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Pathological Effects of Water-Soluble Fraction of Burned Motor Oil in *Tilapia zillii* and *Mugil cephalus* Through Bioremediation Processes

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Abstract: The present study evaluated the ability of four indigenous bacterial and four fungal isolates, isolated from Lake-Temsah, Egypt, to bioremediate the total petroleum pollution that was caused by burned motor oil in the Lake and to record the impact of the bioremediation process on two edible fishes namely *Mugil cephalus* and *Tilapia zillii*. The toxicity and bioremediation studies were carried out on the fishes to evaluate the effects of water-soluble fraction (WSF) of burned motor oil either alone or in combination with the four bacterial and four fungal strains for 30 days on the growth performance and the survival percent of fish in relation to histopathology of liver and gills. Aliquots of burned motor oil was added to Lake water in aquaria and the microbial treatment was carried out using four indigenous bacterial isolates (*Achromobacter* sp., *Bacillus* sp., *Clostridium* sp. and *Pseudomonas* sp.) and four fungal isolates (*Absidia corymbifera, Aspergillus sydowii, Mucor circinelloides* and *Penicillium* sp.). The experiment was extended for 45 days. The impacts of these treatments on the growth performance and the survival percent of fish were evaluated. Histopathological changes were recorded. The results indicated that the used motor oil led to severe lesions of gills and liver of tested fishes. On the other hand, microbial treatment using bacterial or fungal alone and/or combination of both were effective for the remediation of the motor oil contaminated seawater and also for keeping the growth performance and histopathology of the tested fish as similar as the untreated fish. Biodegradation rates were slightly more enhanced by using the combination of bacteria and fungi than bacteria or fungi alone.

Key words: Bioremediation · Histopathological · Motor oil · *Mugil cephalus* · *Tilapia zillii*

INTRODUCTION

Petroleum products are one of the most relevant to aquatic eco-toxicology [1]. Polycyclic Aromatic Hydrocarbons (PAHs) are considered important petroleum products because they are highly resistant compounds under normal conditions as they have strong molecular bonds. These groups of petrochemicals are mainly found in accidental oil spills, pipe leakages and rainwater run-off from roadway. Improper disposal of PAHs can cause environmental pollution as they accumulate in the surrounding soil sediment. Hydrocarbon components have been known to belong to the family of carcinogens and neurotoxic organic pollutants [2]. The histology of liver and gills are good biomarkers to evaluate the toxicity of hydrocarbons [3, 4]. Teleost liver is the primary organ for biotransformation of organic xenobiotics and probably also for the excretion of

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harmful trace metals, food digestion and storage and metabolism of sex hormones [5, 6]. The process of bioremediation of oil is defined as the use of microorganisms to detoxify or remove pollutants of petroleum industry [7]. In addition, biodegradation by natural populations of microorganisms is cheaper than other remediation technologies [8]. Microbial degradation is the major and ultimate natural mechanism by which one can cleanup the petroleum hydrocarbon pollutants from the environment [9, 10]. The microbial biodegradation processes can suggest an effective method where the crude oil is considered as a carbon source for microbial growth which results in the breakdown of the oil to lower molecular weight compounds [11]. Bacteria are the most active agents in petroleum degradation and they work as primary degraders of spilled oil in the environment [12, 13]. Singh [14] also reported a group of terrestrial fungi, namely, *Aspergillus*, *Cephalosporium* and *Pencillium* which were also found to be the potential degrader of crude oil hydrocarbons. The aim of the present study was to evaluate the histopathological alterations induced by burned motor oil toxicosis in fish as well as the efficacy of some bacterial and fungal isolates in biodegradation of the motor oil and their impact on histology of both liver and gills of *Tilapia zilli and Mugil cephalus*.

MATERIALS AND METHODS

Fish Stock: Fishes were captured using fishing nets (1 mm mesh) from Lake-Temsah. Fish fry of *Mugil cephalus* were with average body weight of 0.68g and average total length of 3.38cm and *Tilapia zillii* with average body weight of 4.17 and average total length of 5.29cm. They were transported and acclimatized in 100 L glass tanks filled with seawater from the collecting sites (35ppt salinity and 20±1°C temperature). After 2 weeks of adaptation, eighty individuals (40 mugil and 40 tilapia) were randomly selected and transferred to sixteen aquaria, eight glass aquaria for mugil and eight for tilapia. Each Aquarium was (40 x 40 x 60 cm) and was filled with 30L seawater at densities of 10 fish /aquarium.

Bacterial Strains: A number of well identified bacteria which were isolated from five contaminated sites near the shore of Lake-Temsah (samples were collected from sediments and sub-surface water). Only four isolates of bacteria and four isolates of filamentous fungi were selected on the basis of their ability to utilize burned motor oil as their sole source of carbon and energy. The selected bacterial species were *Achromobacter, Bacillus, Clostricium* and *Pseudomonas*. Bacterial identification was made by using gram and staining motility tests according to Allegrucci and Sauer [15] and biochemical tests according to Holt *et al*. [16].

Table 1: distribution of *M. cephalus* and *T. zillii* in aquaria.

Mugil	Tilapia	Meaning	Content of the aquarium
gp1	gp1	Control	Seawater+ fish
gp2	gp2	Blank	Seawater+ fish + mix bacteria
gp3	gp3	Blank	Seawater+ fish + mix fungi
gp4	gp4	Blank	Seawater+ fish + mix fungi and bacteria
gp5	gp5	Control oil	Seawater+ fish + 1 ml oil/ Aquaria
gp6	gp6	Treatment	Seawater+ fish + mix bacteria +oil
gp7	gp7	Treatment	Seawater+ fish +mix fungi+ oil
gp8	gp8	Treatment	Seawater+ fish +mix fungi and bacteria+ oil

Fungal Strains: Four fungi were isolated from the same five contaminated sites in Lake Temsah. The isolates that were proved the ability to degrade burned motor oil were selected. Fungal strains were *Absidia corymbifera Aspergillus sydowii, Mucor circinelloides and Penicillium* sp. and identified according to Barnett and Hunter [17]. Water Lake was used in this study and both fungi and bacteria were loaded on sodium alginate.

Experimental Design: Sixteen aquaria were used in this experiment, 8 for *Mugil cephalus* and 8 for *Tilapia zillii.* All 8 aquaria distributed as shown in Table 1. The experimental period was extended for 45 days; it was divided into 15 days for acclimation and 30 days for motor oil treatments. The feeding regime was applied as 5% body weight per day throughout the experiment period; the frequency of feeding was maintained as twice a day for six days a week. Dose of motor oil: 1ml, dose of bacteria: 0.01g (dry weight) and dose of fungi: 0.04g (dry weight) per aquarium.

Histopathological Examination*:* Specimens from liver and gills from individual fish of all groups were collected and fixed in 10% neutral buffered formalin for histopathological examination. After fixation the specimens were dehydrated in graded alcohol, embedded in paraffin. Five microns sections were obtained and they were stained with routine Hematoxylin and Eosin stain (H&E) then they were examined by light microscope as described by Bancroft and Stevens [18].

RESULTS

As shown in Table 2, the bacterial treatment led to improvement of the growth performance and survival percentage of the *M. cephalus as* compared to the

Table 2: The effects of the microbial motor oil treatments on the growth performance, survival percentage and feed utilization parameters of *M. cephalus* after 0, 45 days of rearing

Estimated parameters	Control	F & O	F & B & Fun	O & F & B & fun
At zero time (Stocking data)				
Av. Initial weight (g) ± SD	0.68±0.48	0.68±0.48	0.68±0.48	0.68±0.48
Av. Initial length (cm) ± SD	3.38±1.0	3.38±1.0	3.38±1.0	3.38±1.0
After 45 days of rearing				
Av. final weight (g) ± SD	0.89±0.50	0.93±0.41	0.99±0.346	1.67±0.43
Av. final length (cm) ± SD	3.97±0.87	4.2±0.74	4.05±0.80	5.03±0.47
Survival rate (%)	100	60	80	80

F: fish O: oil B: bacteria Fun: fungus

Table 3: The effects of the microbial motor oil treatments on the growth performance, survival percentage and feed utilization parameters of *T. zillii* after 45 days of rearing

Estimated parameters	Control	F&O	F & B & Fun	O & F & B &FUN
At zero time (Stocking data)				
Av. Initial weight (g) ± SD	4.17±2.29	4.17±2.29	4.17±2.29	4.17±2.29
Av. Initial length (cm) ± SD	5.29±1.19	5.29±1.19	5.29±1.19	5.29±1.19
After 45 days of rearing				
Av. final weight (g) ± SD	8.65±2.15	8.4±3.91	8.24±0.45	8.36±3.69
Av. final length (cm) ± SD	7.96±0.58	7.8±1.37	7.95±0.35	7.75±1.05
Survival rate (%)	100	50	80	100

untreated fish (control), while the fungal treatment showed slight impacts on both the growth performance and the survival percentage of *M. cephalus* compared to the control. Also, the consortium of bacterial and fungal isolates showed higher impacts on survival percentage and improvement of the growth performance of the *M. cephalus* compared to the untreated fish (control). Moreover, it was observed that after 45 days of the rearing period the addition of 1ml motor oil in the tested aquaria led to moderate effects on the growth rates of *M. cephalus* on fungal treatment. But the bacterial and fungal treatment have been improved the daily weight gain and the daily length day compared to the control. On the other hand, *P. aeruginosa* (in respect to *M. cephalus*), signed as F&B led to 10.7 and 6.7%, reduction percentage in the daily weight gain and the daily length gain, respectively compared to the control. In addition, the microbial treatment which carried out in the aquaria O & F & B100, O & F & B300 and O & F & B500, it led to a reduction of 7.1, 10.7 and 39.3% in the daily weight gain, respectively and a reduction of 6.7, 20 and 33.3% in the daily length gain compared to the control**.**

The obtained data in Table 3 showed that the bacterial and combination of bacteria and fungi treatments led to slight improvement on the growth performance and good growth on survival percentage of *T. zillii* compared to the untreated fish (control). But the fungal treatments led to mild impact on the growth rate and no effect in survival percentage of *T. zillii* compared to the untreated fish (control). Moreover, it was observed that after 45 days of the rearing period the addition of 1ml motor oil in the tested aquaria, O&F&B led to slight effects on the survival of *T. zillii* but it not affected on the growth rates.

Histopathological Results of *Mugil cephalus*:

Liver*:* Control group 1: The liver of control group that were isolated from lake Temsah revealed moderate alteration of histological architecture consists mainly of focal to diffuse areas of fat degeneration and mild to moderate congestion of blood vessels as shown in (Fig. 1). **Group 2 (B & F)** most livers of this group showed mild focal areas of degenerative changes represented mainly by vacuolar degeneration. Mild congestion of blood vessels was observed. **Group 3 (Fun & F**), livers of this group had mild vacuolar degeneration. **Group 4 (B & Fun & F)** livers of this group showed mild to focal areas of vacuolar degeneration. Congestion of blood vessels was also observed as shown in Fig. 2. **Group 5 (O & F)**: Liver showed diffuse fatty degeneration, moderate to severe areas of coagulative necrosis, severe congestion of blood vessels and focal areas of hemorrhages. (Fig.3&4). Focal areas of mononuclear cell infiltrations were also observed (Fig.5). **Group 6 (B & Fun & F)** most livers of this group showed mild focal vacuolar degeneration and mild congestion of blood vessels. **Group 7 (Fun & F**), livers of this group had mild vacuolar degeneration. **Group 8 (O & F & Fun & B)**, Liver showed pronounced improvement with focal mild degeneration and mild congestion of blood vessels (Fig. 6).

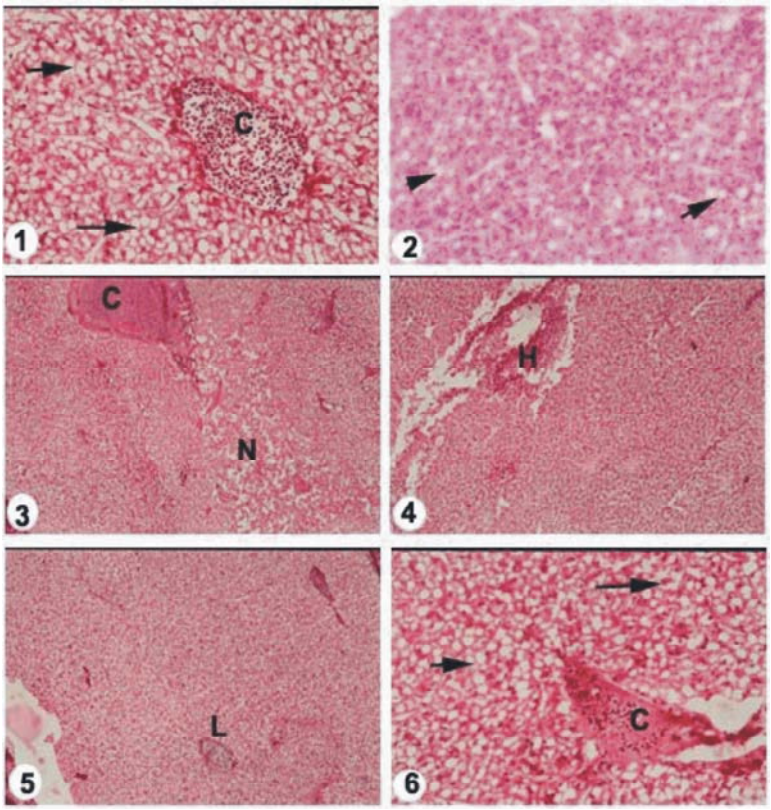

Fig 1: *Mugil cephalus*, liver control group from Lake Temsah water showing congestion (C), fatty vacuolation of hepatocytes (arrows). H&E. X 400.

Fig 2: *Mugil cephalus*, liver received bacteria and fungi (B & Fun & F) showing mild focal areas of vacuolar degeneration (arrows). H&E. X 400.

Fig 3: *Mugil cephalus*, liver received engine oil (O&F) showing congestion (C) diffuse fatty degeneration of hepatocytes and massive necrosis (N). H&E. X 200.

Fig 4: *Mugil cephalus*, liver received engine oil (O&F), showing diffuse fatty degeneration of hepatocytes, in addition to focal area of hemorrhage (H). H&E. X 200.

Fig 5: *Mugil cephalus*, liver received engine oil (O&F) showing focal area of leukocytic infiltration (L). H&E. X 200

Fig 6: *Mugil cephalus*, liver received engine oil and bacterial/fungal combination (O & F & Fun &B) showing fat degeneration (arrow) and congestion of blood vessels (C). H&E. X 400. O=Oil, B=Bacteria, Fun=Fungi, F=Fish

Gills: Control group 1: Gills of control group showed congestion of blood vessels at the base of gill arch and central venous sinuses of primary lamellae (Fig.7& 8). **Group 2 (B & F),** Gills showed mild histological alterations represented by mild vacuolation and mild hyperplasia of the epithelial lining the secondary lamellae. **Group 3 (Fungus & Fish):** Gills showed mild congestion of blood vessels, mild vacuolation and hyperplasia of the epithelial lining the secondary lamellae. **Group 4 (B & Fun & F):** Gills showed mild congestion of blood vessels, vacuolation and hyperplasia of the epithelial lining the secondary lamellae (Fig. 9). **Group 5 (O & F):** Gills showed moderate to severe congestion of blood vessels, in addition to sever vacuolation and loss of secondary lamellar epithelium along with massive leukocytic infiltration mainly lymphocytes (Fig.10 &11). Destruction of plical cells of secondary lamellae was seen. **Group 6 and group 7** showed mild hyperplasia of gill lamellae. **Group 8 (O & F & Fun & B):** Gills of this group showed less sever histological alterations. Mild congestion of blood vessels in both gill arch and primary lamellae was observed. Both primary and secondary lamellae showed mild vacuolar degeneration and hyperplasia (Fig. 12).

Histopathological Results of *T. zillii*:

Liver: Control group 1: The liver of control group revealed moderate degeneration of both hepatocytes and hepatopancreas (Fig. 13). **Group 2 (B&F):** most livers of

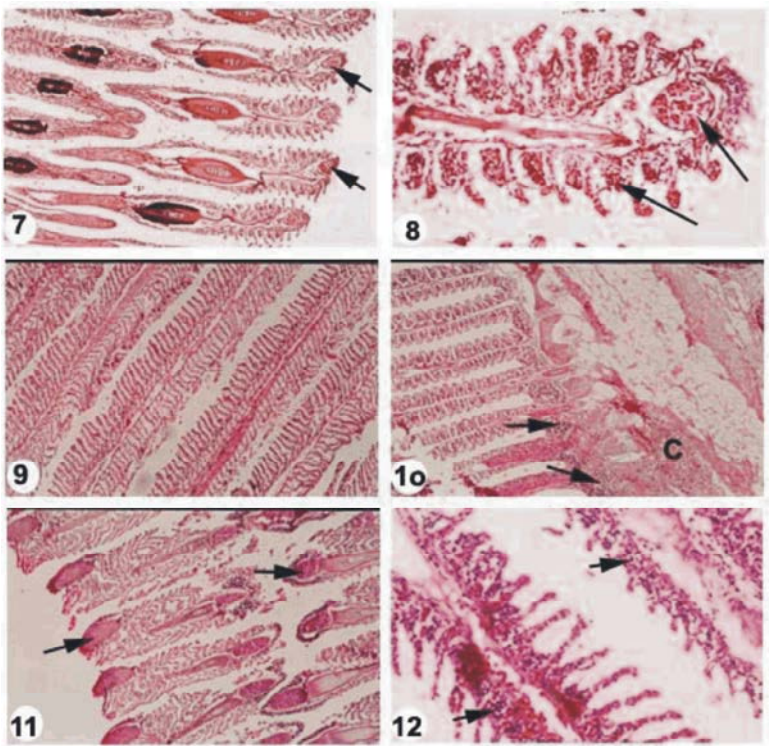

Fig. 7: *Mugil cephalus*, gills of control group showing congestion of blood vessels at the base of gill arch and central venous sinuses of gill lamellae (arrows). H&E. X 200.

Fig 8: *Mugil cephalus*, gills higher magnification of Fig. (7), showing congestion of blood vessels of primary and secondary lamellae (arrows). H&E. X 400.

Fig 9: *Mugil cephalus*, gills bacteria and fungus (B & Fun & F) showing mild vacuolation and hyperplasia of the epithelial lining the secondary lamellae. H&E. X 400

Fig 10: *Mugil cephalus*, gills received oil (O&F), showing moderate to severe congestion of blood vessels at base arch (C) and central venous sinuses, in addition to massive leukocytic infiltration (L) mainly lymphocytes H&E. X 200.

Fig 11: *Mugil cephalus*, gills received oil (O&F), showing hyperplasia of gill lamellae and in addition to dilatation of venous sinuses (arrows). H&E. X 200.

Fig 12: *Mugil cephalus*, gills received oil and fungus/bacteria (O & F & Fun & B), showing mild to moderate hyperplasia of gill lamellae and lymphocytic infiltration (arrows). H&E. X 400.

this group showed mild to moderate focal areas of degenerative changes represented mainly by vacuolar degeneration. Congestion of blood vessels was also observed. **Group 3 (Fun & F)**: livers of this group showed mild to moderate focal areas of degenerative changes represented mainly by vacuolar degeneration. Congestion of blood vessels was also observed. **Group 4 (B & Fun & F):** most livers of this group showed mild to moderate focal areas of degenerative changes mainly vacuolar degeneration. Congestion of blood vessels was also observed (Fig. 14). **Group 5 (O & F):** The liver revealed advanced vacuolar degeneration of the hepatocytes. Other cases showed degeneration and hyperplasia of the hepatopancreas. However, most cases exhibited vacuolar degeneration in the hepatocytes and coagulative necrosis in the hepatopancreas (Fig. 15 &16). **Group 6 and group7** showed mild degeneration of hepatocytes and mild congestion. **Group 8 (O & F & Fun & B)**: liver showed mild congestion of blood vessels, in addition to mild degeneration of hepatopancreas and swelling and vacuolar degeneration in the hepatocytes with activation of melanomacrophages (Fig. 17).

Gills: Control Group 1: Gills of control group showed excessive sloughing, degeneration and severe congestion of central venous sinuses (Fig. 18). **Group 2 (B & Fun)**: Gills showed mild congestion of blood vessels, mild histological alterations represented by vacuolation and

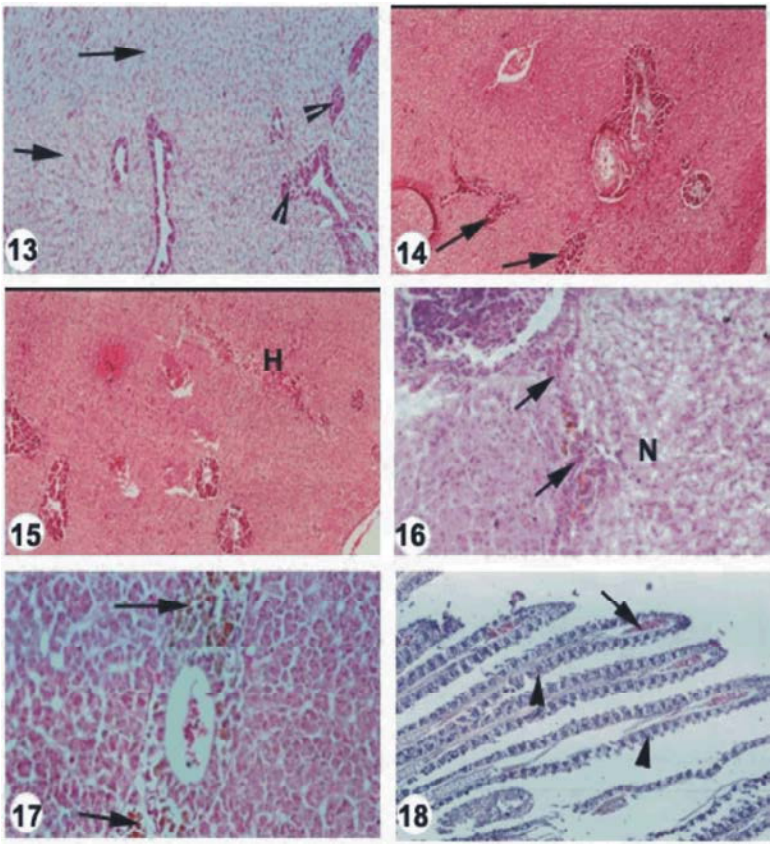

Fig 13: *Tilapia Zillii*, liver, control group from lake Temsah showing moderate vacuolar degeneration of both hepatocytes (arrowe) and hepatopancreas (arrow head) H&E. X 200.

Fig 14: *Tilapia Zillii*, liver received bacteria and fungus (B & Fun & F) showing mild to moderate focal areas vacuolar degeneration and congestion of blood vessels (arrows). H&E. X 200.

Fig 15: *Tilapia Zillii*, liver received oil (O&F), degeneration of the hepatocytes and hyperplasia of the hepatopancreas. In addition to focal hemorrhage (H). H&E. X 200.

Fig 16: *Tilapia Zillii*, liver received oil (O&F), showing vacuolar degeneration and necrosis of the hepatocytes and coagulative necrosis in hepatopancreas (arrows). H&E. X 400.

Fig 17: *Tilapia Zillii*, liver received oil and fungus bacteria (O & F & Fun & B), showing mild congestion of blood vessels, mild degenerative changes in the hepatocytes with activation of melanomacrophages (arrows). H&E. X 400.

Fig 18: *Tilapia Zillii*, gills of control group showing excessive sloughing of gill lamellae, Leukocytic infiltrations (arrow head) and congestion of central venous sinuses (arrows). H&E. X 200.

hyperplasia of the epithelial lining the secondary lamellae. **Group 3 (Fun & F)**: Gills showed mild congestion of blood vessels, mild histological alterations represented by vacuolation and hyperplasia of the epithelial lining the secondary lamellae. **Group 4 (B & Fun & F)**: Gills showed mild congestion of blood vessels, mild histological alterations represented by vacuolation and hyperplasia of the epithelial lining the secondary lamellae (Fig. 19). **Group 5 (O & F)**: The gills of examined tilapia showed congestion and hemorrhage in the gill arch with intravascular hemolysis. Congestion and mononuclear cell infiltrations in the primary lamellae with focal sloughing, shortening and adhesion in the secondary lamellae were evident (Fig.20 & 21). **Group 6 and group 7** showed mild to moderate congestion of blood vessels, mild to moderate vacuolation and hyperplasia of the epithelial lining the secondary lamellae. **Group 8 (O & F & Fun &B)**:

The gills, of *T. zillii* exposed to Oi land treated with combination of bacteria and bacteria revealed mild congestion in the gill lamellae and mild edema in the gill arch (Fig. 22).

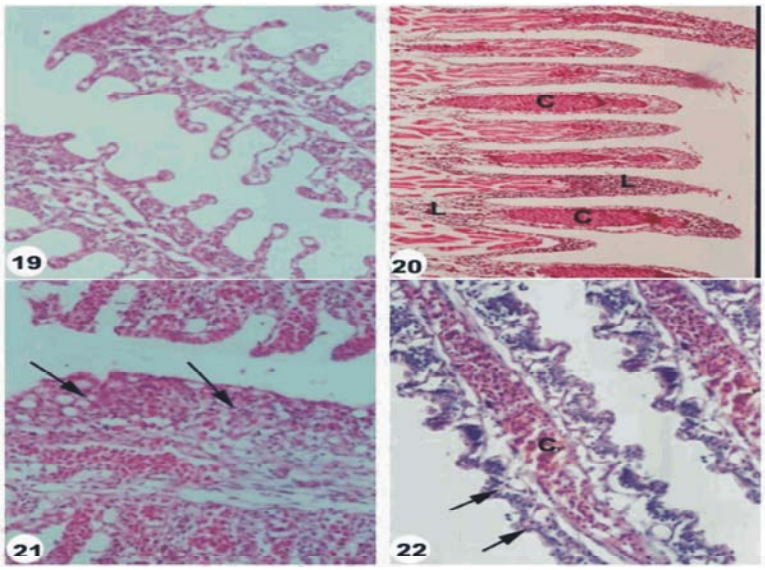

Fig 19: *Tilapia Zillii*, gills (B & Fun & F), showed mild congestion of blood vessels, mild histological alterations represented by vacuolation and hyperplasia of the epithelial lining the secondary lamellae H&E. X 400.

Fig 20: *Tilapia Zillii*, gills received oil (O & F), showing severe congestion and hemorrhage in the gill arch and congestion (C) and mononuclear cell infiltrations (L) in the primary lamellae with focal sloughing. (H&E). X 200.

Fig 21: *Tilapia Zillii*, gills received oil (O & F), showing severe hyperplasia of gill lamellae and shortening and adhesion in the secondary lamellae (arrows). H&E. X 400.

Fig 22: *Tilapia Zillii*, gills received oil and fungus/bacteria (O & F & Fun & B), revealed mild congestion in the gill lamellae and mild atrophy and shortening in the epithelial lining of the secondary lamellae (arrows). H&E. X 400.

DISCUSSION

Polycyclic Aromatic Hydrocarbons (PAHs) are groups of petrochemicals that can cause environmental pollution as they are hazardous to human health due to their carcinogenic, mutagenic and potentially immunotoxicants properties. Cell injury in form of degenerative and neoplastic diseases in target organs results from disturbance of living processes at the molecular and subcellular levels of biological organization by xenobiotics. Moreover, exposure to crude oil and derivatives can induce a variety of toxic signs in experimental animals. Therefore, histopathological biomarkers have been proven to be a useful indicator of toxicity in fish organs [19, 20]. The histology of liver and gills are good biomarkers to evaluate the toxicity of hydrocarbons [3]. Biodegradation is increasingly being considered as a less expensive alternative to physical, mechanical and chemical means of disposing petroleum derivatives and hydrocarbon pollutants. The ubiquitous distribution of fungi and bacteria and their isolation from oil-contaminated environments indicate that they play an important role in the degradation of oil spilled in the environment.

Liver is the main organ of biotransformation and excretion of xenobiotics that can cause structural, biochemical and molecular alterations in the liver [21, 22]. In the present study, histological alteration of hepatic tissue as a result of motor oil toxicity showed severe congestion of blood vessels, focal hemorrhages fat degeneration and focal areas of massive necrosis. These results agreed with histopathological abnormalities obtained by [3, 23] in liver of freshwater and salt water species exposed to petroleum hydrocarbons. These abnormalities were necrosis and cellular inflammatory response of liver tissue. Gills are very sensitive and respond extremely to water pollution caused by petroleum and its derivatives. So, gill morphology is used as a biomarker to the environmental pollution. In the present study, the engine oil induced hyperplasia of the epithelial cells of gill lamellae of both Mugil and Tilapia. These results came in agreement with that of Brand *et al*. [3] who recorded hyperplasia of secondary lamellae of pink salamon *Oncorhynchus gorbusha* exposed to crude oil. Hyperplasia was reported also with lamellar fusion of gills on Nile tilapia (*O. niloticus*) exposed to diesel WSF [24, 25]. Khan [23] observed hyperplasia of epithelial lining the lamellae in three species of marine fish to the

petroleum hydrocarbons. Rupture of pillar cells was also observed along with telangectasis was also observed. Martinez *et al*. [26], Simonato *et al*. [27] and Ricardo *et al*. [28] recorded rupture of pillar cells as a response to xenobiotics with expression of telangectasis. Hyperplasia of epithelial linning and aneurysm was also recorded by Juliana *et al*. [29].

Groups of both mugil and tilapia which treated with the four bacterial isolates (*Pseudomonas*, *Bacillus*, *Achromobacter* and *Clostricium*) showed mild pathological changes and this could be attributed to the effect of bacterial strains in degradation of oil. Many authors used *Pseudomonas* sp. for the decontamination of wastes and such biodegradation processes proved to result in little or no impact on environment [30, 31]. Yakimov *et al*. [32] mentioned that several bacteria are even known to feed exclusively on hydrocarbons. The present results agreed with that of El-Naggar *et al*. [33] who recorded that the bacterial treatment using the marine *P. aeruginosa* led to the degradation of the oil with low impact on the fish in the surrounding medium. The group treated with fungi (*Mucor circinelloides, Absidia corymbifera and Aspergillus sydawii* and *Penicillium* sp.) has evolved the ability to degrade petroleum hydrocarbons where histopathological alterations of both liver and gills were mild and better than that obtained by bacteria. Our results supported by Obire *et al.* [34], who recorded that *Aspergillus s*and *Penicillium* species were the most efficient metabolizes of hydrocarbons**.** In addition to degrading hydrocarbons directly, fungal mycelia can penetrate oil, thereby increasing the surface area available for biodegradation. Davis and Westlake [35] reported that fungi can grow under environmentally stressed conditions such as low pH and poor nutrient status, where bacterial growth might be limited

The group treated with the combination of bacteria and fungi showed mild or no histopathological lesions and this could be attributed to the role of fungal and bacterial combination in degradation of oil. Obire and Amusan [36] mentioned that fungal mycelia penetrate oil and increase the surface area available for degradation by other microbes. Fungi are notably aerobic and can also grow under environmentally stressed conditions such as low pH and poor nutrient status. Okoro [37] showed greater capability in the degradation of PAHs than the pure cultures of bacteria. The cultures of *Penicillium* sp. and *A. niger* used in the study of Andrea *et al.* [38] and Sutherland [39], have reported that fungi are good PAH degraders.

CONCLUSION

In conclusion the use of bacterial or fungal strains isolated from Lake Temsah in treatment of water polluted with petroleum oil fractions led to the degradation of the oil with low impact on the fish histopathology. Moreover, the use of both bacteria and fungi together was more efficient and both gills and liver were more or less similar to the untreated normal fish. So, it could be concluded that it may be useful to use such integrated microbial system (fungi + bacteria) for crude oil bioremediation in marine contaminated areas.

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