nutritional components for marine animals such as tilapia fish**(FAO,2009 and Fiogbé *et al*., 2014)** as well as it has raped growing because it has the ability to fixed nitrogen from atmospheric and also consider biofilters for maintaining aquatic environment quality. One of the most critical factors that cause a break in ecosystem balance is heavy metals such as lead and zinc, which are very toxic to fish and public health **(Huseen and Mohammed, 2019)**. These macrophyte plants can remove heavy metals from aquaculture, such as iron and copper **(Jain *et al*.,1989)**. Fish are sensitive to toxicants even at lower concentrations and affect human public health**(Salma and Naik, 2020)**. So this study aims to evaluate the histopathological effects in Cyprinus carpio treated with Azolla growing on the pond with sheep manure fertilizer.

1. MATERIAL AND METHODS

Fish: The weight of Cyprinus carpio is transferred from 75 ± 10 g Erbil hatchery to the fish laboratory - Department of Animal Production, Faculty of Agriculture - University of Mosul; fish remain in fibreglass (40 *30*30) centimetres for at least 15 days.

2.1 Propagation of Fertilizer

The Azolla spp .are propagated in the pond under the slight shadow with tap water(dechlorinated). Sheep manure was used as fertilizer 70 gm, dissolved in tap water and left for five days. Then the plant was transferred to the pond. After seven days, the growing biomass was harvested, swaying and dried in the air for at least 48 hours and then kept in a plastic bag **(Roshidia *et al*., 2020)**.

2.2 Estimation Heavy Metals (mg/kg)

(1) **Water:** The concentration of zinc and lead was estimated in the filtered water, as 50 ml of it was taken and concentrated nitric acid was added to it at the rate of 1.5 mm/litre of the sample volume and then estimated by Atomic absorption**.**

(2) Azolla: The heavy elements zinc and lead in the plant were estimated based on the dry matter, as 0.5 gm of Azolla was taken, and 5 ml of concentrated sulfuric acid and 2 ml of concentrated pyrochloric acid were added and digested on a hot plate until the solution became clear transparent, left to cool and filtered, then the volume of the solution was completed to 50 ml, and then estimated by Atomic absorption **.**

The absorption percentage of both Pb and Zn was estimated in the Azolla according to the formula

$$\% = \frac{\text{Concentrated heavy metal in the Azolla mg/kg}}{\text{Concentrated heavy metal in the water mg/kg}} \times 100$$

2.3 Experimental design

Fifty carp fish were randomly divided into five groups depending on the level of Azolla, which is added to the commercial fish diet (T_1 0%, T_2 2.5%, T_3 5%, T_4 7.5% and T_5 10%); after the end of the experiment for 60 days, the fish were anaesthetized according to **(AL-Taee *et al*., 2021)** for collecting intestine and liver samples for rotational histopathological technique.

2. RESULTS

Azolla spp. Considered good Phytoremediation, this study's results proved that the percentage of the accumulative concentration of lead and zinc in the sheep manure was (28.5 and139.3)mg/ kg, respectively. Their percentage in the Azolla was (50% and 19 %) for each Pb and Zn, respectively Table (1).

Table (1) Heavy Metals concentrated mg/kg in sheep manure, and it is percentage absorption in the Azolla

Samples	Lead mg/kg	Zinc mg/kg
Sheep manure	28.5	139.3
Azolla	14.5	26.7
% absorption	50%	19%

These heavy metals cause histopathological changes, and the severity depends according to it is concentration and the levels of Azolla, so microscopic examination of intestine fish group feed at 4.5% macrophyte there was only abnormal shape in the villi apex and adhesion between them with infiltration of inflammatory cells with oedema in the muscular layer Fig 1(1-B) as in comparison with control group Fig.1(1-A), while in fish feed with Azolla at levels 9% and concentrated heavy metals (50l% for Pb and 19% Zn) the lesion appear enteritis with oedema and hyperplasia in the goblet cells and abnormal villi folded Fig. 2(1-C). The severity of lesion was clearly in the intestine of fish feed with Azolla at levels 13.5% concentrated Pb and Zn characterized by increased branching and length of midgut villi Fig.3 (3-D) with Increase thichining of villi lead to adhesion between them and infiltration of inflammatory cells also the microscopic examination revealed there was hyperplasia of epithelial cells lying mucus gland with necrosis Fig.3 (3-E) these lesions are more severity at hindgut in fish feed with Azolla at levels 18% a combined with Zenker necrosis myositis Fig. 4(4-F) and damage in the villi of forgetting with necrosis Fig. 4(4-G)

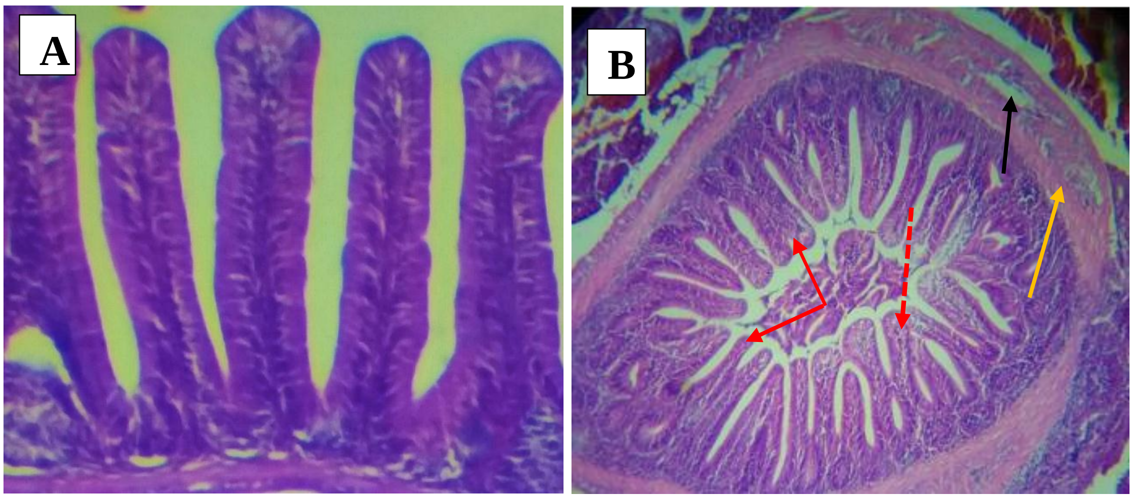

Figure 1 (1-A) Microscopic examination of intestinal fish in control group 40*1.5X, H&E, (1-B) Fish feed with Azolla 4.5% concentrated Zn and Lead abnormal shape (red row)villi apex and adhesion between villi (dot red row) oedema (black row) with infiltration of the inflammatory cell(yellow row)in the muscular layer, 10*1.1X, H&E.

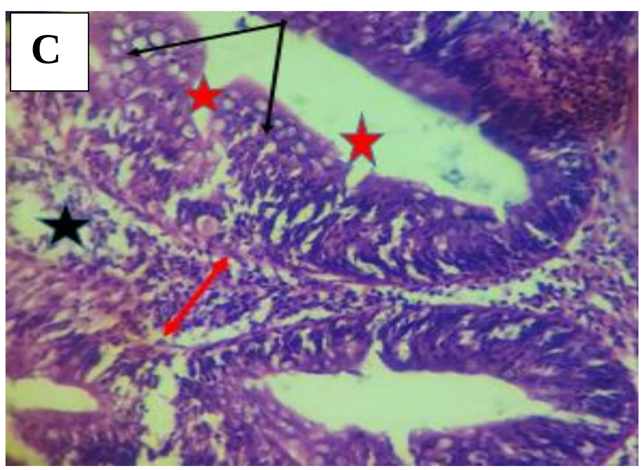

Figure 2 (2-C) Microscopic examination of the intestine of fish feed with 9% Azolla concentrated Zn and Lead showing hyperplasia of goblet cells (black row), severe enteritis (red two head row) with oedema (black star) with abnormal villi folded (red star) 40*1X, H&E.

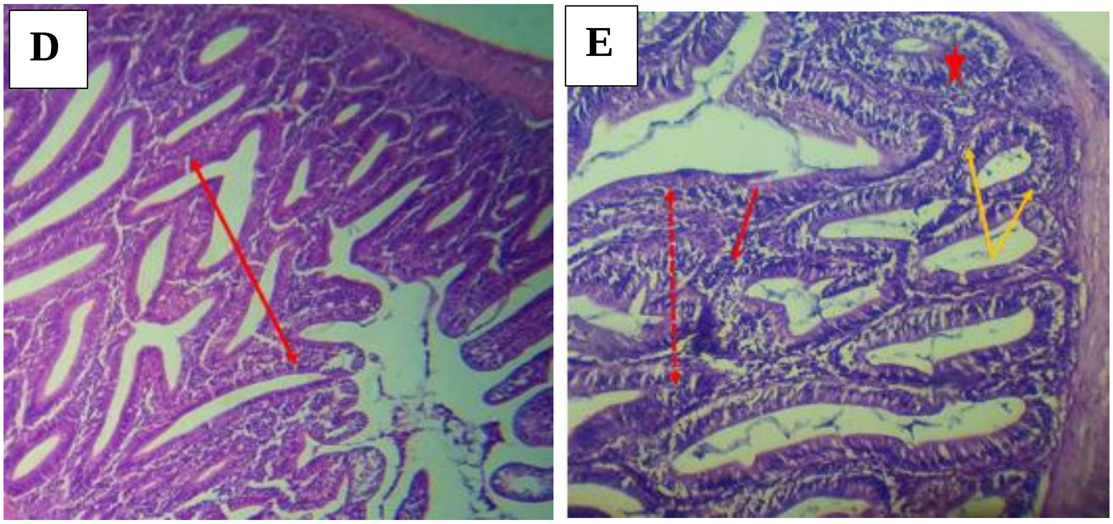

Figure 3 (3-D) Microscopic examination of the intestine of fish feed with 13.5% Azolla concentrated Zn and Lead showing increased branching and length of midgut villi 40*1.5X, H&E, (3-E)Increase thichining of villi lead to adhesion between them (red dot row), infiltration of inflammatory cells(red row) and hyperplasia of epithelial cells lying mucus gland (yellow row) with necrosis of mucus gland (red star),10*1.4X, H&E.

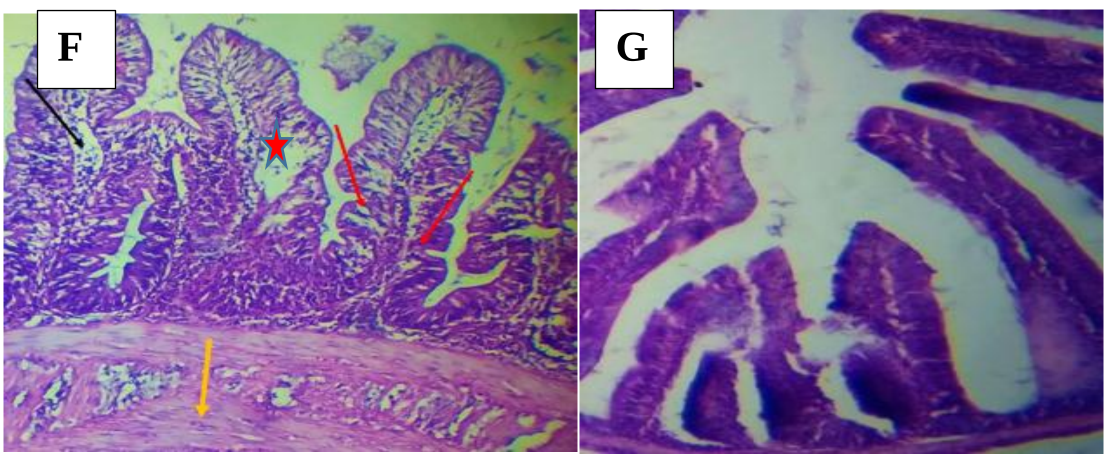

Figure 4 (4-F) Microscopic examination of the intestinal hindgut of fish feed with 18% Azolla concentrated Zn and Lead showing infiltration of the inflammatory cell(black row), oedema (red star) with increased villi folded and necrosis of muscular layer (yellow row), 40*1X, H&E.,(4-G)necrosis and damage of foregut villi 10*2.6X, H&E.

The histopathological changes in the liver, which represents the primary organ for metabolic and detoxification, are characterized by vacuolar degeneration in the hepatocyte and dilatation in the sinusoid Fig. 5(5-B) in fish feed with Azolla at level 4.5%, in contrast, to control group Fig. 5(5-A), in the fish feed with Azolla at percentage 9% there was thickening in the wall of bile duct with inflammation exudate and congestion of hepatic portal vein Fig. 6(6-C) as well as to vasculitis with vasogenic oedema with atrophy of pancreas in the group of fish fed with Azolla at 13.5% with concentrated Pb and Zn Fig. 6(6-D), with vacuolar degeneration and necrotic pancreas Fig. 7(7-E). The microscopic examination of the liver in fish feed with Azolla at the level of 18% and concentrated heavy metals, the lesion is more severity characterized by thrombus formation with severe hepatitis as well as severe vacuolar degeneration Fig. 8(8-E) and necrotic portal area Fig . 8(8-F).

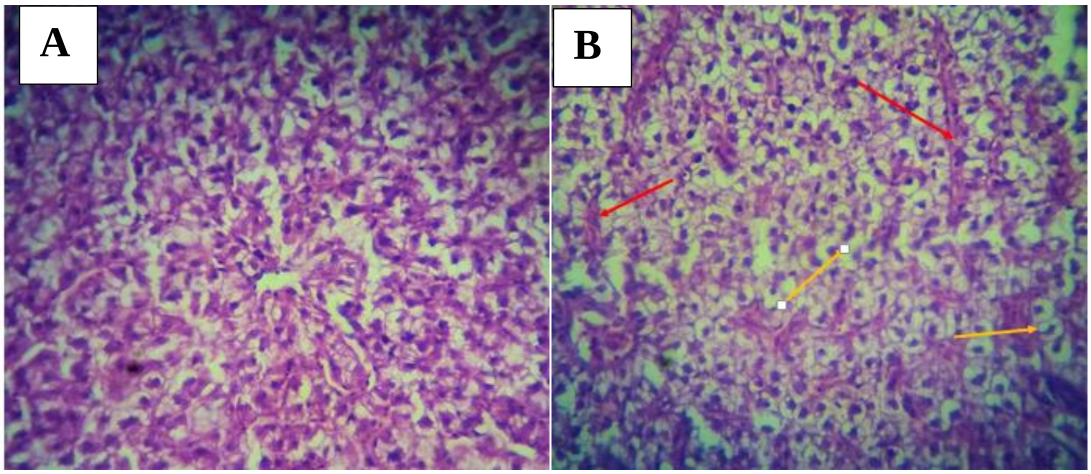

Figure 5(5-A) Microscopic examination of liver in control fish group 40*1.2X, H&E, (5-B), liver of fish feed with 4.5% Azolla concentrated with lead and zinc showing vacuolar degeneration (yellow row), dilation of the sinusoid (red row)40*1X, H&E.

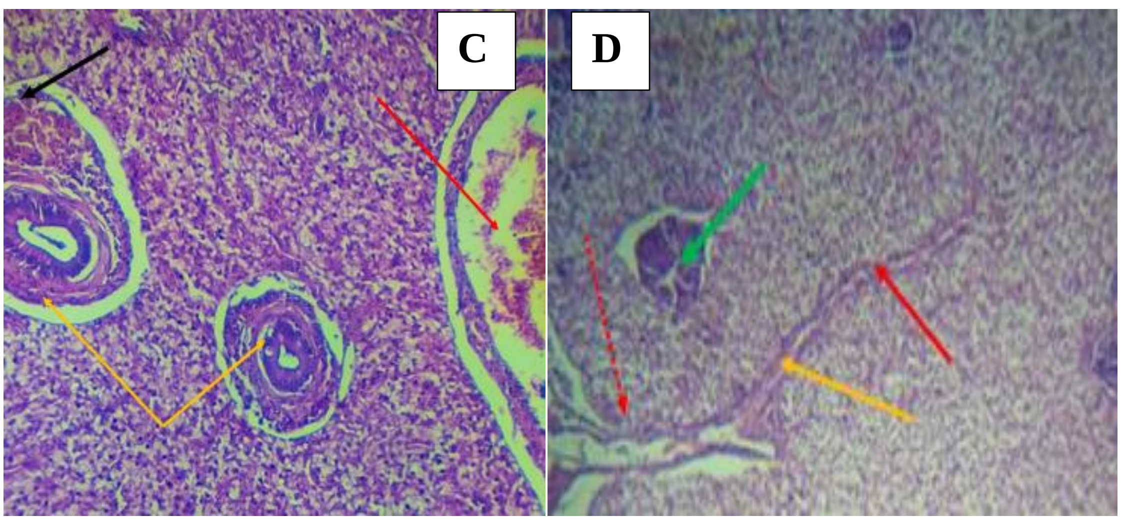

Figure 6(6-C) Microscopic examination of the liver in fish feed with 9% Azolla display of contraction of the hepatic portal vein (red row), 10 * 2.3X, H&E, (5-D), fish feed with 13.5% Azolla. Atrophy H&E of 10*1.8X.

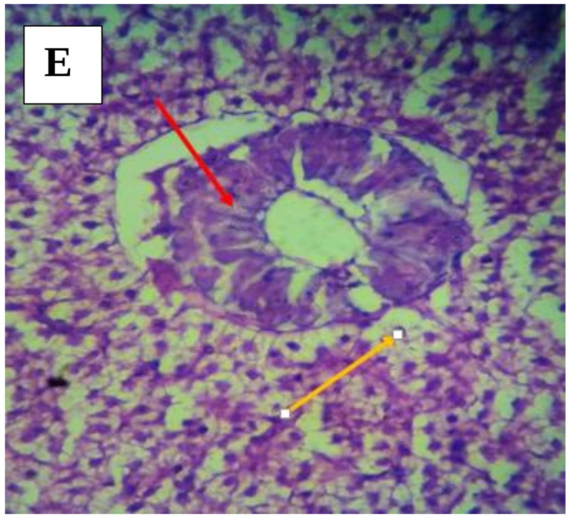

Figure 7(7-E) Microscopic examination of liver in fish feed with 13.5% Azolla concentrated with lead and zinc showing vacuolar degeneration of hepatocytes (yellow row) with necrosis in pancreas tissue (red row) 40*1.3X, H&E.

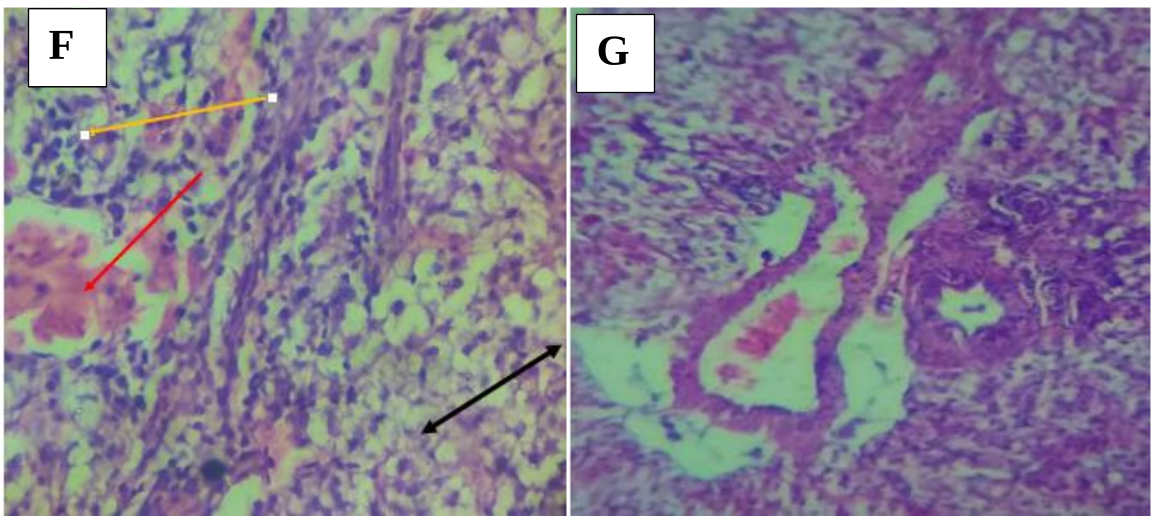

Figure 8(8-F) Microscopic examination of liver in fish feed with 18% Azolla concentrated with lead and zinc showing severe infiltration of inflammatory cells (yellow row) with vacuolar degeneration of hepatocytes (black row), thrombus formation (red row) 1*40X, H&E, (8-G) necrotic hepatic tissue and portal area 10*3.3X, H&E.

DISCUSSION

Inadequate farming environments in aquaculture can alter dilatory fish habits, which are considered an essential factor in improving the immune status and increasing the resistance of fish to diseases on the health of fish **(El Megid *et al*., 2020)**. Heavy metals hit aquatic ecosystems, and, as a result, humans relying on marine products for food can accumulate in the tissues of ecosystems and aquatic organisms, causing toxicity to fish and humans (Shelke and Wani, 2016). Heavy metals, such as lead, have considerable hazardous effects on fish. They are significant contaminants for fish because they are not removed from aquatic systems by natural ways, such as organic pollutants, and are abundant in mineral organic compounds. Heavy metal occurrence varies among fishes regarding age, development, and other physiological factors. Fish are a population that can be severely harmed by hazardous contaminants **(Huseen and Mohammed, 2019)**. Phytoremediation is a recent technique involving plants extracting, degrading, or absorbing pollution from soil and water. Azolla spp is one of the Phytoremediation has the ability to tolerance and concentration capability for several metal ions. Hence, the result of this study revealed that Lead and Zinc from sheep manure fertilization could accumulate in the Azolla which was in agreement with the development **(Ahmed *et al.*, 2016 Kakkalameli *et al.*, 2018)**. Even the result of **(Hasanein *et al.*, 2018)** reported that blue-green algae could chelate heavy metals and decrease their toxic Effects on aquatic organisms. Also, (**Faˇckovcová *et al.*, 2020)** said that heavy metals such as Zn and Pb may accumulate in the Azolla macrophytes after three days of cultivation.

Fish exposure to heavy metals in two ways through skin and gills rout which are represented indirect way or directly a way through consuming contaminated water or food, so it is effected may be present clearly in the intestine and liver, and even at low concentrations may cause tissue damage **(Yousif *et al.*, 2021 and Jaber *et al.*, 2021)**, so the histological changes reported in our study in both the intestines and liver of common carp fish treated with a different percentage of Azolla may be due to the concentration of lead and zinc in Azolla. Then their accumulation in the fish organs, Endogenous waste products as well as externally generated liver detoxifies poisons such as heavy metals, so the pathological alteration in the liver may be caused by the cumulative Effect of metal concentration in the liver. The liver's cellular deterioration could be attributed to a lack of oxygen and effects of heavy metals in the circulation system, and disturbances in the blood vessels' permeability **(Mohamed, 2001).**

Metal ion's interaction with functional groups on the cell membrane leads to toxicity. It may cause the formation of reactive oxygen and nitrogen species (R.O.S. and NO) such as hydrogen peroxide, superoxide anion radicals, and hydroxyl radicals through different mechanisms such as Fenton and Haber-Weiss reactions, causing a variety of problems such as enzyme replacement complexes and transcription factors, antioxidant enzyme inhibition, cellular redox imbalance, ionic transport imbalance, D.N.A. damage, and protein oxidation **(Doherty *et al.*, 2010, Lushchak, 2011)**.

At the same time, these histological changes may be related to plant-based diets have different effects on fish gut architecture depending on the species and the dietary low inclusion levels because they contain less fibre digestion **(Mosha *et al.*, 2020)**, so the pathological effects intestine and liver have more clearly in fish treated with Azolla higher than 2.5%. The adverse effects may be related to a composition of tannin and another phenolic compound. This result does not agree with the development of (**SHAIMA *et al.*,**

2020), who reported that natural Azolla (5.00 and 7.5 percent g kg-1 diet) had considerable physiological, nutritional, and biological impacts on common carp.

CONCLUSION

Azolla macrophytes are one of the essential Phytoremediation, which plays a role in removing heavy metals from the aquatic environment; this heavy metal may be transmitted through ingested contaminated Azolla to fish and other marine organisms and cause histopathological alteration in the fish tissue. More studies should be done to determine the toxic effects of heavy metals concentration in the Azolla consumed by the fish.

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