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Removal of Lead and Oil Hydrocarbon from Oil Refining-Contaminated Wastewater Using *Pseudomonas* spp

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Abstract

The main objective of this study is to investigate the potentiality of using three bacterial species {*Pseudomonas florescence* (PF), *Pseudomonas paucimobilis* (PP) and *Pseudomonas* sp. (PQ)} for the removal of lead and crude oil from oil-contaminated wastewater of an oil refining company, Alexandria, Egypt before discharging into open environments. Bacterial candidates were employed in a batch mode as free-living individuals or mixture. Bioremediation assays were run with three different oily wastewater/bacteria ratios (1:1, 1:2 and 2:1). At each batch, residual concentration (RC) of the selected parameters ($Pb+^2$, oil content, BOD and COD) were determined and their removal efficiencies (RE %) were calculated. Results revealed high removal efficiency for Pb (90.97% from initial concentrations 307.9 mg/l) and oil (56.8 and 68.8% from initial oil concentrations 25 and 500 mg/l respectively) from physically treated oil refinery wastewater. In addition, partial removal of COD (30.98% from 720 mg/l) and BOD (18.98% from 590 mg/l) was achieved. *Pseudomonas florescence* (PF) was the most active reaching the highest removal of most of the tested parameters and wastewater/bacteria ratio of 1:2 was the most convenient while the ratio 2:1 was the least suitable and was inhibitory in most cases for the contaminants removal. Results highly recommend using *Pseudomonas* spp especially Pseudomonas florescence (PF) as efficient, low cost remediation method in oil refineries and similar industries where metallic and organic contaminants are included. It is also suggested treating wastewater using fixed *Pseudomonas florescence* in a continuous system, which can greatly enhance bacterial performance for biodegradation of organic and accumulation of inorganic contaminants.

Key Words: Accumulation, Biodegradation, Industrial Wastewater, Lead, Organic Matter, Petroleum Oil, *Pseudomonas* spp.

1. Introduction

Because of the severity of heavy metal contamination and potential adverse health impact on the public, tremendous efforts have been made to purify waters containing toxic metal ions. Many industries such as coating, automotive, aeronautical, oil refining and steel generate large quantities of wastewater containing various concentrations of lead and other heavy metals (Stellman *et al.* 2008). Lead (Pb^{+2}) is widely used in applications such as storage battery manufacturing, printing, pigments, fuels, photographic materials and explosive manufacturing. Permissible limits for lead in drinking water given by the U.S. Environmental Protection Agency (USEPA) is 0.015 mg/l and for wastewaters is 0.1 mg/l, given by both USEPA and Bureau of Indian Standards (BIS). According to the World Health Organization (WHO), the accepted range of Pb^{+2} in water is 0.01 ppm (Sharma 2009).

Lead is present in natural deposits and may enter soil through (leaded) gasoline leaks from underground storage tanks or through a waste stream of lead paint or lead grindings from certain industrial operations. Emitted lead into the atmosphere can be inhaled, or ingested after it settles out of the air. It is rapidly absorbed into the bloodstream and is believed to have adverse effects on the central nervous system, the cardiovascular system, kidneys, and the immune system (Lane *et al.* 2005). Petroleum refineries discharge large volumes of water including cooling, surface water runoff and sanitary wastewaters. The quantity of wastewaters generated and their characteristics depend on the process configuration, which is approximately 3.5–5.0 m^3 of wastewater per ton of crude generated when cooling water is recycled. Polluted wastewaters generated from oil refineries containing approximately 150–250 biochemical oxygen demand (BOD), 300–600 mg/l chemical oxygen demand (COD), phenol (20–200 mg/l), oil (100–300 mg/l) in desalted water and up to 5,000 mg/l in tank bottoms, benzene (1–100 mg/l), benzo(α)pyrene (less than 1 to 100 mg/l), heavy metal (0.1–100 mg/l for chrome and 0.2–10 mg/l for lead) and other pollutants. Refineries also generate solid wastes and sludges (ranging from 3 to 5 kg per ton of crude processed), 80% of which may be considered hazardous because of the presence of toxic organics and heavy metals. Accidental discharges of large quantities of pollutants can occur because of abnormal operations in a refinery and potentially pose a major local environmental hazard (Sharma 2009).

Lead poisoning in humans, documented ages ago in ancient Rome, Greece, and China (Goyer & Chisolon 1972), can affect almost every organ and system in the body. It may cause severe damage to the kidneys, reproductive system, liver, and brain. Severe exposure to lead is also associated with sterility, abortion, stillbirth, and neonatal deaths. Like mercury, Pb is a potent neurotoxin that accumulates in soft tissues and bone over time. It can damage nervous connections (especially in young children) and associated with delayed puberty in girls (John *et al.* 1998). Lead exposure has been linked to learning disabilities (Singh & Stapleton 2002) and has been shown many times to permanently reduce the cognitive capacity of children at extremely low levels of exposure and there no detectable lower limit, below which lead has no effect on cognition (Sharma 2009). Lead exposure causes blood and brain disorders, weakness in fingers, wrists, or ankles, small increases in blood pressure, particularly in middle-aged and older people as well as anemia. Exposure to high lead levels can severely damage the brain and kidneys in adults or children and ultimately cause death. In pregnant women, high levels of lead exposure may cause miscarriage. Chronic, high-level exposure in men can damage the organs responsible for sperm production (Sharma 2009). Most cases of adult elevated blood lead levels are workplace-related (Subijoy 2002). In the human body, lead inhibits porphobilinogen synthase and ferrochelatase, preventing both porphobilinogen formation and the incorporation of iron into protoporphyrin IX, the final step in heme synthesis. This causes ineffective heme synthesis and subsequent microcytic anemia. At lower levels, it acts as a calcium analog, interfering with ion channels during nerve conduction, a mechanism by which it interferes with cognition (Shree & Tripathi 2007; Stellman *et al.* 2008). Not only humans, but also microorganisms are subjected to metals toxicity due to displacement of essential metals from their native binding sites. Metals can bind to functional groups of biological molecules with varying affinities, and can be classified as either hard or soft (Gadd 2007). Pb (soft metal) is a large cation, very polarizable due to their large number of electrons (Hughesm & Pooler 1989) and preferentially bind to legends containing sulfur such as sulfhydryl ($-SH_2$) groups found in protein. Toxicity of Pb is a consequence of its ability to interfere with several enzymes. Lead has been reported to inhibit acidogenisis, nitrogen transformation or litter decomposition but have stimulatory effects on methanogenisis in anoxic salts sediments (Capone *et al.* 1997).

Thus, it becomes mandatory to remove lead from drinking and wastewaters. However, the conventional methods have some disadvantages such as incomplete removal, high reagent and energy requirements, and generation of toxic sludge or other waste products that require disposal. A variety of methods, e.g. precipitation, coagulation, ion-exchange membrane processing, and electrolytic technologies are used to remove these toxic substances from effluents and industrial wastewater. The search for alternative and innovative treatment techniques has focused attention on the use of biological materials such as algae, fungi, yeast, and bacteria for the removal and recovery technologies. This has gained importance during the recent years because of the better performance and low cost of such biological materials. Bio-sorption presents an alternative to traditional physicochemical means for removing toxic metals from ground waters and wastewaters.

Remediation of metals often involves five general approaches: isolation, immobilization, mobilization, physical separation, and extraction. A combination of more than one approach may be used to properly treat metal-contaminated sites. This combination can be cost-effective (Evanko & Dzombak 1997; Raskin & Ensley 2000; NABIR 2003; Gadd 2007). Microorganisms reduce metals when utilizing them as terminal electron acceptors for anaerobic respiration. Microbial methylation such as transformation of lead to dimethyl lead observed in various contaminated environment especially soil plays an important role in the biological cycle of the metal because methylated compounds are often volatile (Pongratz & Heumann 1999). The ability of bacteria to degrade, accumulate or transform a variety of organic and inorganic compounds is remarkable and has been used in waste processing and bioremediation (Singh & Ward 2004; Atlas & Philip 2005). Many microorganisms are capable of adsorbing metal ions from aqueous solution even with dead cells (Brady & Duncan 1994). Microorganism may uptake metals through either fast, unspecific route (driven by chemo-osmotic gradient across the cytoplasmic membrane of bacteria) or slow, highly specific route that often uses ATP hydrolysis as an energy source and is only produced by cell in time of need. Pb is uptaken through specific slow route to inside the cell (Fagan & Saier 1994). The outer membrane of Gram-negative bacteria such as *Pseudomonas* effectively complexes metals including calcium, nickel and lead (Beveridge & Doyle 1989).

It was documented that some members of the genus *Pseudomonas* are able to metabolize chemical pollutants, therefore, can be used for bioremediation. Biodegradation abilities were shown for polycyclic aromatic hydrocarbons by *P. alcaligenes* (Riis *et al.* 1998), toluene by *P. mendocina* and *P. putida* (Diane & Katherine 1994; Wild *et al.* 1996), carbazole by *P. resinovorans* (Atlas & Philip 2005), a variety of simple aromatic organic compounds by *P. veronii* (Singh & Ward 2004) and carbon tetrachloride by *P. stutzeri* strain KC. Moreover, *P. pseudoalcaligenes* was found able to use cyanide as a nitrogen source (Juana & Edward 1998). In the present study three *Pseudomonas* spp. were investigated on individual and mixed cultures basis for biosorption of lead from contaminated wastewater of an oil refinery, Alexandria, Egypt before discharging into open environments.

2. Materials & Methods

2.1 Microorganisms

Three different bacterial species of *Pseudomonas* {*Pseudomonas florescence* (PF), *Pseudomonas paucimobilis* (PP), *Pseudomonas sp* (PQ)} were kindly provided by IGSR (Institute of Graduate Studies & Research, Alexandria University) collection. They were originally isolated from heavily polluted wastewater and environments and exhibited superior ability for organic matter and heavy metals remediation (El-Bestawy 2005; El-Bestawy & Ibrahim 2005). In the present study, they were used as individual and mixed culture (Pmix) for batch bioremediation of Pb-contaminated wastewater from an oil refinery, Alexandria-Egypt.

2.2 Samples

Samples were collected from water-oil separator (physical treatment unit) of an oil refinery located in Alexandria, Egypt before entering any biological treatment.

2.3 Media and Incubation Conditions

Dehydrated nutrient broth (NB) and agar (NA) used during the present study were supplied by HIMEDIA. NB medium contained (g/l) peptic digest of animal tissue, 5.0; sodium chloride, 5.0; yeast extract, 1.50 and beef extract, 1.50 with 15.0 g/l agar in case of NA medium. They were prepared by dissolving 13.0 and 28.0 g/l from NB and NA dehydrated media respectively. Media pH was adjusted to 7.4, sterilized by autoclaving at 121ºC for 20 min and freshly used for growth experiments as well as bioremediation assays.

2.4 Lead Bioaccumulation

For each species one loop-full of cells were transferred from a fresh slant culture into 50-ml NB placed in a 100-ml conical flask, three replicates each. After inoculation, flasks were incubated under 30°C ± 1 and 120 rpm for 48 hours to allow entering the log phase and getting enough bacterial growth. Nine 250-ml Erlenmeyer flasks containing 50 ml distilled water each with 50 mg/l Pb (II) ion was inoculated with 5% (v/v) of each of the bacterial inocula and incubated under the same conditions. Samples were collected at 24 h interval for 7 days. After collection, samples were centrifuged at 4000 rpm for 20 minutes where bacterial pellets were discarded. Residual concentration of lead present in the clear supernatant was determined using Atomic Absorption Spectrophotometer (Varian spectraAA200). The percent metal accumulated was taken to be the difference between the control and the final concentration of metal in the supernatant.

2.5 Bioremediation Bioassay

Selected bacterial candidates (PP, PF and PQ) were employed in a final volume of 500 ml and run with different ratios of the bacterial inocula (B) and the contaminated wastewater (W) in the following arrangements:

1- 1:1 Oily Wastewater : Bacteria {250 ml (W) + 250 ml (B)}
2- 2:1 Oily Wastewater : Bacteria {333.3 ml (W) + 166.7 ml (B)}
3- 1:2 Oily Wastewater : Bacteria {166.7 ml (B) + 333.3 ml (W)}

For each flask, 5% (v/v) inoculum from 24 h old culture was used. Thus, in case of 250 ml bacterial suspension, 12.5 ml from the pre-grown culture was taken and completed with 237.5 ml nutrient broth (NB). Similarly, with the other ratios when 166.7 ml bacterial suspension is needed 8.3 ml from 24-h culture is added to 158.4 ml NB and in case 333.3 ml bacterial suspension 16.66 ml from pre-grown culture was inoculated in 316.6 ml NB. In addition a 500 ml-bottle was used as a control sample (blank) and run along with other cultures. It contains 250 ml oily-wastewater and 250 ml nutrient broth (NB) free of bacteria. Treated effluent was sampled for 7 days (24 h interval), then residuals of Pb and organic matter (BOD and COD) were determined at each exposure time and their removal efficiency were calculated to determine the effectiveness of the remediation process.

2.6 Analysis of the Raw and Treated Industrial Effluent

2.6.1 Determination of Lead

Concentration of lead in the raw as well as treated samples was determined using an atomic absorption spectrophotometer (Varian Spectra AA200). Sometimes raw concentrated samples had to be diluted and other times synthetic wastewater was prepared when oil samples were not available.

2.6.2 Determination of Petroleum Oil

Oil contents in wastewater samples were extracted using trichloroethane and determined by colorimetric method using Hack spectrophotometer (Hack DR 2000) at 450 nm wavelength.

2.6.3 Biochemical Oxygen Demand (BOD5)

Method 5210 B was used for BOD_5 determination as described in the Standard Methods for Examination of Water and Wastewater (Clesceri *et al.* 1999). BOD_5 can be calculated as follows:

$$\text{BOD}_5\ (\text{mg/l}) = \frac{D1 - D2}{P}$$

Where D1 = DO of diluted sample immediately after preparation in mg/l,
D2 = DO of diluted sample after 5- day incubation at 20 °C in mg/l,
P = Decimal volumetric fraction of sample (300 ml).

2.6.4 Chemical Oxygen Demand (COD)

Closed Reflux Colorimetric Method 5220 D was used for COD determination using potassium dichromate as chemical oxidant as described in the Standard Methods for Examination of Water and Wastewater (Clesceri *et al.* 1999). Colour developed in the samples as well as blank and standards was measured at 620 nm using DR/5000 HACH spectrophotometer and the concentration was calculated from the slope of the standard curve.

3. Results

Bioremediation assays were run with three different oily wastewater/bacteria ratios (1:1, 2:1 and 1:2). At each batch, residual concentration (RC) of the selected parameters (Pb, oil content, BOD and COD) were determined and their removal efficiencies (REs %) were calculated. Since time of sampling was different at the different applying wastewater/bacteria ratios, the initial concentrations (Zero Time) of the tested parameters were different. Regardless bacterial strains, there was a general trend where REs % of all the tested parameters were proportionally correlated with exposure time reaching the highest removals at the end of experiment duration (7 days). Also, at each wastewater: bacteria ratio, clear variation was achieved among the tested species.

3.1 Removal of Lead

Oil wastewater samples were drawn from the API separator (oil-water separation basin) and were tested for the presence of Pb ion that showed varied concentrations according to the production and operational scheme. Only samples with high Pb concentration were collected and subjected to bioremediation using the tested bacteria, otherwise, synthetic wastewater with the required Pb content was prepared and used. Initial lead concentrations in the contaminated effluent ranged between 94 and 307.9 mg/l during sampling time, which considered quite high levels if reached any water source. Comparison of lead removal among the tested *Pseudomonas* spp. either as individual or mixed cultures during the experiment duration at the tested wastewater/bacteria ratios (Table 1 and Fig. 1) revealed the following points:

1. As individuals, PF considered the most efficient (90.3%) followed by PP (74.7%) and finally PQ (45.0%) with the lowest efficiency.
2. The individual cultures of the tested bacteria were most efficient at wastewater/bacteria ratios of 1:1 for strain PF, 1:2 for strain PQ and at 2:1 for strain PP.
3. However, according to the maximum permissible limit (MPL) of Pb stated by the Egyptian Environmental Law (48/1982), none of the cultures reached safe limit (0.05 mg/l) for discharging the effluent. A range of 13.7-25.5 mg/l was recorded as the lowest RC of the Pb after seven treatment days, which is equivalent to 274-510 folds higher than MPL of Pb.
4. Using a mixed culture of the 3 tested species remarkably enhanced Pb removal especially at the highest investigated Pb concentration (307.9 mg/l) reaching 90.97% RE (27.8 mg/l). Such result highly recommends using of the mixed culture especially when handling highly contaminated effluents in addition to either increasing the exposure time or doubling the inoculum size to reach acceptable limits for safe discharge

3.2 Removal of COD

COD values ranged between 700 and 720 mg/l in the raw wastewater samples. COD removal using the selected bacteria concluded the following points: the least efficiency (19.4%) all after 7 exposure days.

1. At 1:1 wastewater/bacteria ratio (Fig. 2A), PP showed the highest COD removal (30.55%) followed by PQ (22.22%) and finally PF with the least efficiency (19.4%) all after 7 exposure days.
2. At 1:2 wastewater/bacteria ratio (Fig. 2B), PF showed the highest COD removal (30.98%) with almost no significant difference with PP (30.28%) and finally strain PQ (23.94%) after seven

Table 1. Comparison of Residual Concentrations (RC) of Pb (mg/l) and the Highest Removal Efficiency (RE %) Using ***Pseudomonas*** spp. Cultures at Different Wastewater/Bacteria Ratios

Exposure Time (Days)	Bacterial Culture												
	PP			PF			PQ			P Mix			Blank
	1:1	1:2	2:1	1:1	1:2	2:1	1:1	1:2	2:1	1:1	1:2	2:1	1:1
0	141	94.0	188	141	94	188	141.0	94.0	188	230.9	154	307.9	230.9
1	121	87.1	181	129	87.3	168	130.0	83.0	169.0	228.1	153	304.3	
2	92.8	64.5	140	92.6	62.6	128	120.0	64.0	151.0	215.6	152	259.7	
3	72.3	41.7	84.6	69.1	58.4	64.7	109.0	56.0	145.3	156.4	143	86.8	180.6
4	60.4	36.4	71.4	34.6	36.8	52.0	105.0	55.6	131.2	134.1	134	73.5	168.4
5	53.7	33.1	63.2	26.1	32.8	34.8	104.2	54.3	128.2	127.3	113	56.7	
6	48.2	28.6	51.3	20.3	26.7	26.7	101.0	54.0	123.7	120.5	106	33.9	
7	46.3	25.5	47.5	13.7	25.5	25.5	100.7	51.7	120.3	117	103	27.8	131.6
RE%	67.2	72.9	74.7	90.3	72.9	86.4	28.58	45.0	36.01	49.33	33.1	90.97	43%

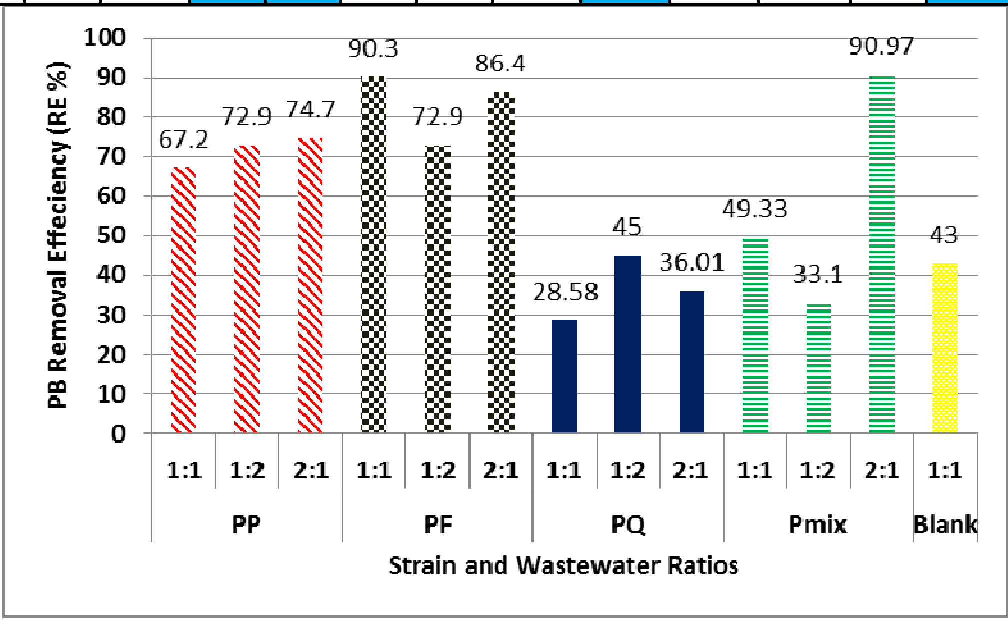


Figure 1. Comparison of Biological Lead Removal among ***Pseudomonas*** Cultures

exposure days. At this ratio although PP had no efficiency change compared to the first wastewater/bacteria ratio (1:1) but the other 2 (strains PF in particular) exhibited significant increase in COD RE%. These results confirmed the influence of the inoculum size of those strains where higher bacterial densities have led to enhancement in their biodegradation capabilities.

3. At 2:1 wastewater/bacteria ratio (Fig.2C), COD removal by all the tested strains was significantly reduced recording 19.72% as the highest RE by PQ after 7 days followed by 16.90 and 14.08% by PP and PF respectively at the same exposure. This is mainly attributed to the toxicity of the wastewater components when the amount was doubled.
4. Using the mixed *Pseudomonas* spp. culture (PMix) (Fig. 3) achieved COD removal ranged between a maximum of 26.39% and a minimum of 19.72% at wastewater/bacteria ratios of 1:2 and 2:1 respectively.
5. PP was the most active for COD degradation at all the tested wastewater/bacteria ratios while PMix culture showed relatively lower REs% compared to individual cultures especially at 1:1 and 1:2 ratios.
6. Wastewater/bacteria ratio of 1:2 considered the best choice for COD removal by individual or mixed cultures due to the presence of enough bacterial cells performing the degradation process.
7. The lowest recorded RC of COD recorded 490 and 495 mg/l achieved by PF and PP respectively after 7 days at 1:2 wastewater/bacteria ratio. These values are almost 5 fold higher than MPL of COD (100 mg/l).

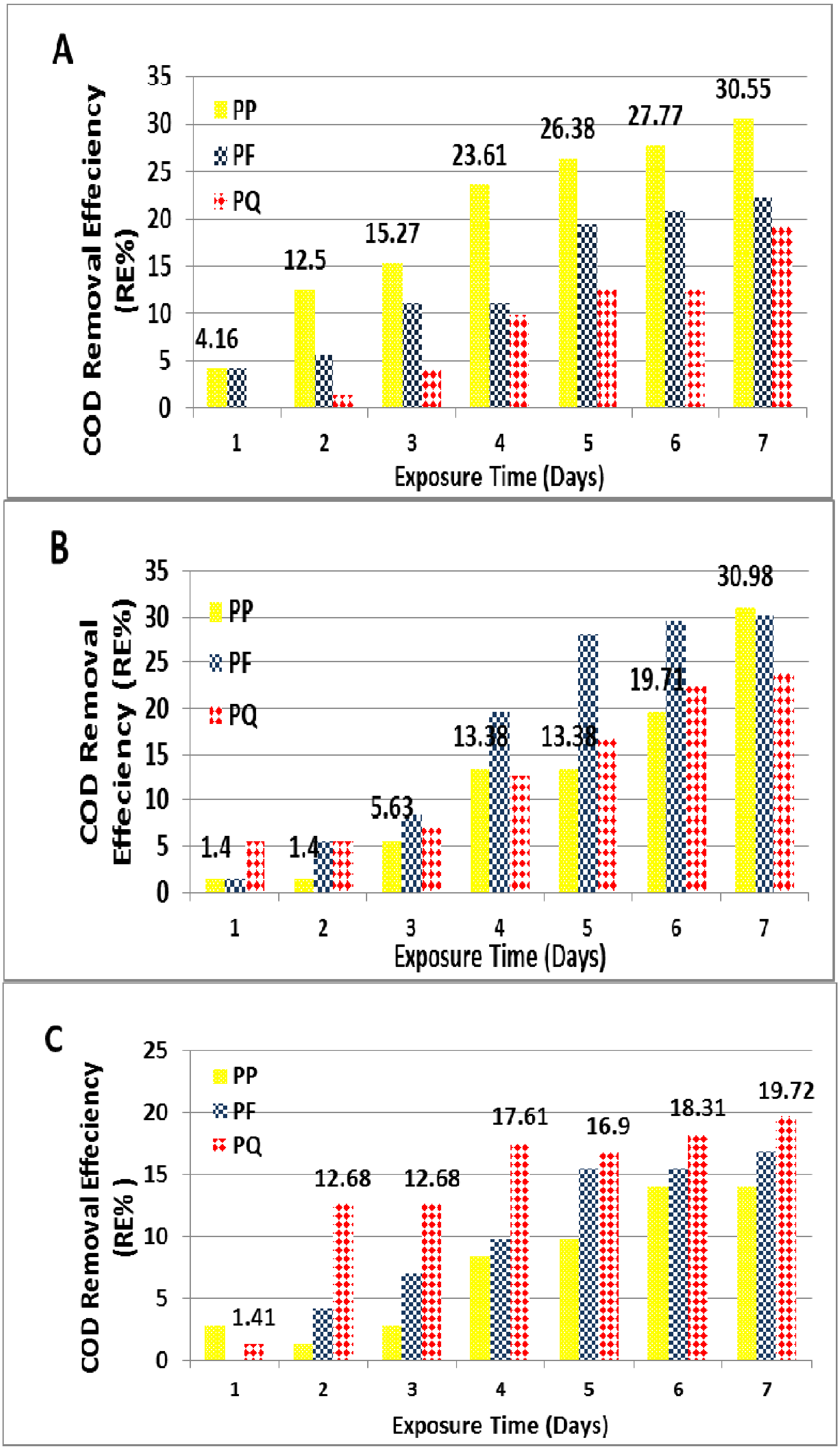


Figure 2. Removal Efficiency (RE %) of COD Using the Selected ***Pseudomonas*** spp. at (A) 1:1; (B) 1:2 and (C) 2:1 Wastewater/Bacteria Ratios

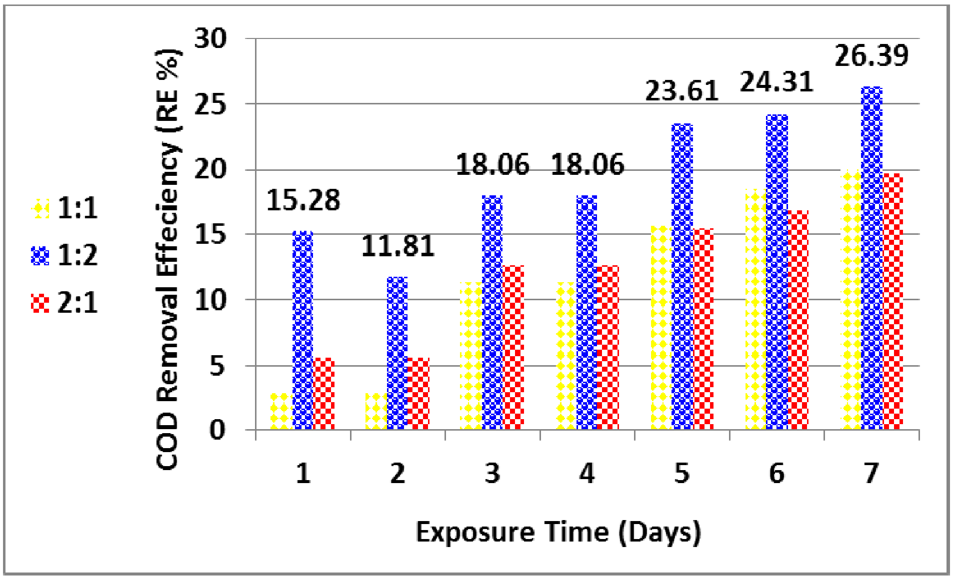


Figure 3. Removal Efficiency (RE) of COD Using the Mixed Culture of Pseudomonas spp. (PMix) at Different Wastewater/Bacteria Ratios

3.3 Removal of BOD

BOD levels in the raw contaminated effluent ranged between 482 and 887 mg/l. BOD removal using *Pseudomonas* spp. (Fig. 4) revealed the following:

1. Generally, low removals for BOD were recorded. Moreover, due to the high toxicity of wastewater on the tested bacteria, BOD was increased occasionally compared to that recorded in the raw wastewater (zero time). These increases are attributed mainly to the death of the involved bacteria, which in turn increased the organic load (BOD).
2. RE of the BOD ranged between a minimum of 0.18% and a maximum of 18.98% both recorded by Pmix after 5 days at 1:1 and 1:2 wastewater/bacteria ratios respectively. PQ also achieved as high as 18.82% RE of BOD after 5 days at 2:1 wastewater/bacteria ratio.
3. Wastewater/bacteria ratio of 1:2 stills the best ratio for organic matter (BOD & COD) removal due to the highest bacterial density at this ratio to overcome wastewater toxicity. This was confirmed by the very low removal and even BOD increases at the 1:1 and 2:1 wastewater/bacteria ratio.

In conclusion and in addition of being highly promising candidates for Pb removal, *Pseudomonas* spp. could remove almost one third of the organic load (BOD & COD) which is definitely an additional advantage.

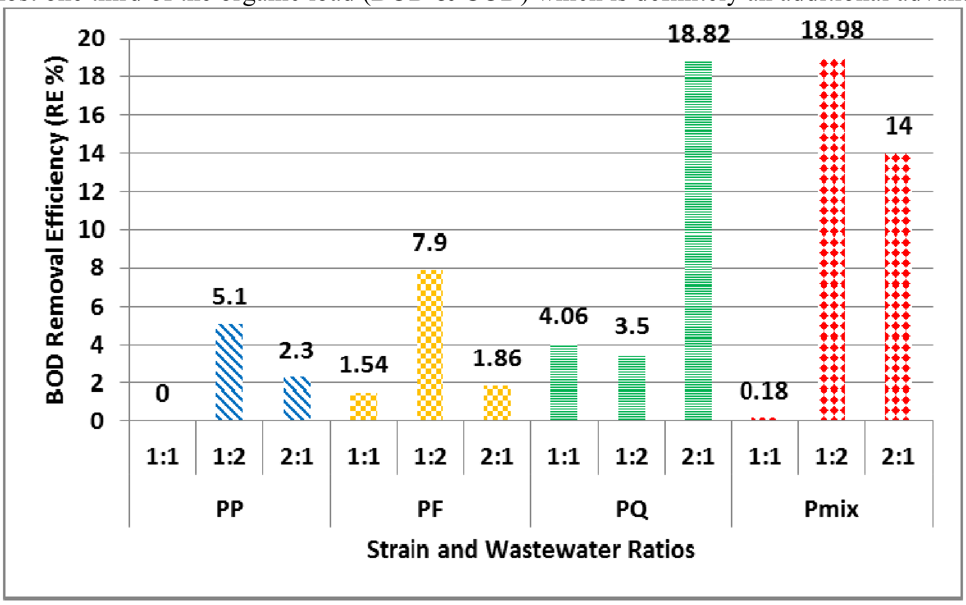


Figure 4. Removal Efficiency (RE) of BOD Using the Selected *Pseudomonas* spp. at Different Wastewater/Bacteria Ratios

3.4 Removal of Oil

Removal of oil by the tested bacteria was investigated at two concentrations 25 and 500 mg/l at different wastewater/bacteria ratios and exposure time.

3.4.1 At Low Oil Concentration (25 mg/l)

Oil content in the wastewater was adjusted at 25 mg/l that was modified to 12.5, 8.33 and 16.7 mg/l due to the dilution in 1:1, 1:2 and 1:3 wastewater/bacteria ratio respectively. Comparison among the tested bacteria in the removal of crude oil at its lowest (25 mg/l) applied concentration (Table 2 and Fig. 5A) revealed the following points:

1. PF showed the highest oil RE (56.8%) followed by PP (53.6%), Pmix (43.2%) and finally PQ (35.0%) all after 7 exposure days.
2. Again, the highest RE of oil took place at 1:2 followed by 1:1 wastewater/bacteria ratio while the lowest oil RE by all the tested bacteria was recorded at 2:1 wastewater/bacteria. This is mainly attributed to the toxicity of the wastewater, which is magnified when small bacterial inoculums size is used (2:1).
3. However, control sample (bacteria-free wastewater) showed extremely low oil removal (4.0%) after seven exposure days confirming the efficiency of the augmented species for oil biodegradation.
4. According to MPL of the crude oil (5 and 10 mg/l) in wastewater discharged into fresh/ground water and fresh surface water respectively stated by law, the treated wastewater had lower recorded RC (3.6 mg/l) and can be safely discharged.

3.4.2. At High Oil Concentration (500 mg/l)

Oil content in the wastewater was adjusted at 500 mg/l that was modified to 250, 166.7 and 333 mg/l due to the dilution in 1:1, 1:2 and 1:3 wastewater/bacteria ratios respectively. Comparison among the tested bacteria in the removal of crude oil at its highest applied concentration (Table 3 and Fig. 5B) revealed the following points:

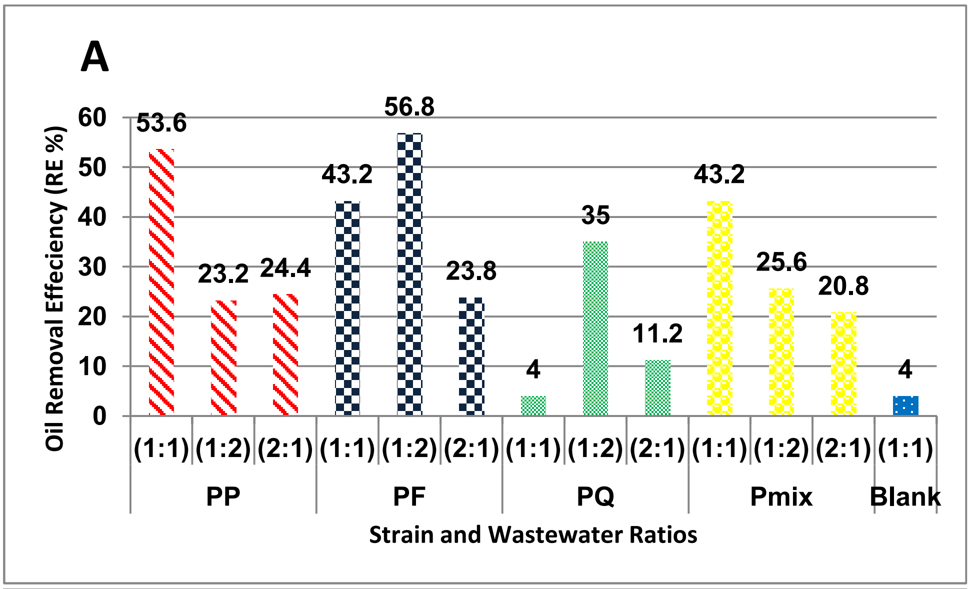
1. Increasing oil concentration 20 folds significantly enhanced oil RE due to the induction of the required enzymes. Again PF showed the highest RE of oil (68.8%), followed by PP (64.1%), Pmix (56.8%) and finally PQ (55.3%) all after 7 days.
2. At this oil concentration, all the highest removals were achieved at 1:2 wastewater/bacteria ratio confirming the importance of bacterial size or density during the treatment process.
3. Control wastewater also showed enhancement in oil removal (32.0%) after seven exposure days.
4. The lowest recorded RC (52.0 mg/l) is slightly (5.2-10.4 fold) higher than the MPL. To reach acceptable discharging limits, wastewater can be treated using fixed bacteria in a continuous system, which can greatly enhance bacterial performance for either biodegradation and/or accumulation of organic and inorganic contaminants.

Table 2. Residual Concentration (mg/l) and Removal Efficiency of Crude Oil in the Raw and Treated Wastewater at Different Wastewater/Bacteria Ratios at the Lowest Tested Oil Level

Exposure Time (Days)	PP			PF			PQ			Pmix			Blank
	1:1	1:2	2:1	1:1	1:2	2:1	1:1	1:2	2:1	1:1	1:2	2:1	1:1
0	**12.5**	**8.33**	**16.7**	**12.5**	**8.3**	**16.7**	**12.5**	**8.3**	**16.7**	**12.5**	**8.3**	**16.7**	**12.5**
1	**12.2**	**8.1**	**16.1**	**12.3**	**7.1**	**16.2**	**12.5**	**7.9**	**16.4**	**12.4**	**8**	**16.2**	**12.5**
3	**11.6**	**7.8**	**14.9**	**11.9**	**6.3**	**15**	**12.3**	**7.1**	**15.9**	**11.9**	**7.5**	**15.3**	**12.5**
5	**8.6**	**7.1**	**13.8**	**9.7**	**5**	**13.9**	**12.1**	**6.2**	**15.4**	**9.8**	**6.8**	**14.2**	**12.2**
7	**5.8**	**6.4**	**12.6**	**7.1**	**3.6**	**12.7**	**12**	**5.4**	**14.8**	**7.1**	**6.2**	**13.2**	**12.0**
RE%	**53.6**	**23.2**	**24.4**	**43.2**	**56.8**	**23.8**	**4.0**	**35.0**	**11.2**	**43.2**	**25.6**	**20.8**	**4.0**

Table 3. Comparison among the Selected Bacteria in the Removal of Oil from Wastewater at its Highest Tested Concentration at Different Wastewater/Bacteria Ratios

Exposure Time (Days)	PP			PF			PQ			Pmix			Blank
	1:1	1:2	2:1	1:1	1:2	2:1	1:1	1:2	2:1	1:1	1:2	2:1	1:1
0	250	166.7	333	250	167	333	250	167	333	250	167	333	250
1	245	165	328	242	142	319	242	164	329	241	160	323	246
2	233	154	321	237	133	305	234	162	326	233	156	320	----
3	216	131	302	198	118	293	208	147	313	184	150	280	214
4	146	95.6	276	162	95.6	262	165	107	291	159	112	252	195
5	113	74.8	253	146	88.2	251	151	98.6	281	143	89.3	241	----
6	106	61.3	247	118	71.6	234	133	86.4	271	129	74.8	230	182
7	93.7	59.8	246	110	52.0	219	117	74.5	258	115	72.0	228	170
RE%	62.5	64.1	26	56	68.8	34	53	55.3	22.6	54	56.8	31	32%



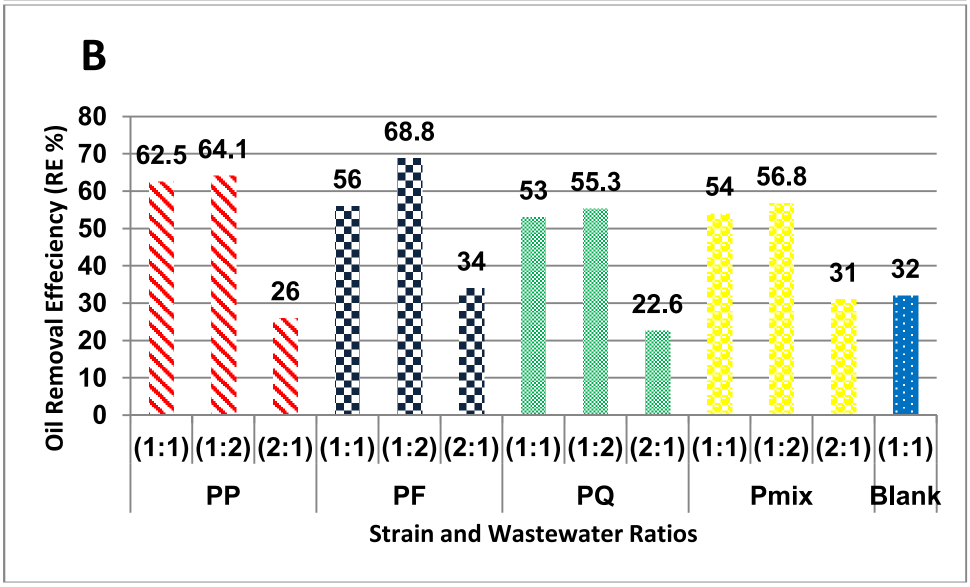


Figure 5. Removal Efficiency (RE) of Oil at its Lowest (A) and Highest (B) Tested Concentration Using the Selected Pseudomonas spp. at Different Wastewater/Bacteria Ratios

4. Discussion

The increase in population initiates rapid industrialization and consequently increases the discharge of industrial and domestic wastewater into aquatic ecosystems. Metal as well as oil pollution in aquatic ecosystems is well known as a serious environmental and public problem. Conventional methods for the removal of heavy metals from wastewaters are often costly and less efficient, therefore, biological removal considered as a cheap treatment method, which proved high capability for pollutants removal.

The three promising *Pseudomonas* bacterial candidates (PP, PF and PQ) used in the present study were selected according to their high ability for the removal of organic and inorganic pollutants (El-Bestawy *et al.* 2005). Genus *Pseudomonas* includes the opportunistic human pathogen *P. aeruginosa*, plant pathogenic bacteria, plant beneficial bacteria, ubiquitous soil bacteria with bioremediation capabilities and other species that cause spoilage of milk and dairy products. *P. fluorescens* (PF), one of the present bacterial selections has multiple flagella indicating high motility. It has an extremely versatile metabolism, and can be found in the soil and in water. It is an obligate aerobe, but certain strains are capable of using nitrate instead of oxygen as a final electron acceptor during cellular respiration**.** Optimal temperatures for growth of *P. fluorescens* are 25-30 ºC. It tests positive for the oxidase test. *P. fluorescens* is also a non-saccharolytic bacterium**.** Heat-stable lipases and proteases are produced by *P. fluorescens* and other similar pseudomonades (Riis *et al.* 1998). During the present study, the selected *Pseudomonas* spp. showed superior ability to bioaccumulate Pb and biodegrade crude oil from the contaminated effluent and use it as a source of carbon and energy. In addition, they could remove one third of the organic load (BOD & COD) which considered lower efficiency compared to the removal of Pb and oil but it is definitely an additional advantage in the present case. The rate of their metabolism for contaminants removal from contaminated media either synthetic or raw industrial effluent was found to be a function of bacterial species, contaminants concentration, and ratio of wastewater: bacteria used and finally exposure time. The marvelous resistance and superior potentiality of *Pseudomonas* for biodegradation of toxic organic pollutants and biosorption of heavy metals have been extensively proved by many authors (Aislabie & Lloyd 1995; De Souza *et al.* 1998; El-Bestawy & Ibrahim 2005; El-Bestawy & Albrechtsen 2007).

Considerable variations were detected in Pb removal by the individual cultures of the tested bacteria according to bacterial species and wastewater/bacteria ratios but they all showed their maximum removal at the last exposure day. In that respect, PF considered the most efficient followed by PP and finally PQ with the lowest efficiency. They were most efficient at wastewater/bacteria ratios of 1:1 and 2:1 (strain PF) and at 1:2 (PP and PQ). Using a mixed culture (3 tested species) remarkably enhanced Pb removal especially at the highest investigated Pb concentration (307.9 mg/l) reaching 90.97% RE (27.8 mg/l). A range of 13.7-25.5 mg/l was recorded as the lowest RC of the Pb after seven treatment days using the tested bacteria, which is 274-510 fold increase in the Pb content allowed for safe discharge (0.05 mg/l).

These results are in agreement with other workers (Srivastava & Majumder 2008) where growth of three freshwater microalgae {*Phormidium ambiguum* (Cyanobacteria)*, Pseudochlorococcum typicum* and *Scenedesmus quadricauda var quadrispina* (Chlorophyta)} was enhanced (chlorophyll a and protein) at lower concentrations of Pb^{2+} and Cd^{2+} (5–20 mg/l) while elevated concentrations (40–100 mg/l) were inhibitory. The highest bioremoval of Pb^{2+} (70%) was achieved by *P. typicum* within the first 30 min exposure (Foster 1982). Comparing with the present results showed that the selected *Pseudomonas* spp. exhibited much higher bioremoval efficiency especially at much higher Pb concentration (307.9 mg/l) which is more than three folds the highest tested concentration in Foster's study.

The Neem tree leaves, known for its drought metals resistance could remove 76.8, 67.5, 58.4 and 41.45% for Cu^{2+}, Ni^{2+}, Zn^{2+} and Pb^{2+} ions respectively from synthetic wastewater after 120 minutes (Emmanuel & Thomas 2009). Again, *Pseudomonas* spp. in the present showed higher Pb removal in addition to minimizing organic content of the effluent. These results confirmed that biosorption is an effective and versatile method and can be easily adopted at low cost to remove heavy metals from industrial wastewaters**.**

Among the tested strains applied as individuals, *Pseudomonas paucimobilis* (PP) was the most active for COD degradation (30.55%) at all the tested wastewater/bacteria ratios. On the other hand, mixed *Pseudomonas* culture showed relatively lower REs% compared to individual cultures especially at 1:1 and 1:2 ratios. These results confirmed the influence of the inoculum size of those strains where higher bacterial densities have led to enhancement in their biodegradation capabilities. The lower COD efficiency is mainly attributed to two factors; first, most of the metabolic energy was directed towards Pb removal. Second, *Pseudomonas* spp. showed high efficiency for crude oil even at its very high concentration tested where they used crude oil as the main carbon and energy source while using other organic content in the effluent as secondary source in a co-metabolic metabolism. Dincer *et al.* (2008) reported that batch oxidation reactions using H_2O_2/Fe^{+2} could achieve 86% COD reduction (from 21000 to 2980 mg/l) in 60 minutes. To achieve this removal 200.52 g/l H_2O_2 and 23.16 g/l Fe^{+2} concentrations were used to reach an optimal mass ratio of 8.658 g. Using UV/H_2O_2 recorded lower (39%) COD reduction (from 21000 to 12730 mg/l). Although higher COD removal could be achieved using chemical oxidation, huge amount of chemicals (H_2O_2 and Fe^{+2}) are required especially for the treatment of highly contaminated oil recovery industrial wastewater.

Generally, low removals for BOD were recorded and may even increase compared to the initial raw wastewater value due to death of the tested bacteria because of the high toxicity of wastewater, which in turn increased the organic load. RE of BOD ranged between a minimum of 0.18% and a maximum of 18.98%. Wastewater/bacteria ratio of 1:2 still the best ratio for organic matter (BOD & COD) removal due to the highest bacterial density at this ratio which is partially overcome wastewater toxicity. Although the cost of removing BOD/COD through chemical oxidation with hydrogen peroxide is typically greater than that through physical or biological means, it has an advantage of high efficiency of BOD and COD removal. However, H_2O_2 considered high cost treatment. In addition, the residual hydrogen peroxide in the sample will liberate oxygen over the test period, resulting in a "false low" BOD value (1 mg/l H_2O_2= 0.5 mg/l DO). In the standard COD test, residual hydrogen peroxide will react with the potassium dichromate reagent, resulting in a "false high" COD value (Steiner 1992). Although high organic matter (BOD & COD) removal can be achieved through chemical oxidation, such reactions considered costly and add huge amount of chemicals into the environment, a character that must be avoided to keep the ecosystem balance. Biological treatment overcomes such disadvantages since no chemicals are used; instead, naturally occurring microorganisms are employed that possess high metabolic activity to deal efficiently with the included contaminants.

Pseudomonas florescence (PF) considered the most active in oil removal at the lowest and highest tested concentration (25 and 500 mg/l respectively). Significantly, higher removals were recorded at the highest tested oil concentration, which is mainly attributed to the active induction of more enzymes upon increasing oil concentration 20 folds, which in turn enabled the tested bacteria to degrade and remove more oil achieving higher efficiencies. At both concentrations, the highest REs of oil took place at 1:2 wastewater/bacteria ratio due to the high bacterial density while the lowest REs were achieved at 2:1 wastewater/bacteria ratio due to the toxicity of the wastewater. Augmented species showed significantly higher oil removal at the tested oil levels compared to bacteria-free wastewater. Such bacteria were able to bring oil content in the wastewater at both concentrations tested to levels below its MPL. In a similar study, bulked soil with indigenous or augmented microbial flora showed more rapid degradation of oil compared to all other amendments. During the experimental period (90 day), wheat bran-amended soil showed 76% hydrocarbon removal compared to 66% in the case of inorganic nutrients-amended soil associated with increase in the number of bacterial populations was also noticed. Addition of the bacterial consortium in different amendments significantly enhanced the removal of oil from the petroleum sludge at the different treatment units (Rajaram & Vasudevan 2001). Although good removal efficiency for oil from soil was obtained, a very long duration (90 day) of time was required compared to the 7 days required in present study to achieve similar results.

Microorganisms are capable of degrading different types of hydrocarbons. Each organism has a preference kind of hydrocarbon and may have a spectrum of optimal degradation activity among related hydrocarbons. Generally, the aerobic degradation of simple aromatic compounds follows different metabolic pathways based on the enzyme system present in the microorganisms. Biodegradation of phenol, for example, is initiated by the formation of catechol, which later undergoes ring cleavage via either meta fission (Hermann et al. 1995) or ortho fission (Ahamad & Kunhi 1996) to intermediates of the central metabolism. On the other hand, five different biochemical pathways have been characterized for BTEX degradation by aerobic bacteria (Cerniglia 1992).

Among microorganisms, bacteria are usually the choice for bioremediation technology because they have more rapid metabolic rates and numerous metabolic pathways of various organic pollutants have been determined in bacteria. Bacterial genera including *Pseudomonas, Aeromonas, Bacillus, Flavobacterium, Corynebacterium, Micrococcus* etc. were reported for their ability to degrade oil hydrocarbon. Previous observations have identified the *Pseudomonas* genus as most efficient among hydrocarbon degrading microorganisms. *Pseudomonas aeruginosa* was the most active hydrocarbon utilizer in crude oil and tolerating high concentrations (up to 50% v/v) of crude oil. It was able to utilize compounds such as aliphatic and mono-aromatic hydrocarbons as well as alcohols as substrates (Das & Chandran 2011). In a recent study, *Pseudomonas stutzeri* was the most efficient and cost-less in the degradation of petroleum hydrocarbons (ALGodah 2012). Enhanced oil degradation, particularly high-molecular-weight n-alkanes and alkylated PAHs, suggesting an increase in the microbial bioavailability of these compounds (Kasunga & Aitken 2000).

5. Conclusion

The selected ***Pseudomonas*** spp. exhibited high efficiency for removing Pb (90.97%) and oil (56.8 and 68.8% from initial oil concentrations 25 and 500 mg/l respectively) during remediation of oil refinery wastewater. In addition, partial removal of COD (30.98%) and BOD (18.98%) was achieved. ***Pseudomonas florescence*** (PF) was the most active reaching the highest removal of Pb, oil and COD during the experimental period while Pmix and PQ showed the highest BOD removal. Wastewater: bacteria ratio of 1:2 was the most convenient where amount and density of bacteria was doubled compared to the wastewater helping to efficiently handle the treatment and increasing removal efficiency. On the other hand, using 2:1 wastewater/bacteria ratio was the least suitable and was inhibitory in most cases due to high toxicity exerted on the augmented bacteria, which is magnified when small bacterial inoculums size is used. In addition, the short period of the treatment (7 days) considered behind the unacceptable limits of the tested parameters for safe discharge. Therefore, results highly recommend using ***Pseudomonas*** spp, especially ***Pseudomonas florescence*** (PF) can be manipulated as efficient, low cost remediation method in oil refineries and similar industries where metallic and organic contaminants are available. To overcome being not compile with law and to reach acceptable discharging limits wastewater can be treated using fixed ***Pseudomonas florescence*** in a continuous system, which can greatly enhance bacterial performance for biodegradation of organic and accumulation of inorganic contaminants.

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