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Oil pollution in Lake Timsah, detection and bioremediation through rearing of *Mugil cephalus* and *Tilapia zillii*

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Abstract

This research assessed the capacity of bioremediation the complete pollution of petroleum produced by burned motor oil in the lake Timsah, Egypt using four indigenous bacteria and four isolates of fungi, separated from Lake and to record the effects on two species of fish, Mugil cephalus and Tilapia zillii, Fish to assess the impacts of the water-solution (WSF) fraction of burning motor oil on the growth efficiency and percentage of the fish's survival with regards to their clinical signs, lengths and weights alone and in combination with 4 bacterial and 4 fungal strains, have been carried out on the toxicity and bioremediation research. Burnt motor oil liquids was added to Aquarium water and four bacterial (Achromobacter sp., Bacillus sp., Clostridium sp. and Pseudomonas sp.), four fungal Isolates (Absidia corymbifera, Aspergillus sydowii, Mucor circinelloides, and Penicillium sp.) were taken to treat the water in Lake aquarium with a microbial application. The treatment was prolonged for 45 days. The results showed the great disparity between both types of fish, mullet and tilapia, where the resistance of tilapia over mullet for each of the oil and microorganisms has led the oil added to the aquaria is none treated to the rate of death of 40% of the mullet fish, and 50% of the tilapia processing. A comparison between treatment and blank aquaria the results has shown that the rate of death was more in blank which proves that the micro-organisms have been used in the aquaria of oil additive treatment had improved and grown well. Microbiological analysis was conducted to the muscles and liver of both types of fish and the results showed that the average of CFU of bacterial colonies in fish treated with bacteria is much less compared with untreated fisf. This explain that the growth rate of the tested fish keeping a similar as untreated groups. On the other hand, the GC / FID showed a decrease in the concentration of (PAHs) and this promotes better fish in the treatment groups compared with the control and also do not see any of the compounds of (PAHs) in the aquaria for the treatment of tilapia fish with fungi and bacteria. The results proved that the microbial treatment using combination of fungi and bacteria are more effective for the remediation of the burned motor oil contaminated lake-water than fungi or bacteria alone.

Keywords: oil pollution, bioremediation, bacteria, fungi, Lake Timsah, *Mugil cephalus and Tilapia zillii*..

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Introduction

Crude oil production constitute a major sours of energy in the wide world and produce serious environmental problems due to accidental oil spillage, leakage and break pipelines[1]**.** Moreover, many studies reported that the oil contamination occurs due to the transportation of oil, surface runoff, illegal bilge water discharges and port activity[2]**.** Oil

spills can cause deleterious impacts to aquatic **and terrestrial environment and human health3.** However, its effects on aquaculture could be serious on the grounds that oil tends to bio-accumulation in the tissues of mollusks, fish and mammals[4]. For the most part take-up of marine oil pollutants by fish can happen through bioconcentration from water and additionally bioaccumulation from diet or potentially suspended sediments[5]. The U.S. Environmental Protection Agency (EPA) lists 16 PAHs as priority pollutants[6]. The environmental concern about PAH is due to their potential to form highly carcinogenic and mutagenic derivatives such as diols and epoxides[7,8]. The water shows limited solvency of PAHs so it has been shown that contact of water with PAH-pollutants sediment that could tend to toxicity of the water. Moreover, Polluted sediment in surface waters represents a continuing resource of pollution in the aquatic food chain. Indeed, even minor convergence of numerous PAHs could be complemented through fish, representing a possible danger to man, being at the head of the food chain[9,10]. PAHs entering the water framework can be first collected in fine-grained sediments, suspended particles, then remobilized in the seawater, at that point become bioavailable to local organisms Lastly accumulate in biota that has high rates of take-up or is unfit to effectively process the parent mixes (for example mussels and many invertebrates)[11]. In the water biological ecosystems, fungi assume a significant role with their capacity in expelling risky compounds from the water. Sediment particles polluted with hydrocarbons from oil spills is one of the desired ecological specialties to fungi which use them as carbon source[12].

The microorganisms involved bacteria, yeast and fungi represent the important groups in the degradation of hydrocarbons[3,13]. There are two ways for bioremediation: bioaugmentation and biostimulation[14,15]. Whereas, the bioaugmentation defined as the addition of highly efficient oil-degrading bacteria to improve and enhance the degradation, biostimulation means stimulate indigenous bacteria activity by modification of the environment conditions. All of these important bioremediation considers promise techniques to be better alternatives, saver and eco-friendly, than physicochemical methods. In co-culture degradation of PAHs, it has been suggested that the procedure is started by the release of fungal extracellular proteins that separate particles that are too enormous to even think about passing through bacterial cell dividers, achieving a halfway oxidative corruption of the PAH. In co-culture degradation of PAHs by fungi, the process is initiated by the release of fungal extracellular enzymes that break down molecules that are very large to pass through bacterial cell walls, accomplishing a partial oxidative degradation of the PAH[16]. This initial ring oxidation increases the potential for degradation and mineralisation by bacteria as the oxidised metabolites have increased water solubility and reactivity thereby eliminating this initial ring oxidation as the rate-limiting step for bacteria[17]. This process not only addresses the inability of the bacteria to transport the molecule into the cell, it also prevents the accumulation of the fungal metabolites. Previously fungal metabolites have been reported to accumulate and exhibit an inhibitory effect on degradation[18]. Before the development of the Suez Canal in the nineteenth century, the Bitter Lakes were moderately little hyper-saline inland lakes encompassed by salt-encrusted sabkha. After the lakes were associated with both the Mediterranean and the Red Sea by the Suez Canal, they turned into a solitary marine body; their size expanded, and their saltiness diminished, coming to somewhere in the range of 43 and 46 ppt[19]. The northern more extensive finish of the water body is known as the Great Bitter Lake, while the southern smaller part is known as the Little Bitter Lake. The base is sandy and sparsely secured with vegetation. Agrarian land, traveler advancement and intermittent zones of salt bog outskirt the lakes on the western side, while the eastern side is generally sandy desert. Lake Timsah, one of the little lakes that establish the Bitter Lakes and situated on the north of the Suez Canal, is a land-inundated embayment with an all out region of 15 km2. Lake Timsah is the most important

asset in Ismailia city. It lies on Suez Canal at the mid-path between Port Said and Suez urban communities; The Lake is the end-purpose of different outlets that release huge volumes of rural, civil and modern wastewater. The lake is limited by Ismailia, the primary city of the district that releases portions of its crude and somewhat rewarded metropolitan waste into the lake. The lake is the primary wet dock of the city, a little port that likewise harbors an assortment of marine works, including the upkeep work of the Suez Canal Authority and its partnered sea works and serves the significant wellspring of fish which spread Ismailia city and its neighbored region. As of late there are an incredible mindfulness that the lake water get a lot of releases which include a huge scope of contaminations, for example, substantial metals, harmful natural mixes and others. All sort of contaminations begin from cultivating and mechanical exercises in the region of the lake. These toxins are harmful to both sea-going biota and people. The disintegration of the lake has reached out to a genuine level where pressing activity is required promptly to reestablish the lake biological system[20].

As the principal oil productive zone of the world is the Middle East zone, the red Sea is considered, still and will be for long time, the primary transportation course for unrefined petroleum. On account of this reality, the hazard and capability of oil contamination are parallely increased in the Red Sea condition[21] what's more, thus Suez Canal, and Lake Timsah. A portion of the channels releasing into the lake are heavy with an assortment of modern contaminations beginning from mechanical zones in Cairo.

The lake is likewise the end-sink of aliphatic and fragrant hydrocarbons that start from delivery exercises, ballasting water, upkeep and sea works in the few harbors around. The lake underpins fishing and the travel industry businesses that utilize countless neighborhood residents and give a critical segment of the locale incomes. A few investigations were led to screen determined natural poisons in the distinctive segment of the lake, including diverse marine organism[22]. Polycyclic aromatic hydrocarbons (PAHs) were the compounds mapped out in these studies[23]. Generally take-up of marine oil pollutes by fish can happen through bioconcentration from water as well as bioaccumulation from diet as well as suspended sediments[5]. Also, the Lake Timsah presented to some of extra oil contamination sources, from the boat building organizations which encompassing the lake, for example, El-Timsah transport building organization, El-Timsah shipyard, El-karakat workshop, and Arab Contractors shipyard work shop[20]. The quality of life in the lake has been a major concern for the local authorities for the last few years. High contamination level is the cause behind the decline in the lake biodiversity and the decline in fish quality harvested from the lake[23,24].

The present study aimed to Isolation and identification of some of the indigenous microorganisms (bacteria and fungi) capable to biodegradation of polycyclic aromatic hydrocarbons (PAHs) and evaluate the ability of four bacterial and four fungal strains to bioremediate the problems caused by motor oil pollution through rearing *M. cephalus* and *T. zillii*.

MATERIALS AND METHODS

Isolation and identification of Bacteria and Fungi

The objective: Isolation and identification of species of bacteria and fungi able to biodegradation of oils.

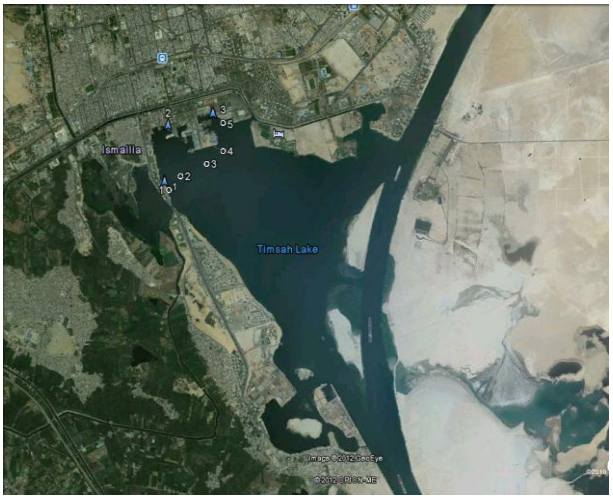

photo.1: *Lake Timsah map showing sampling sites* ***(Google-earth)***

○: sites of water collection (NO. from 1-5),

▲: sites of sediment collection (NO. from 1-3)

Preparing Media for Isolation of Bacteria and Fungi

The following medium was used for isolation and enumeration of bacteria and fungi (half-Strength minimal medium). The composition of the medium per liter was $NaNO_3$ 2.0g, K_2HPO_4 1.0g, $MgSO_4.7H_2O$ 0.5g, KCl 0.5g, $FeSO_4$ 0.01g, Agar 20g, 500 ml distilled water. For isolation of fungi, Chloramphenicol 250mg/l and Rose-Bengal were added to prevent bacterial growth, autoclaved at 121^{o}C for 20 min. The components were allowed to cool to about 45^{o}C under aseptic conditions, and 500ml sterile lake-water was added and mixed thoroughly and then dispensed into sterile Petri-dishes.

Isolation of bacteria and filamentous fungi from sediment

The procedure of isolation was as described by[25], with some modifications.10g (wet weight) of each sediment sample was aseptically transferred to 250ml Erlynmyer-flask, containing 90ml sterile d. H_2O and agitated for 10 min at 150 rpm, then 1ml was aseptically removed and spread of on to half-strength minimal medium plates (5 replica each). Plates were incubated at 21^{o}C for 3 days for enumeration of fungal growth and overnight at 37°C for examination of bacterial growth. After incubation, the developed colonies were counted (as counts of total viable bacteria/ fungi (CFU).

Sampling: Surface water samples were collected in wide neck glass bottles. Surface sediment (1-5cm) samples were collected using a hand shovel (trawel) and stored in sterile glass jars. Five surface water and three surface sediment samples were collected from different sites at Lake Timsah. **(photo.1).**

Isolation of Bacteria and Filamentous Fungi from Water

10ml of each water samples was concentrated by centrifugation at 7000 rpm for 10 min then precipitate was aseptically removed with a sterile pipette and spreaded of onto half-Strength minimal medium plates (5 replica each). Plates were then incubated at 21^{o}C for 3 days for enumeration of fungal growth and overnight for recognizing of bacterial growth. After incubation, the developed colonies were counted (as counts of total viable bacteria/fungi (CFU).

Purification of Microorganisms: The procedure of purification of bacteria and fungi was undertaken as described by[26] with some modifications. Bacterial and fungal colonies were purified and subcultured on LB agar and Czapeks-dox agar plates respectively and incubated at 21^{o}C for 2-3 days for fungi and at 37^{o}C overnight for bacteria. The isolates were stored in refrigerator to be used later.

Identification of Fungi: Growing fungi were identified on agar plates and microscopic examinations were carried out according to the recommendations stated in compendium of soil fungi[27].

Identification of Bacteria: Bacterial isolates were identified using various morphological and biochemical characteristics. For identification of bacterial isolates to genus level, gram staining, motility tests[28] were carried out. In addition to Morphological parameters as colony color, size, shape and margin were carried out according to[29]. Biochemical tests such as catalase, oxidase, indole, methyl red, Vogus Presuker, glucose fermentation, citrate, urea and nitrate reduction were performed using the taxonomic scheme of Bergey's Manual of Determinative Bacteriology[30].

Biochemical tests – according to the Bergey's Manual of Systematic Bacteriology[30].

Extraction of total hydrocarbons from sediments

Soxhlet apparatus was used. 50-100g of wet weight sediment was extracted with 200ml of n-hexane-dichloromethane mixture (1:1) v/v. Mixture was then wormed to 40^{o}C in water bath for 7 hours. The extract was then filtered through watt-mar filter paper in the presence of 2- 6g of anhydrous sodium sulphate, and the extract was then concentrated to 2ml as discussed previously.

Extraction of total hydrocarbons from water: Water samples (1L each) were extracted with two volumes of 30ml n-hexane in a separating funnel with shaking thoroughly for 15min at 150 rpm on a horizontal shaker. The extract was dried on anhydrous sodium sulphate (2-6g) and then concentrated to 1ml as explained above.

Cleaning-up and fractionation process: Cleaning-up and fractionation was performed prior to gas chromatograph/flame ionization detector (GC/FID). The extracted volume was passed through the silica column prepared by slurry packing with 10 g of silica, followed by 10 g of alumina and finally 1g of anhydrous sodium sulphate. The aliphatic fraction (F1) was sequentially eluted from the column using 25 ml of hexane. However, the unsaturated aromatic fraction (F2) was eluted with 60ml of hexane and dichloromethane (80:20; V/V). Both of F1 and F2 fractions were concentrated using a gentle stream of pure nitrogen to about 0.2 ml, before being injected into GC/FID**.**

GC/FID analysis - according to[31]**.** The extracted oil samples were analyzed using Aglient model 6890 plus gas chromatography at the National Research Center. The condition of analysis was as follows:

Chromosorb W-AWDCMS (100/120mesh) coated with 3% OV-17 packed in a 1.8 m long x 2 mm ID glass column with nitrogen carrier gas at 40 ml/min flow rate. Column temperature held at 100^{o}C for 4 min, then programed at 8^{o}C/min to final hold at 280^{o}C. Detector: flame Ionization Detector. Injector: splitless injection.

Experiment for bioremediation of motor oils by bacteria and fungi through rearing fishs

Objective: 1- Assessment of The efficiency of bacteria and fungi and combination of both in the process of bioremediation of motor oil through the period of experiment.

2-The impact of oil pollution on representative samples of living organisms by histopathological effects of motor oil in some organ of the fishes such as livers and gills.

Sampling: Fish samples of fingers *Mugil cephalus* (av. Weight 0.68g& av. length 3.38cm) and *Tilapia zillii* (av. Weight 4.17& av. length 5.29cm) were obtained from fishermen at the shore of Lake Timsah over all the periods of this study.
1000 L of of Lake Timsah Water (**pH:** 7.93 - 8.56 and **salinity:** 30 – 35ppm)
Water collected from site 1.
Dose of oil: 1ml motor oil, **Dose of bacteria**: 0.01g (dry weight), **Dose of fungi:** 0.04g (dry weight) and **Temperature:** 20 – 25 ^{o}C
Fish stock

Fishes were captured using fishing nets (1 mm mesh) from Lake Timsah. They were transported and acclimated in two 100 L glass tanks filled with lake-water from the collecting site (30-35ppt salinity, and 20-25ºC

temperature). The tanks were equipped with constant aeration and kept under natural photoperiod (13 L: 11 D). The fishes remained in the tanks for two- four weeks, according to[32]. Before the fishes were distributed in the experimental aquaria, 10 individuals were randomly selected for the detection of the initial weight and length. Then were transferred to eight glass aquaria (40 x 40 x 60 cm) filled with 30 L lake-water at densities of 10 mullet/aquarium.

Experiment design : The experiment design was planned as the methods described by[33] with some modifications. 16 aquaria used in this experiment, 8 for *Mugil cephalus* and 8 for *Tilapia zillii.* All 8 aquaria distributed **as a followin**

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

Mug. , Tilap.		Meaning	Content of the aquarium
A1,	**A1/**	Control	Lake-water+ fish
A2,	**A2/**	Control+Oil	Lake-water + fish +oil
B1,	**B1/**	blank	Lake-water + fish +mix bacteria
B2,	**B2/**	blank	Lake-water + fish +mix fungi
B3,	**B3/**	blank	Lake-water + fish +mix fungi and bacteria
C1,	**C1/**	Treatment	Lake-water + fish + mix bacteria +oil
C2,	**C2/**	Treatment	Lake-water + fish +mix fungi+ oil
C3,	**C3/**	Treatment	Lake-water + fish +mix fungi and bacteria+ oil

One as a normal control (sea water and Fish), one as a poisonous control (sea water, Fish and oil burned of tractor 1ml), three as blank (sea water, Fish and mix of bacteria or fungi or mix of bacteria & fungi) and final three aquaria used as treatment (sea water, Fish, mix of bacteria or fungi or a consortium of bacteria & fungi and (motor oil 1ml) **photo2.**

The experimental period was extended for 45 days; it was divided into 15 days for adaption and acclimiation, 15 days for oil burned treatments and 15 days for washing process. The feeding regime was applied at 5% body weight per day throughout the experiment, the frequency of feeding was maintained as twice a day for six days a week. The artificial diet was analyzed for moisture, crude protein, ether extract and ash according to standard AOAC methods[33]

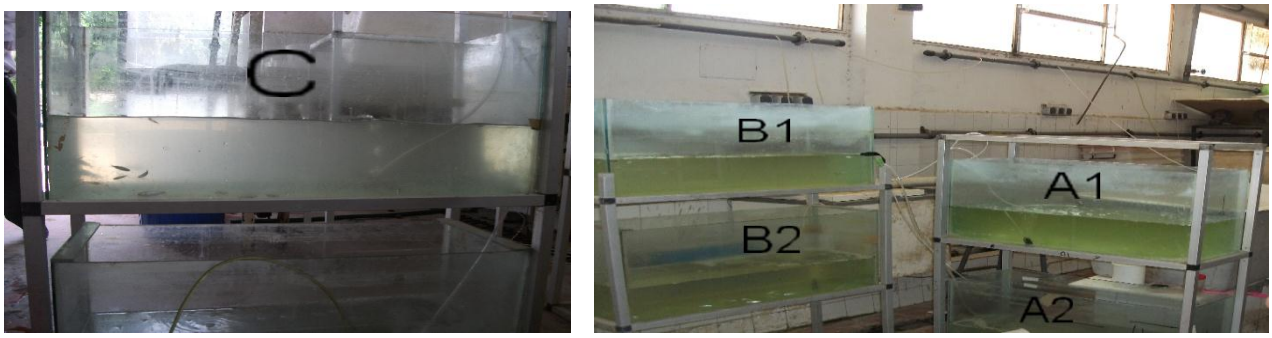

photo 2. Photographs (a, b, c) shows experimental frame work and the used glass aquaria filled with untreated lake-water (control) (A, A/); the bacteria in contact with M. cephalus, T. zillii blank (B1, B1/) respectively; the oily contaminated lake-water under treatments (C1,C2, C3) with M. cephalus and (C1/, C2/, C3/) with T. zillii. The feeding regime

Sampling processes and microbial examination processes in the oily contaminated lake-water

Water samples for microbiological analyses were taken regularly from the rearing fish aquaria after zero, 3, 5, 10, 15, 21 and 30 days of the treatment. 1ml of these lake-water samples was used aseptically to inoculate 9ml of sterile culture medium (nutrient broth) and then incubated for 24hr at 37ºC. Then, the dry weight of the bacterial growth was estimated in mg/100 ml according to[34].

Examination processes of fish in aquarium

1. Examination of skin: The skin of three treated fish per aquarium were swapped and resuspended in 5 ml sterile phosphate-buffered saline (PBS), which is composed of (g/l): 8.0 of NaCl; 0.3 of KCl; 0.73; of NaH_2PO_4 and 0.2 of K_2HPO_4. Complete to 1 liter with dis H_2O, adjust pH to 7.4. All samples were 10-fold diluted, squeezed by hand for few minutes and then spreaded onto nutrient agar plates which composed of (g/l): 3; beef extract; 5 peptone and 20 agar using a glass spreader (5 cm). The total bacterial count , [colony forming unit (cfu/ml)] was estimated in each sample according to[35]. Sterile gloves, bags, swabs, and glass beakers were used for sampling.

2. Examination of muscultre and liver: 1 g of the muscle part (or all the liver) of each examined fish was removed under sterile condition, using sterile forceps, and transferred to sterile tubes each, contains 1 ml PBS. All samples were 10-fold diluted, squeezed by hand for a few minutes and spread onto nutrient agar plates as mentioned above. cfu/ml was estimated in each sample according to[35].

Determination of PAHs through rearing Fish: Water samples for PAHs analyses were taken regularly from the rearing fish aquaria after 1, 2, and 4 weeks, 1litr of these lake-water samples used for determination of hydrocarbons by the method described above.

Analyses

Gain in weight (g/fish) = Av. final weight (g) – Av. Initial weight (g)

Daily weight gain = Gain in weight (mg/fish) / Days

Daily length gain (mm/fish/day) = Gain in length (mm/fish) / Days

Specific growth rate (SGR)(% day-1) = [(ln final weight(g) – ln initial weight (g))/days of the expr.] *100

Instant Daily Growth: IDG= 100 * (Ln final weight- Ln initial weight) / days

Statistical analyses

The statistical analyses of the data were carried out in triplicates using ANOVA test and the least significant difference L.S.D.

RESULTS AND DISCUSSION

RESULTS

Isolation of Bacteria and Fungi from several contaminated sites

In this study, it was attempted to demonstrate the potential degradability of motor oil and a range of hydrocarbon substrates by microorganisms isolated from contaminated sites of surface sediments and water around Lake Timsah. Total count of bacteria isolates from contaminated sites in Lake Timsah presented in **Table 2** ranged from 63.66±2.51 to 195±66.64 of water and samples ranged from 37.33±36.35 to 110.33±8.73 of sediments samples.

Table 2 *Enumeration of Bacterial colonies in Water and Sediment samples.*

Site	Sample	Isolates	Dilution	10^2(CFU)
1	water	Bacteria	10^{-2}	97.7±9.29
2	water	Bacteria	10^{-2}	92.66±2.51
3	water	Bacteria	10^{-2}	63.33±25.16
4	water	Bacteria	10^{-2}	143±68.46
5	water	Bacteria	10^{-2}	195±66.64
1	sediment	Bacteria	10^{-2}	102.33±22.50
2	sediment	Bacteria	10^{-2}	110.33±8.73
3	sediment	Bacteria	10^{-2}	37.33±36.35

Total count of Fungi isolates from contaminated sites (**Table 3)** ranged from 2.33±0.57 to 56±57.23 of water and samples ranged from 6±3 to 57±55.83.

Table 3 *Enumeration of fungal colonies in Water and Sediment samples*.

Site	Sample	Isolates	Dilution	10^2(CFU)
1	water	Fungi	10^{-2}	9.67±6.11
2	water	Fungi	10^{-2}	56±57.23
3	water	Fungi	10^{-2}	2.33±0.57
4	water	Fungi	10^{-2}	7.33±4.04
5	water	Fungi	10^{-2}	16.67±7.24
1	sediment	Fungi	10^{-2}	57±55.83
2	sediment	Fungi	10^{-2}	6±3
3	sediment	Fungi	10^{-2}	8.33±3.21

Identification of Bacterial Strains (Gram Stain and Biochemical Tests)

Seven bacterial isolates *Pseudomonas* sp., *Achromobacter* sp., *Acinetobacter* sp., *Pseudomonas aerogenosa, Bacillus* sp., *Clostridium* sp., and *Aeromonas* sp. were isolated from five contaminated sites around Lake Timsah from water and sediment. Morphological and biochemical characteristics of all bacterial isolates were determined by Gram's staining and biochemical tests according to the Bergey's Manual of Systematic Bacteriology**.** All the isolates were tested for selective biochemical tests which are presented in **Table 4.**

Table 4 *Biochemical tests of selected bacteria Species used in the Treatment*

Source	Location	Species	g.s	s.f	Shape	Cat.	O	In.	Nit.	G.F	V.P	m.r	Cit.	U	M
a & b	1,4,5	***Clostridium sp***	+	+	Rods	-	+	-	+	+	-	+	+	-	-
a & b	3,5	***Bacillus sp***	+	+	Rods	+	+	-	+	+	+	+	+	+	+
B	3	***Pseudomonas aerogenosa***	-	-	Short bacilli	+	+	-	Zn+	-	-	-	-	+	+
A	1,4	***Achromobacter***	-	-	Rods	+	+	-	+	-	+	-	+	-	+
B	3	*Acinetobacter* sp	-		Bacilli	+	-	-	-	+	-	+	-	+	+
B	1,3	*Pseudomonas* sp	-	–	Mono,bi.poly,bacilli	+	+	-	Zn+	-	-	-	-		-
A	5	*Aeromonas sp*	-	–	Short,mono,bi,bacilli	+	+	+	-	-	-	+	-	-	+

Foot notes: a = water, b = sediments, g.s = gram stain, s.f = Spore forming, cat. = catalase, O = oxidase, In = Indole, Nit. = nitrate, V.P = Voges-ProskauerG.F = glucose fermentation, m.r = Methyl red ,Cit. = citrate, U = urea , M = motility .

Whereas, 10 fungal isolates *Pencillium* sp., *Mucor hiemalis, M. mucedo, M. circinelloides, Aspergillus flavus, Alternaria* sp., *Absidia corymbifera, Fusarium* sp., *A. sydawii, Rizopus stolonifer,* were identified on agar plates and microscopic examinations were carried out according to the recommendations stated in compendium of soil fung

Extraction and detection of PAHs from sediments and water in Lake Timsah

Resuts of extraction and detection of PAHs from sediments and water in Lake Timsah are presented in Figures (1 and 2) and Table 6.

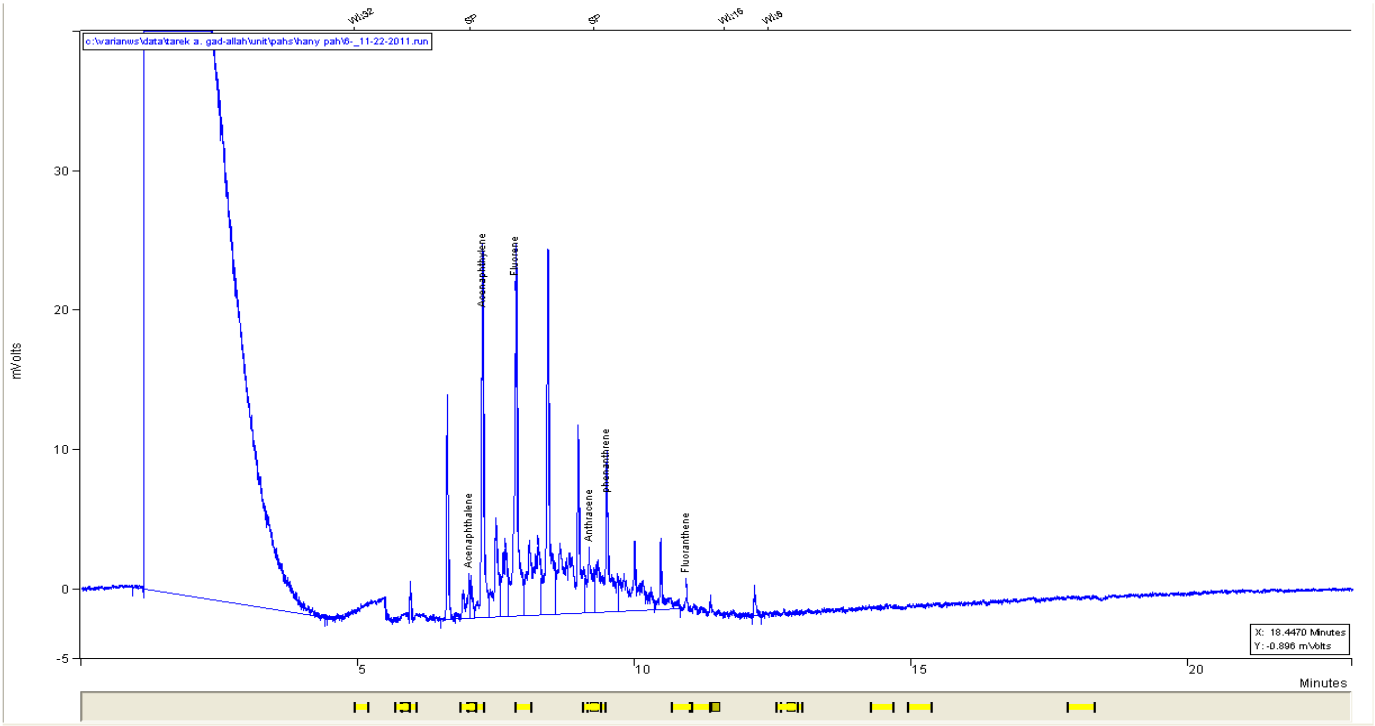

Fig.1 *GC/FID chromatogram of PAHs content of a sediment sample from site 2 in Lake Timsah*.

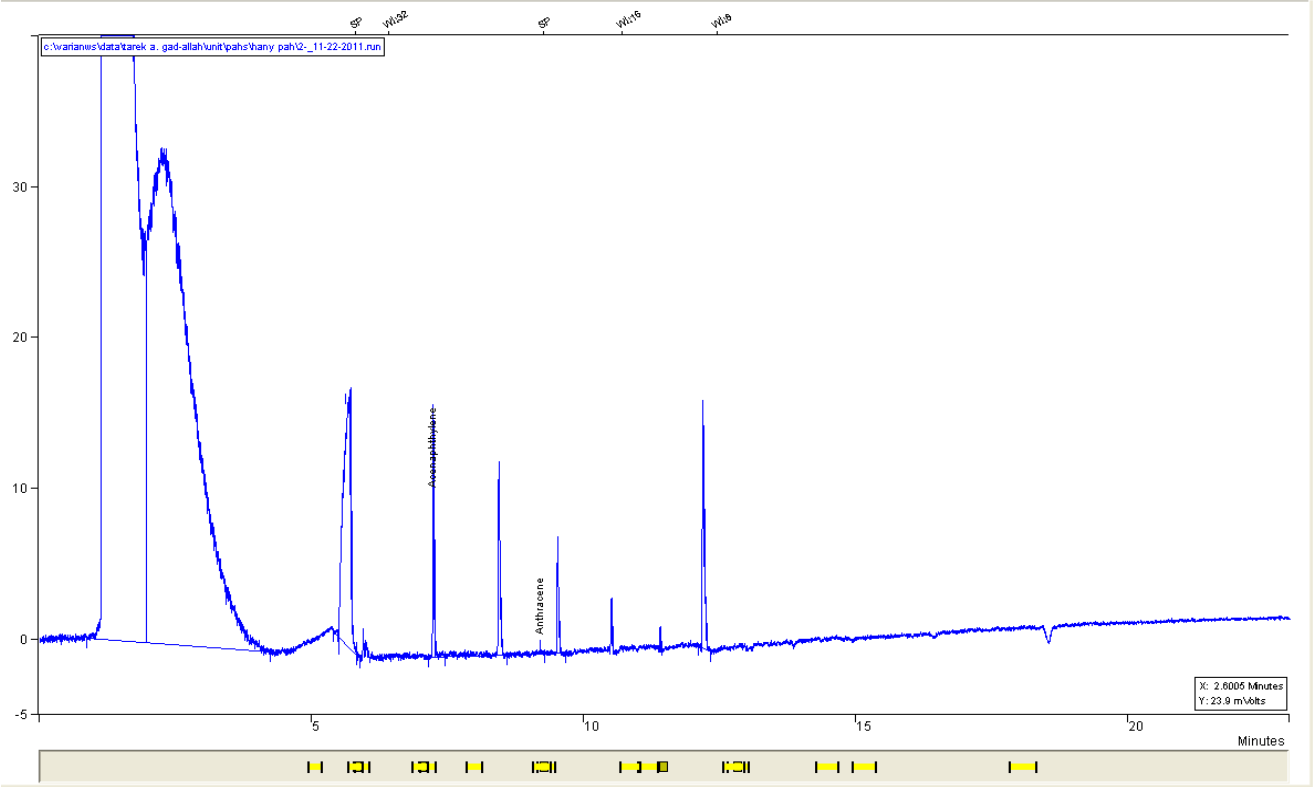

Fig.2 *GC/FID chromatogram of PAHs content of a water sample from site 2 in Lake Timsah.*

Table.6 PAHs content in sediment and water samples from site 2 in Lake Timsah.

Standard PAHs	Sediment (µg/L)	Water(µg/L)
Naphthlaene	N.D	N.D
1-methylnaphalene	N.D	N.D
2-methylnaphalene	N.D	N.D
Acenaphthalene	29.31	N.D
Acenaphthylene	42.26	13.17
Fluorene	65.94	N.D
Anthracene	48.60	4.17
Phenanthrene	62.05	N.D
Fluoranthene	14.22	N.D
Pyrene	N.D	N.D
Benzo(a)anthracene	N.D	N.D
Chrysene	N.D	N.D
Benzo(b)anthracene	N.D	N.D
Benzo(k)fluoranthene	N.D	N.D
Indopyrene	N.D	N.D
TOTAL	261.93	17.34

Experiment for bioremediation of motor oils by bacterial and fungal strains through raring fishes

4.5. Estimation of growth performance and the survival percentage of *M. cephalus* and *T. zillii*

The effect of the microbial motor oil treatments on the growth performance, survival percentage and feed utilization parameters of M. cephalus and *T. zillii,* after 0, 45 days of rearing are presented in **(Table 7., 8)** respectively.

Table: (7) The effect 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.

	Measured parameters	control	F&O	F&B	F&Fun	F&B&Fun	O&F&B	O&F&fun	O&F&B&fun
At zero time	Av. Initial weight (g) ± SD	0.68±0.48	0.68±0.48	0.68±0.48	0.68±0.48	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	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	1.74±0.71	0.84	0.99±0.346	1.59±1.05	0.96±21	1.67±0.43
	Av. final length (cm) ± SD	3.97±0.87	4.2±0.74	4.88±0.86	4.4	4.05±0.80	4.29±0.83	3.80±0.42	5.03±0.47
	Gain in weight (g/fish)	0.21	0.25	1.06	0.16	0.31	0.91	0.28	0.99
	Daily weight gain (mg/fish/day)	4.67	5.56	23.56	3.56	6.89	20.22	6.22	22
	Daily length gain (mm/fish/day)	0.13	0.18	0.33	0.23	0.15	0.20	0.09	0.37
	specific growth rate (SGR)	0.47	0.56	2.36	0.36	0.69	2.02	0.62	2.2
	Instant daily growth (IDG)	0.6	0.7	2.09	0.47	0.83	1.89	0.77	2
	Survival rate (%)	100	50	70	80	80	100	70	80

Foot notes F=Fish, O=Oil, B=Bacteria, Fun=Fungi

Table 8 *The effect of the microbial motor oil treatments on the growth performance, survival percentage and feed utilization parameters of T. zillii after 0, 45 days of rearing*

	Measured parameters	control	F&O	F&B	F&Fun	F&B&Fun	O&F&B	O&F&fun	O&F&B&I
At zero time	Av. Initial weight (g) ± SD	4.17±2.29	4.17±2.29	4.17±2.29	4.17±2.29	4.17±2.29	4.17±2.29	4.17±2.29	4.17±2.2
	Av. Initial length (cm) ± SD	5.29±1.19	5.29±1.19	5.29±1.19	5.29±1.19	5.29±1.19	5.29±1.19	5.29±1.19	5.29±1.1
After 45 days of rearing	Av. final weight (g) ± SD	8.65±2.15	8.4±3.91	9.51±3.23	8.06±1.65	8.24±0.45	8.66±2.66	7.82±3.36	8.36±3.6
	Av. final length (cm) ± SD	7.96±0.58	7.8±1.37	7.94±1.11	7.62±0.79	7.95±0.35	8.01±0.96	7.05±1.68	7.75±1.0
	Gain in weight (g/fish)	4.48	4.23	5.34	3.89	4.07	4.49	3.65	4.19
	Daily weight gain (mg/fish/day)	99.55	94	118.67	86.4	90.4	99.78	81.11	93.11
	Daily length gain (mm/fish/day)	0.59	0.55	0.59	0.52	0.59	0.60	0.39	0.55
	specific growth rate (SGR)	9.96	9.4	11.87	8.64	10.4	9.98	8.11	9.3
	Instant daily growth (IDG)	1.62	1.56	1.83	1.46	1.51	1.62	1.40	1.55
	Survival rate (%)	100	60	80	100	80	80	100	100

Foot notes F=Fish, O=Oil, B=Bacteria, Fun=Fung

The data presented in **Fig. 3 (A, B)** showed that with 1ml oil in these tested aquaria the maximum fungal dry weight in *M. cephalus* and *T. zillii* was increased to 0.08g/L and it was obtained mainly after the 4th day of treatment.

The data presented in **Fig. 4 (A, B)** showed that with 1 ml oil in these tested aquaria the maximum bacterial dry weight in *M. cephalus* was increased to 0.05g/L and it was obtained mainly after the 7th and 13th day of treatment.

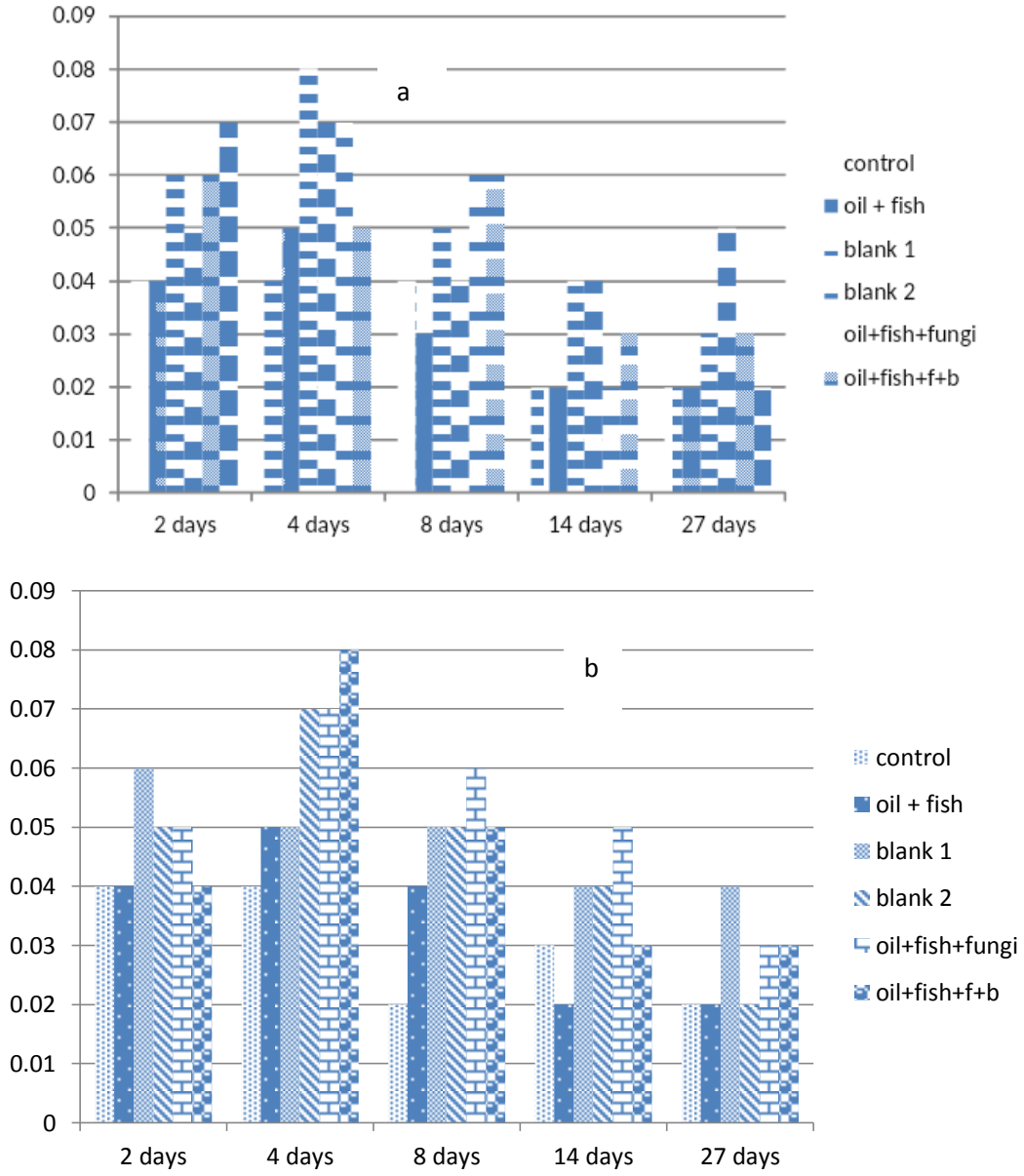

Fig.3 *The fungal dry weight (g/L) in the surrounding media of the contaminated aquaria (O&F, O&Fun&F, O&F&Fun&B, F&Fun, F,B,Fun and the control) during 30 days of oil treatment of (a) M. cephalus and (b) T. zillii.*

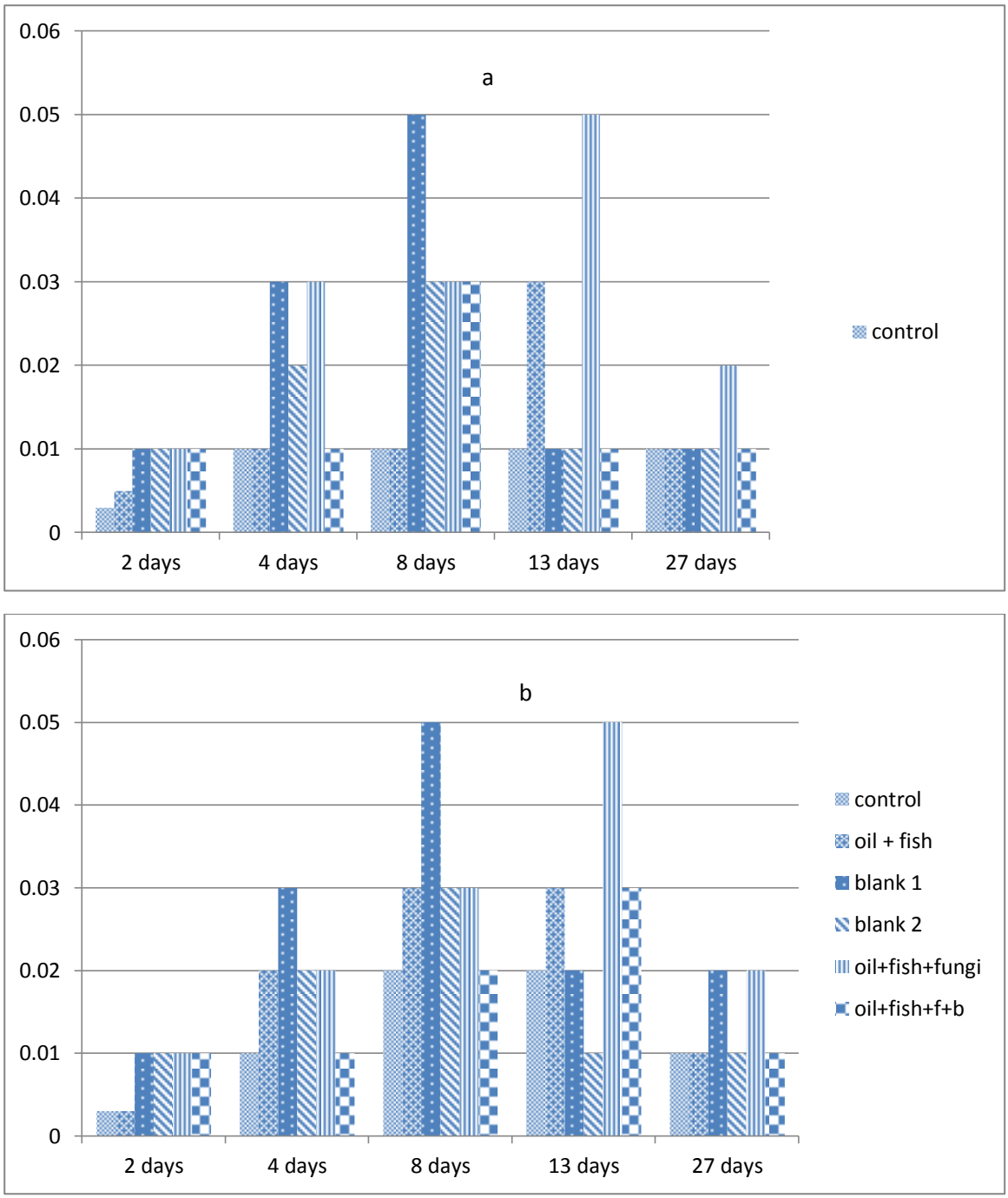

Fig.4 *The bacterial dry weight (g/L) in the surrounding media of the contaminated aquaria (O&F, O&Fun&F, O&F&Fun&B, F&Fun, F,B,Fun and the control) during 30 days of oil treatment of (a) M. cephalusand (b) T. zillii.*

The microbiological examinations of *M. cephalus* and *T. zillii* after 45 days of rearing shwed in **Table 9** and **Table 10** respectively.

Table 9*. The microbiological examinations of M. cephalus after 45 days of rearing.*

treatments	Bacterial count CFU × 10^2/100 ml			
	Skin	Musculature	Liver	Mean*
Control	27.00	28.00	17.00	24[a]
F&B	0.13	0.64	0.83	0.53[b]
F&B&Fun	0.57	1.19	0.63	0.80[b]
O&F&B	1.08	0.10	6.53	2.57[b]
O&F&B&Fun	0.15	0.03	4.74	1.64[b]
Mean*	5.79[c]	5.99[c]	5.95[c]	

**Mean values in the same column or in the same row which have the same letter are insignificantly different at P < 0.05 while the mean values with different letters are significantly different at P < 0.05. F=Fish, B=Bacteria, Fun=Fungi, O=Oil.*

Table 10 *The microbiological examinations of T. zillii after 45 days of rearing.*

treatments	Bacterial count CFU × 10^2/100 ml			
	Skin	Musculature	Liver	Mean*
Control	25.00	24.00	20.88	23.29[a]
F&B	0.14	2.40	9.80	4.11[b]
F&B&Fun	0.11	0.07	0.09	0.09[b]
O&F&B	0.03	2.79	3.71	2.18[b]
O&F&B&Fun	27.24	28.20	26.00	27.15[a]
Mean*	10.50[c]	11.49[c]	12.10[c]	

**Mean values in the same column or in the same row which have the same letter are insignificantly different at P < 0.05 while the mean values with different letters are significantly different at P < 0.05.*

Bioremediation of some fractions of PAH in treated aquaria

Table 11: *Bioremediation of motor oil using selected isolates of bacteria and fungi* ***(****Oil & fungi & Bacteria & T. zillii)*

	After 1 week of treatment	After 2 weeks of treatment		After 4 weeks of treatment
Sample	Additive(µg/L)	B+Fun+O+F(µg/L)	Remediation rate	B+Fun+O+F
methylnaphalene	96.907	28.541	70.55%	N.D
Acenaphthalene	71.478	0.293	99.59%	N.D
Fluoranthene	42.094	9.419	77.26%	N.D
TOTAL	**335.063**	**38.253**	**88.58%**	**N.D**

B= bacteria, Fun= fungi, O= oil, F= fish

Table 12: *Bioremediation of motor oil using selected isolates of fungi (Oil & fungi & T. zillii).*

	After 1 week of treatment	After 2 weeks of treatment		After 4 weeks of treatment
Sample	Additive(µg/L)	Fun+F+O(µg/L)	Remediation rate	Fun+F+O
Acenaphthylene	13.841	12.551	9.36%	N.D
Fluorene	25.102	1.831	92.71%	N.D
Phenanthrene	27.195	14.380	47.12%	N.D
Pyrene	11.042	4.602	58.33%	N.D
TOTAL	**77.18**	**33.364**	**56.77%**	**N.D**

The Fig .5 Showed results of GC/FID chromatogram of PAHs content of a water samples standard sample, additive sample, and treated sample by B & Fun after two week, and treated sample by Bacteria & Fungi after four week from aquaria of *M cephalus*

Table 13: *Bioremediation of motor oil using selected isolates of bacteria (Oil & bacteria & M. cephalus).*

	After 1 week of treatment	After 2 weeks of treatment		After 4 weeks of treatment
Sample	Additive(µg/L)	B+F+O(µg/L)	Remediation rate	B+F+O
Acenaphthalene	71.478	50.327	29.59%	25.311
Acenaphthylene	3.841	7.535	45.56%	4.226
Fluorene	25.102	10.128	59.64%	6.394
Anthracene	40.845	7.882	80.71%	4.663
TOTAL	**151.266**	**75.872**	**49.84%**	**40.594**

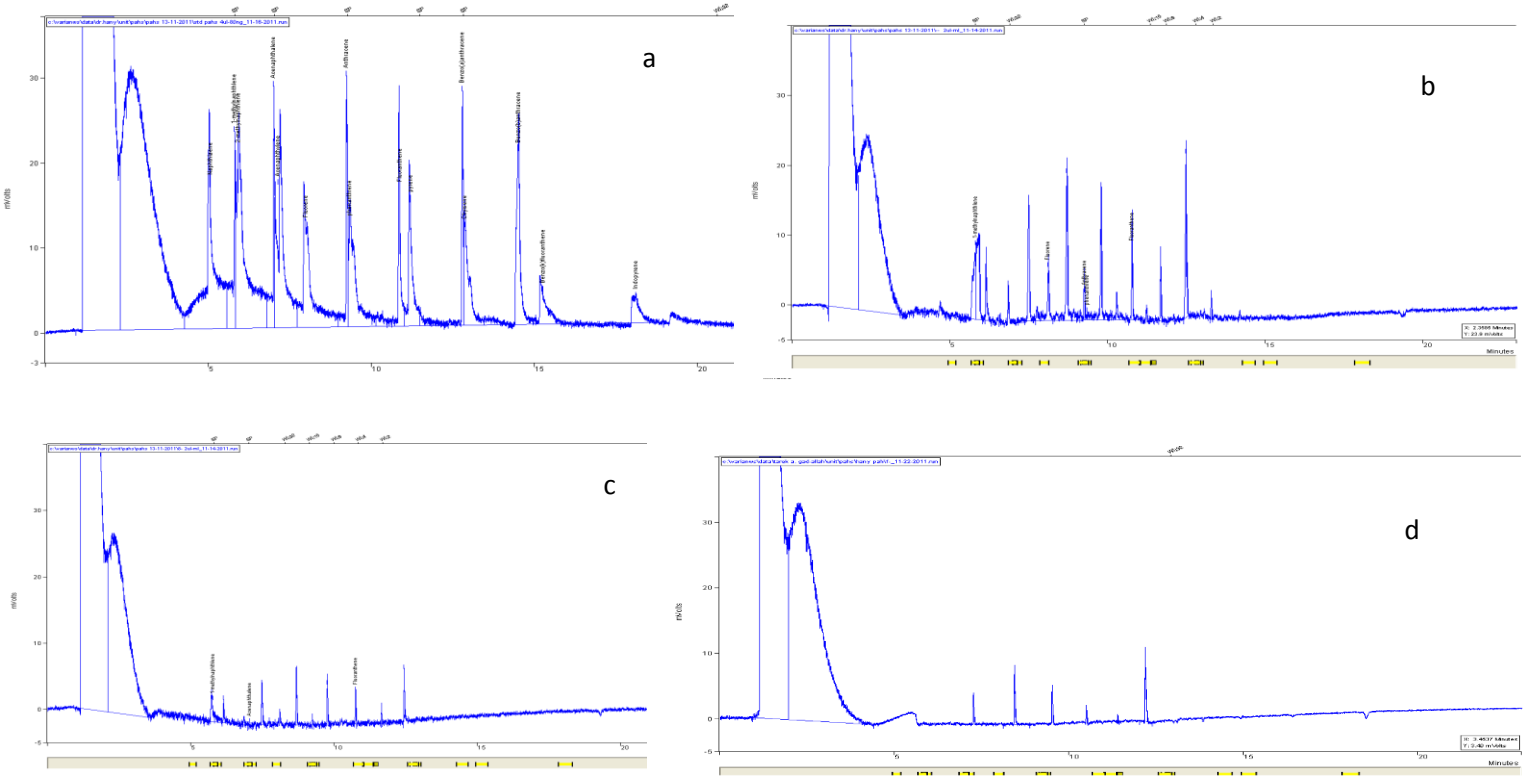

Fig. 5 *GC/FID chromatogram of PAHs content of a water samples (a) standard sample, (b) additive sample, (c) treated sample by B & Fun after two week, (d) treated sample by Bacteria & Fungi after four week from the aquaria.*

Discussion

The effect of oil contamination and its removal from the environment considered one of strategies for environmental restoration of oil polluted sites. The oil involves hydrocarbons that naturally occurring organic compounds and the ability to utilize hydrocarbons are widely distributed among diverse microorganisms. several researchers documented lists containing bacteria and fungi that can remove a wide range of contaminants[36,37]. moreover, several authors illustrated that the fungi play an significant role in the bioremediation products of oil and the most of these fungi attributes to the following genera: *Candida*, *Cephalosporium*, *Cladosporium*, *Alternaria*, *Aspergillus*, *Fusarium*, *Geotrichum*, *Gliocladium*, *Paecilomyces*, *Penicillium*, *Pleurotus*, *Polyporus*, *Rhizopus*, *Torulopsis*, *Mucor*, *Rhodotolura*, *Saccharomyces*, and *Talaromyces*[38-42]. This work has shown the occurrence of pure strains of various bacteria and fungi **Tables (4, 5)** from contaminated sediment and lake water. Surface water and sediment samples were collected from contaminated sites allover Lake Timsah (Ismailia, Egypt). The contaminating hydrocarbons were extracted from each sample to be analyzed qualitatively and quantitatively using standard methods. Results obtained indicate that generally all sites are contaminated with PAHs which were affected by weathering to different degrees as detected by gas chromatographic (GC/FID)[23]. who reported that the levels of PAHs residues detected in the sediment samples from Lake Timsah a variety of 15 PAHs were detected in all sampling sites, with total concentrations ranging from 54.6 mg kg-1 to 27,784 mg kg-1. In the study of[20]**,** for the nature, origin and distribution of the listed reference US Environmental Protection Agency (EPA) priority pollutants; 16 polycyclic aromatic hydrocarbons (PAHs) were investigated in water, sediment and fish of Timsah Lake (Suez Canal, Egypt) using high performance liquid chromatography (HPLC) have reported that the concentration ranged from 52.46–3393µg/L, 585.9-8592.8µg/L for water and sediment, respectively. [43] are also reported the total petroleum hydrocarbon (TPH) content in water of Lake Timsah samples reaching 103mg/l, in sediment samples reaching 635mg/kg. In This study amounts of pollutants are somewhat alarmingly high; the total PAHs content in sediment sample reaching 261.93 µg/L **(Fig.1** and **Table.6**) Whereas total PAHs content in water samples reaching 17.34 µg/L **Fig.2** and **Table.6** PAHs are contaminants of marine coastal sediments because of their hydrophobic character (water solubility between 10-10 and 10-13 mol/l) they are easily sorbed onto suspended particulate[44,45]. In this form, they are more persistent to biodegradation in comparison to dissolved PAHs[45,46]**.** This explains why their concentration in sediments could be higher than that in the overlaying water samples. [47] reported that the *P. aeruginosa* (in respect to *M. cephalus*), (F&B) let to 6.7and 10.7 %, decrease percentage in the daily length gain and the daily weight gain respectively, compared to the control. Furthermore, the biological treatment using microbes which carried out in the aquaria (O&F&B) also led to a reduction in the daily weight gain and a reduction in the daily length gain compared to the control**.** In this study, the survival percentage and growth performance of *M. cephalus* and *T. zillii*were estimated in the examined oily contaminated lake-water after 30 and 45 days of the rearing process are presented in **(Tables 7, 8).** The obtained data in **(Table.7)** showed in general that the bacterial treatment led to improve on the survival percentage and the growth performance of the *M. cephalus* compared

to the untreated fish (control), while the fungal treatment showed slight impacts on both the survival percentage and the growth performance of *M. cephalus* compared to the control. Moreover, the mix of bacterial fungal treatment showed slight impacts on survival percentage and improve on 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 improved the daily weight gain and the daily length day compared to the control. [47] who recorded that the microbial treatment using *P. aeruginosa* is more effective for the remediation of the crude oil contaminated seawater and also for keeping the growth performance of the tested fish as similar as the untreated fries. The obtained data in **(Table8.)** showed that the bacterial and combination of bacteria and fungi treatments led to slight improve on the growth performance and good growth in 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. In contrast, the addition of 1ml motor oil in the untreated fish showed mild to serius effects in both *M. cephalus* and *T. zillii*. The data presented in **Fig.3 (a, b)** showed that with 1ml oil in these tested aquaria the maximum fungal dry weight in *M. cephalus* and *T. zillii* was increased to 0.08g/L and it was obtained mainly after the 4th day of treatment.

The data presented in **Fig.4 (a, b)** showed that with 1 ml oil in these tested aquaria the maximum bacterial dry weight in *M. cephalus* was increased to 0.05g/L and it was obtained mainly after the 7th and 13th day of treatment. The impact of the microbial treatments on the total bacterial count of the muscle, skin and the internal organs of the tested *M. cephalusand* and *T.* zilliiwere presented in Table 6.8 & 6.9 respectively. And the impact of the microbial treatments on the total bacterial count of the muscle, skin and the internal organs of the tested M. cephalus was observed that the most affected fish part was the skin followed by the internal organs.

Table 9 Showed *Mugil* was observed that the most affected fish part was the muscle followed by the liver. The lowest bacterial count was estimated in the fish skin. The microbial treatment led to decrease the fish susceptibility towards the bacterial accumulation compared to the control. It showed a high significant difference at $P < 0.05$ in accumulating the bacterial counts in these examined fish the mean bacterial count was 2.57×10^2 cfu/100 ml and 1.64×10^2 cfu/100 ml in bacterial and bacterial &fungal treatment respectively compared to that of the control (20×10^2 cfu/100 ml).

Table 10 Showed *T. zillii*was observed that the most affected fish part was the liver followed by the muscle. The lowest bacterial count was estimated in the fish skin. The microbial treatment led to decrease the fish susceptibility towards the bacterial accumulation compared to the control. It showed a high significant difference at $P < 0.05$ in accumulating the bacterial counts in these examined fish the mean bacterial count was 2.18×10^2 cfu/100 ml in bacterial treatment compared

to that of the control (23.29×10^2 cfu/100 ml). However, it showed insignificant difference at $P < 0.05$ in accumulating the bacterial counts in these examined fish the mean bacterial count was 27.15×10^2 cfu/100 ml in bacterial treatment compared to that of the control (23.29×10^2 cfu/100 ml). The lowest bacterial count was estimated in the fish muscle.

Biodegradation is one of the major means by which hydrocarbon pollutants can be removed from the environment[48]. A wide range of organisms are involved in this process, often acting as consortia. The polycyclic aromatic hydrocarbons, especially the high molecular weight PAHs, are regulated contaminants at sites polluted with crude oil[49]. It is often difficult to find organisms that will individually degrade all the fractions of crude oil (aliphatics, alicyclic, and aromatic). The four organisms isolated of bacteria (pseudomonas sp., bacillus sp., Clostricium sp and Achromobacter sp) and isolated of fungi (Aspergillus sydawii, Mucor sp., Pencillium sp., Fusarium sp., and Absidia corymbifera) in this study which were identified as species of bacteria and fungi possess the ability of grow on aromatic fraction of petroleum. These are of great interest because previous findings have demonstrated broad substrate spectra of the genus not only on hydrocarbons but also on diverse range of xenobiotic compounds[50,51]**.** [52] who recorded that *Aspergillus* and *Penicillium* species were the most efficient metabolizers of hydrocarbons. In addition to degrading hydrocarbons directly, fungal mycelia can penetrate oil, thereby increasing the surface area available for biodegradation. [53] reported that fungi can grow under environmentally stressed conditions such as low pH and poor nutrient status, where bacteria growth might be limited. In the present study. GC/FID analysis showed that the use of both bacteria and fungi in the bioremediation of oil was more efficient compared to using bacteria in the treatment aquaria **(Fig.5).** Fungi also showed more efficient for bioremediation of PAHs than bacteria.

Table 11 infers that when selected isolates of bacteria and fungi were used for bioremediation of motor oil through rearing ***T. zillii***, 71% of 1-methylnaphalene, 100% of Acenaphthalene and 77% of Fluoranthene fractions of PAH were removed after 2 weeks of the beginning of the experiment while no residues were detected by GC/FID after 4 weeks **(Fig.5).** This result also presented in **Table 12** showed that the fungi removed 56.77% of total PAHs after 2 weeks of the beginning of the experiment while no residues were detected after 4 weeks. For *M. cephalus* results from the polycyclic aromatic hydrocarbon analyses are summarized in **Table 13** which showed Lower bioremediation rate 49.84% after 2 weeks of the beginning of the experiment but 40.594 µg/L residues were detected after 4 weeks when bacteria were worked together in the aquaria of *M. cephalus***.** In another study, strains were isolated from petroleum polluted soil and identified as Pseudomonas pseudoalcaligenes, Bacillus firmus, Bacillus alvei, Penicillium funiculosum, Aspergillus sydowii and Rhizopus sp., and they removed 79%, 80%, 68%, 86%, 81% and 67% of TPH respectively[54].

[55] reported that the Genus Stenotrophomonas, Bacillus, Brevibacillus, Nocardiodes and Pseudomonas were used in combination and give a degradation rate of 67% after only 12 days of treatment. On the other hand the bioremediation rates were relatively lower when selected isolates of bacteria were used alone. Histopathological changes in liver and gills were reported in published paper[56].

CONCLUSION

The use in the treatment of water polluted with oil fractions of the bacterial or fungal strains isolated from Lake Timsah has led to the bioremediation of the polycyclic aromatic hydrocarbons (PAHs) as important fraction of motor oil and Improvement of the condition of the fish tissues with decrease of microbial accumulation. In addition, bacteria and fungi were more effective to be used together. The conclusion could be, therefore, that the use of such an embedded microbial scheme (fungi & bacteria) for the bioremediation of crude oil in marine contaminated fields could be helpful.

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