Method development for evaluating the effectiveness of hydrocarbons on BOD, UBOD and COD removal in oily wastewater

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ABSTRACT

The effects of different operating parameters on the treatment efficiency of oily wastewater in terms of biological oxygen demand (BOD) and chemical oxygen demand (COD) were measured.

The analyses of BOD using OxiTop biosensors are reviewed regarding performance characteristics like linearity, response time, precision, agreement between BOD28 values obtained from the biosensors and the ultimate BOD (UBOD), as well as toxic resistance and COD. The wastewater samples were seeded with the bacteria, which were isolated in the current study from Kuwaiti oil-contaminated sand, such as Bacillus mycoides and Bacillus subtilis. After 18 days, the margin for saponin solution and oily wastewater using either Rhodococcus (R), a mixture of Bacillus mycoides and Bacillus subtilis (M) or a mixture of R&M exhibited the maximum rate of BOD. It was found that the corresponding COD of the saponin solution (SS) ranged from 1,525 mg/l to 3,890 mg/l by distilled water and the mixture (RM), respectively. The COD of oily wastewater (WW) ranged from 2,900 mg/l to 4,450 mg/l by distilled water and the mixture of (RM), respectively. Moreover, the higher values of BOD28 were recorded when mixtures of bacteria were added together with the saponin solution or oily wastewaters. Furthermore, the average values of UBOD for the oily wastewater with RM or with amendment substance were increased by about 33.5% and 49.5%, respectively. However, BOD28/COD ratios for all the selected have been found to be less than 0.4, indicating low aerobic degradability.

Key words | COD, BOD, UBOD, seed

HIGHLIGHTS

- The effects of different operating parameters on the treatment efficiency of oily wastewater in terms of biological oxygen demand (BOD) and chemical oxygen demand (COD) were measured.
- The biological oxidation of organic compounds was nearly completed after 20 days with oily wastewater or saponin solution, the microbial seeds needing that long to oxidize the carbon compounds may be due to the high toxicity of the tested samples.
- Generally, there has not been success in viewing any major differences in the rates of oil degradation among the microorganisms. The outcome shows that the ultimate BOD values were slightly increased after 28 days for most of the seeded samples.
- This provides an indication that the constituents in the sample effluents were somewhat non-biodegradable. This will probably cause high toxicity in the samples that could affect aquatic life.
INTRODUCTION

Biological oxygen demand (BOD) is an empirical method carried out using predetermined laboratory procedures in assessing the relative oxygen requirements of wastewater, polluted water and effluents. The values of BOD signify the quantity of biodegradable organic material (carbonaceous demand) and the oxygen required to oxidize inorganic materials, for instance ferrous iron and sulphides. It can also be used to assess the amount of oxygen required to oxidize nitrogen in reduced forms (nitrogenous demand) provided that an inhibitor is utilized to prevent their oxidation. The determination of BOD has been performed in a conventional way by properly aerating a water sample by placing it in an airtight bottle and incubating the sample in a dark room at a temperature of 20 °C for a preset duration. The oxygen consumed in the water is then determined upon completion of the incubation period (Cossu et al. 2017). This ratio provides a gross index of proportion of the organic matter present that can be degradable aerobically within a specified period, such as 5 days for BOD5 (Cong et al. 2019). Therefore, it is essential to determine the ratio between BOD and COD, so that biodegradability can be estimated by using the result of (BOD/COD), once BOD values are known. The ratio of BOD5/COD has been widely utilized as a benchmark for improvement in biodegradability, whereby a naught value signifies non-biodegradability with an increase in the ratio indicating improvement in biodegradability (Alvares et al. 2001). Low value of BOD5/COD ratio of 0.1 or lower typically shows that their resistance to conventional treatment is excellent (Imai 1998; Koch 2002; Raffaello et al. 2012).

A study was undertaken by Chun & Yizhong (1999) on photo-catalytically treated wastewater contaminated with azo dyes from the wool manufacturing industry. They discovered that with a BOD5/COD ratio of higher than 0.3, the biodegradability of the effluent was enhanced. Similar conclusions were presented for a BOD5/COD ratio of 0.4 employing non-biodegradable substituted aromatic compounds (Gilbert 1987). Furthermore, a few other authors (Hamoda & Al-Attar 1995; Hashad et al. 2005; Rodrigo et al. 2020) proposed that sodium chloride above 3.0% will lower the performance of the biological process in wastewater treatment plants, with quick changes in salt concentration triggering more glitches than gradual changes. As stated by Salvadó et al. (2001), the tolerance boundaries in the variation of salinity differ substantially for the organisms present in the sludge. There has been contradictory information in the literature on the impacts of salinity towards...
the efficiency of microorganisms in organic removal during treatment procedures. Some researchers claim that the high salinity of sodium chloride may have some serious effects on the performance of biological processes in the treatment of wastewater. It is essential to perform the analysis on the COD and BOD of the wastewater as the results are required to determine the empirical relation to easily convert the COD to BOD. The outcome of the exercise will offer a means to monitor and evaluate the effluent effectively according to the monitoring agencies and specified industries. A number of samples such as industrial wastewater, disinfected water or heated wastewater do not possess an adequate population of microbial. In this case, the oily wastewater is to be seeded by means of introducing a micro-organism population. These samples shall be seeded by adapting microbes collected from a wastewater treatment plant. In the case of unavailability of seed sources, seeds can be developed and adapted in a laboratory. This can be performed by isolating the bacteria from Kuwait oil residual to extract the initial population of microbes. Several reports on the mechanisms of hydrocarbons toxicity to microbial membranes were studied and reviewed (Zunino & Zygdal 2004; Nazzaro et al. 2013; Lopez-Romero et al. 2015; Vermaas et al. 2018; Badawy et al. 2019). The accumulation of toxic hydrocarbons increased membrane fluidity and permeability, causing a loss of membrane function and ultimately cell death (Norman et al. 2004; Zahir et al. 2006). Many of these tolerant bacterial species that grow in hydrocarbon systems have been isolated (Segura et al. 2008), including Gram-negative and Gram-positive bacteria. Gram-negative bacteria include Pseudomonas putida or Pseudomonas sp., while Gram-positive bacteria include Bacillus (Segura et al. 2008; Nazzaro et al. 2013; Badawy et al. 2019). Rhodococcus (Paje et al. 1997) were hydrocarbon-tolerant. Based on the various studies in the published literature, there are limited investigations into the mechanisms of hydrocarbon tolerance. In order to evaluate whether the microbial population is sufficient, an experiment shall be undertaken to assess how the seed in the BOD test sample performs. The seed adaptation was monitored by using OxiTop to test biodegradability of oily wastewater. The main aims of this paper were to examine the potential employment of the microorganisms to biodegrade the hydrocarbons of oily wastewater. Also, to investigate the potential of isolating the bacteria from Kuwait oil residual to enhance the biodegradation rate. The outcome of this research would be beneficial to science as well as aiding in the construction and enhancement of bio-treatment techniques for water that is polluted with oil.

**The mathematical equation of the BOD**

Wastewater or natural water has a population of microorganism, which are capable of biodegrading organic matter. Various complicated reactions could occur in the BOD bottles, such as first order, half order, second order reaction or a mixture of these reactions. In a BOD test, the rate at which microorganisms utilized organics was considered as a first order reaction, so the equation is: the rate of organics matter utilized by microorganism is proportional to the amount of organics available, this can be described mathematically by a first-order kinetics equation (Davis & Cornwell 2008). Mathematically, this can be expressed as follows:

\[
\frac{dLt}{Lt} = -k dt
\]

Integrating on both sides

\[
\int_{Lo}^{Lt} \frac{dLt}{Lt} = -k \int_{0}^{t} dt
\]

\[
\ln \frac{Lt}{Lo} = -kt
\]

\[
Lt = Lo e^{-kt}
\]

(1)

The \( Lo \) represents the total oxygen equivalent to the total mass of organics at \( t = 0 \), the term \( Lt \) is the amount remaining at time \( t \), while \( k \) is the reaction constant. The oxygen equivalent consumed is equal to the BOD exerted, which can be found from the difference between the value \( Lo \) and \( Lt \). So the equation is:

\[
\text{BOD exerted} = \text{Ultimate BOD} - \text{BOD remaining at that time}
\]

\[
yt = Lo - Lt = Lo - Lo e^{-kt} = Lo(1 - e^{-kt})
\]

\[
yt = L(1 - e^{-kt})
\]

(2)

where: \( yt \) = the BOD consumed (mg/l), \( L \) = the ultimate first stage BOD (mg/l), \( k \) = the rate constant (t\(^{-1}\)) to the base e, \( t \) = time in days.

**Ultimate BOD (UBOD) and oxygen consumption rate (k)**

The ultimate biochemical oxygen demand (UBOD) is the total amount of oxygen required for the total biochemical reaction of organic compounds by microorganisms. In
mathematical models, (UBOD) and the oxygen consumption rate \( k \) are used to estimate the effect of toxic metals on receiving bodies such as rivers and lakes. Therefore, the oxygen consumption rate in the analytical test is often determined along with the (UBOD) value. Various methods are commonly used to determine \( k \) and UBOD \((L_0)\) from the results of BOD test, such as the log differences method, the least-squares method, the series method, the slope method, the method of moments, and the graphical method. In 1937, Thomas had developed the graphical slope method and this method was used for many years for computing and evaluating the kinetics parameters and the constants of the BOD curve. The method proposed by Thomas (1950) had originated from the similarity between two series’ mathematical functions. The Thomas method depends on the equation of BOD rate:

\[
\text{BOD}_t = L_0 (k t) [1 + (1/6) kt]^{-3}
\]

Cube root of both sides

\[
\left( \frac{t}{\text{BOD}_t} \right)^{1/3} = \frac{1}{(kL_0)^{1/3}} + \frac{(k/2)^{2/3}}{6(L_0)^{1/3}}
\]

Plots \( t/\text{BOD}_t \) as ordinate against \( t \) as abscissa, and linearizes the data into a straight line with intercept \( a \) and slope \( b \). The slope \( (b) \) and the intercept \( (a) \) of this line are determined by substituting:

\[
k = 6 \left( \frac{B}{A} \right) \text{ and } L_0 = \left( \frac{1}{ka^{3}} \right) \text{ to } k \text{ and estimate the UBOD.}
\]

The Thomas method was employed in this study based on validity, accuracy, ease of implementation and the results can be achieved faster (Penn et al. 2004; Oke & Akindahunsi 2005).

**MATERIAL**

**Oily wastewater samples**

Oily wastewater was obtained from washing weathered Kuwait oil sand using specially designed and fabricated test equipment invented by the author (Patent registered (A) No. US20170138135 (B) US201833100.78 & (C) GCC 2017-33018 (Patent A 2018; Patent B 2018; Patent C 2017) for remediation of oil contaminated sand (OCS). The washing parameters used in this study were 0.5 wt% of saponin, soil/solution ratio of 10 kg/10 L. All samples were stored in polypropylene bottles and maintained at 4 °C until being experimented on. The samples were analyzed for pH, EC, SO₄, NO₃, Na, K, TOC, TDS, TSS, TPH according to United States Environmental Protection Agency (USEPA) using the standard methods for Examination of Water and Wastewater. The effluent sampling was performed in triplicate. The water samples were collected from seawater (Portsmouth sea front, UK).

**Preparation of saponin**

Pure saponin (98%) purchased from Fisher Scientific Ltd, Loughborough (UK) was used as supplied in this study. Five grams of saponin was added to 1 L of artificial seawater to obtain 0.5% (w/v) saponin and stirred for 10 min at a constant speed of 200 rpm to ensure complete dissolution.

**Dilution of samples**

To obtain the appropriate depletion of COD in the incubated samples, the optional dilution of samples was prepared in 25 ml conical flask by using (25:1) ratio of distilled water to sample, and shaken manually for 5 min. While the oily wastewater was prepared in 100 ml conical flask by using (100:1) ratio of distilled water to oily wastewater, and shaken for 5 min as well.

**Microbiology preparation**

**Nutritional requirements**

Isotonic solution with 0.9% of NaCl was used throughout the investigation. The nutrient broth and 0.45 μm of charged nylon membrane were supplied by Oxoid, UK and Sigma UK, respectively.

**Preparation of solid media**

Standard conical flask of 250 ml was used for preparation of solid media. The flask was filled with 100 ml distilled water and 2.8 g of nutrient agar (Oxoid, UK). The conical flask was placed on the hotplate stirrer (CB302) to boil the solution to dissolve completely. Then, the mixture was sterilized using an Autoclave (TOMY SX-500) at 121 °C for 15 min. The solution was then cooled to room temperature and pipetted carefully into the Petri dishes to avoid bubbles to the extent possible. Subsequently, it was allowed to solidify at room temperature for 20 min and 10 ml of nutrient agar was dropped into the petri dish.
Preparations of liquid media

A conical flask of 500 ml was used for the preparation process of the liquid media. The media was prepared by using 200 ml of distilled water with 2.6 g of nutrient broth to provide the required characteristics. The solution was mixed with orbital shaking action for 10 min to distribute the nutrient broth in the conical flask. Then, the mixture was sterilized using an Autoclave (TOMY SX-500) at 121 °C for 15 min. Solution was cooled at room temperature for 30 min.

Amendment substance

This experiment made use of adapted seed; for instance, the influent from the biological purification phase of a wastewater treatment plant. Usually, it is not necessary for specimens obtained from wastewater treatment plants to be seeded with bacteria. The samples can normally be used straight away as the measurement solution. Most wastewater obtained from the municipal treatment plants have adequate minerals, trace elements and nutrients to degrade the carbon compounds at optimum level. In this experiment, primary settled sludge (PSS) taken from the Petersfield Sewage Works (Southern Water) (PSS) and Liquid Plant Fertilizer (LPF) were utilized as the amendment substance. Furthermore, all the bacteria employed in the current research were extracted from the Kuwait oil residual, while *Rhodococcus* (R) was supplied by Kuwait Institute of Scientific Research (KISR). 

DNA extraction

The purification of Genomic DNA was performed from pure bacterial cultures using a Wizard Genomic DNA purification kit, while the fluorometry model TK 100 fluorometer was used for quantification. The extracts of DNA were kept at −20 °C and used to amplify 16S rRNA from the extracted DNA aided by 27F and 1,492 primers. All experiments were performed in volumes of 25 μl comprising 12.5 pmol of each primer, 200 μM of each deoxyribonucleoside triphosphate, 2.5 μl of 10x PCR buffer (100 mM Tris-HCl, 15 mM MgCl2, 500 mM KCl; pH 8.3), and 0.5 U of Taq DNA polymerase (Applied Biosystems), and made up using sterile water to 25 μl volume. Based upon the following programme, PCR was conducted in a Therмocycler (Applied Biosystems, Warrington, UK): 5 min denaturation at 95 °C, followed by 30 cycles of 1 min denaturation at 95 °C, 1 min annealing at 55 °C, 1 min extension at 72 °C, and a final extension step of 5 min at 72 °C. Products from PCR were visualized by means of electrophoresis in 2% (wt vol⁻¹) agarose gels and stained using ethidium bromide (0.5 μg ml⁻¹).

The purification of amplified DNA was performed with QIAquick PCR cleanup kit (Qiagen, Inc., Valencia, California, USA), and the determination of DNA concentrations was carried out according to the previous description. Roughly 10 ng quantity of 16S rRNA were used as the template in dye terminator cycle sequencing reactions (Applied Biosystems PRISM dye terminator cycle sequencing kit). The results from the 16S rRNA sequences were analysed by means of the National Center for Biotechnology Information (NCBI; Bethesda, Maryland, USA) BLAST. The sequences were assigned to representatives that have been identified from the main eubacterial lineages in accordance with the highest score and 16S sequence similarity of 97 and 99% for *Bacillus subtilis* and *Bacillus mycoides*, respectively.
Measuring BOD by OxiTop

It has become feasible to measure the BOD throughout a long period of time due to the respirometric BOD system, for example OxiTop. The utilization of the OxiTop system has made the measurement of BOD possible as the measured values are kept automatically within the system. During the incubation, microorganisms use up oxygen and release carbon dioxide and these gases remain dissolved. The values as measured can be documented throughout long periods of time, even after a few days to a few weeks. In reality, this would aid assessment of the treatment system's efficiency as well as the strength of the wastewater. In comparison to the other BOD measurement methods, the microbial biodegradation in the OxiTop bottle could be able to represent the degradation process in natural conditions. Furthermore, the oxygen needed for the operation is supplied by the graduated measuring flask, and consists of dissolved oxygen and the amounts of oxygen above the solution. Moreover, a good exchange of gases between the gas phases and aqueous phase were maintained through using constant stirring. As such, the sample ought to be diluted prior to incubation in an effort to bring the demand and supply of the oxygen into the correct balance <4,000 mg/l, which is considered as a maximum permissible concentration of BOD values for a determination by OxiTop. Therefore, the BOD value was required to be estimated before analysis.

Respirometry measurement

The standardized biodegradability test, such as that of the Organisation for Economic Co-operation and Development (OECD 2008) and International Organisation for Standardisation (ISO 1999) were used to ensure reproducible and comparable results can be achieved by using the OxiTop control system. All samples were collected in 250 ml reagent amber bottles and kept in an incubator to prevent exposure to light for a period of 28 days at 20 °C ± 1 °C.

So as to ensure maximum mixing of the samples, magnetic bar stirrers were included in the bottle. A rubber sleeve was used to seal the bottle top to ensure it was leak proof. All bottles were fitted with wet seals covered with caps which acted as vapour seals over the top of the bottle seals in order to guarantee that no evaporation takes place. As an additional measure, sodium hydroxide (NaOH) pellets, two pellets for each measurement, were utilized to absorb carbon dioxide. The nitrification bacteria were inhibited in the present experiment by adding one drop of N-Allylthiourea (C4H8N2S) ATU to the measurement solution. Once the samples were secured tightly and water sealed, they were incubated. The components of the OxiTop measuring system. For a total stabilization, the sample may need a very long incubation period, which was impractical.

Preparation of stock solution

The rates of the reaction to pollutant concentrations differ between microorganisms; these impacts can be minimized or even eradicated by proper dilution of the samples. The prepared representative dilutions are totally dependent upon the anticipated value of the BOD. Samples having high values of BOD have to be diluted extensively to ensure the adequacy of the oxygen in the used bottles. In order to supply trace elements to the population of microbes so that it can be within the range of quantifiable BOD, diluted water samples are needed. Distilled water was used to dilute the sample with a ratio of 1:10. Two stock solutions were prepared during this study using 250 ml volumetric flasks. The first stock was prepared using saponin and distilled water at a ratio of 1:10 (A), and second stock was prepared using oily wastewater and distilled water at a ratio of 1:10 (B). All samples were manually shaken. In addition, primary settled sewage (PSS) taken from the Petersfield Sewage Works (Southern Water) and liquid plant fertilizer (LPF) were utilized as the amendment substance during the BOD test. A set of 4 sub-stocks were prepared using 50 ml volumetric flasks by adding 2.5 ml of amendment substance into the volumetric flasks and making up to 50 ml using stock solutions A and B. Exactly 22.7 ml of the respective substock was transferred using a graduated pipette into the BOD bottles.

Conventional seeding material (CSM)

In the case of unavailability of seed sources, seeds can be developed and adapted in a laboratory. This can be performed by isolating the bacteria from Kuwait oil sand to extract the initial microbial population, as explained in the section Aerobic growth. This approach is considered as one of the appropriate methods for biodegradation enhancement (Dua et al. 2002). In order to evaluate whether the microbial population is sufficient, an experiment was undertaken to assess how the seed in the BOD test sample performs. The seed adaptation was monitored by using OxiTop to test biodegradability of oily wastewater. The main purpose of seeding the samples is to make certain that the activity and survival of microorganisms is sufficient.
A number of samples contain components which are usually not degradable by the microorganisms present in wastewater. In order to achieve the required microbiology for oil degradation, the wastewater samples were seeded with the bacteria. It is generally accepted that inoculating microorganisms into an established environment is not a straightforward task (Kragh et al. 2018; Hermans et al. 2020). In the current research, the microbial populations were allowed to be grown in nutrient broth to make the seeding adaption easily and more accurate. The culture was obtained from an agar plate of bacteria by using a sterilized inoculating loop. The isolated colonies of bacteria were allowed to grow under standard conditions of nutrient broth (Oxoid, UK). The colony was spread in conical flasks of liquid media. Then, the conical flasks were incubated at 37 °C for 48 hr. The liquid media was required to transfer the same amount of bacteria into OxiTop bottles. For formulation of BOD-CSM, microorganisms were isolated from oil-contaminated soil, as explained in the next section.

**Measuring chemical oxygen demand by colorimetric method**

COD was measured to estimate the oxidizable organic matter in the water sample. 100 ml of sample were homogenized for 5 min in an overhead stirrer (CP Cole-Parmer). The oily wastewater was diluted (distilled water to oily wastewater, 100:1) in a 100 ml conical flask, and manually shaken for 5 min. The cap from the COD vial provided in the kit was removed and 2 ml of diluted sample was micro-pipetted into the vial. The lids were sealed tightly and the vial mixed gently several times to mix the contents. The vial was placed in the preheated digester block and allowed to react with the COD vial (0–1,500 ppm) containing 86% sulphuric acid and potassium dichromate. The vials were digested in a COD reactor hot block (DRB200, Hach, UK), and heated for two hours at 150 °C. The block was turned off and allowed to cool for 15 min. The digested samples and reagent blanks were measured in a pre-programmed colorimeter (DR890, Hach, UK) photometer. The results are expressed as the number of milligrams of oxygen consumed per litre of sample (mg/l COD). Subsequently, the colorimeter reading was multiplied by the ratio factor.

### RESULTS

Proper samples of oily wastewater were collected from washing of Kuwaiti oil residual throughout a certain duration at various intervals. The wastewater samples have pH values of between 6.6 and 8.5. Among the properties of oily wastewater are the COD, which ranges between 36,000 mg/l and 50,000 mg/l, and the range of conductivity, which was 60,800 μS/cm. They were stored in polypropylene bottles and maintained at 4 °C until experimentation. The effluent sampling was performed in triplicate and the results are shown in Table 1.

The biodegradability test was carried out to determine the BOD of oily wastewater and saponin solution by using the OxiTop method. Aerobic biodegradability was investigated by measuring the amount of oxygen used by bacteria to decompose organic compounds. The samples were seeded by means of introducing a micro-organism population. As mentioned in the section Aerobic growth, the bacteria employed in the current research were extracted from the Kuwaiti oil contaminated soil such as *Bacillus mycoides* and *Bacillus subtilis*, except *Rhodococcus* which was brought from KISR, which have been widely acknowledged as effective cultures in degrading petroleum organic material.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Samples range</th>
<th>Limiting range</th>
<th>Method used</th>
</tr>
</thead>
<tbody>
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<td>—</td>
<td>8.27</td>
<td>6.5–8.5</td>
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</tr>
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<td>EC</td>
<td>μS/cm</td>
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<td>SO₄</td>
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<tr>
<td>NO₃</td>
<td>mg/l</td>
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<td>50</td>
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<td>Na</td>
<td>mg/l</td>
<td>2,155</td>
<td>200</td>
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<tr>
<td>K</td>
<td>mg/l</td>
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<td>12</td>
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<tr>
<td>TOC</td>
<td>mg/l</td>
<td>37,898</td>
<td>No abnormal change</td>
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<td>TDS</td>
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<td>250–850</td>
<td>USEPA 2540, evaporation at 180 °C</td>
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<td>TSS</td>
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<td>558</td>
<td>0.3 mg/l</td>
<td>USEPA 418.1</td>
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</table>
compounds (Yudono et al. 2009; Ali et al. 2011). Other inoculums were obtained from a wastewater treatment plant PSS.

Generally, the bacteria in an aerobic process use oxygen to degrade organic compounds, where the amounts of oxygen used and amount of metabolized organic compounds are in direct proportion. Initially, 28 days was selected as the incubation time since that is the longest travel time of oily wastewater, and all tested samples were examined by using OxiTop, as explained in the section Measuring BOD by wastewater, and all tested samples were examined by using OxiTop. Details of these samples will be given in the Discussion section. The BOD28, initial COD value (CODi) and BOD28/COD ratio values are listed in Table 2.

As illustrated in Table 2, the mean of BOD28 values for the saponin solution using distilled water D and mixture of Rhododcoccus, Bacillus mycoidesis and Bacillus subtilis RM were 71 mg/l to 1,653 mg/l respectively. While the mean of BOD28 values for the oily wastewater using D and RM were 56 mg/l to 1,792 mg/l respectively. The mean of CODi values for the saponin solution using D and RM were 1,968 mg/l and 4,850 mg/l, respectively. While the mean values of CODi for oily wastewater using D and RM were 2,955 mg/l and 3,988 mg/l respectively. Moreover, the higher values of BOD28 were recorded when a mixture of bacteria seed was added together with the saponin solution or oily wastewaters. The BOD28 values for oily wastewater and saponin solution proposed that the bacteria may not be able to biodegrade the oily wastewater. Different BOD values were obtained from oily wastewater and saponin solution due to the concentration of organic compounds in the tested samples.

These samples contain toxic materials that may decrease BOD values, therefore these samples were diluted to a ratio of 1:10 to reduce the toxic substances or minimize their effects. However, the outcome of this work recognized that the UBOD and k are very similar for different samples regardless of reactivity status (see Table 2). The maximum of removal rate k is 0.35 day for saponin solution, which is almost similar to the value of 0.36 day for oily wastewater. The outcome shows that BOD removal rate k is in agreement with those found by Orhon et al. (2000) for carbonaceous deoxygenation in most industrial waste waters, with mean k 0.32–0.45 day at 20 °C. Also, maximum values of ultimate BOD for saponin solution and oily wastewater by using RM seed were recorded in the range of 2,864 to 3,136 mg/l and in the range of 3,351 to 3,867 mg/l respectively. In Table 2, the minimum UBOD for saponin solution and oily wastewater were obtained by not using microbial seed. Referring to Figure 1, there is a slight difference for the BOD samples tested with varying seeds. Generally, the rate of the oxygen consumption decreases as BOD decreases. This study suggested that the hydrocarbon compounds reduced the activity of microbes.

The biological oxidation of organic compounds was nearly completed after 20 days with oily wastewater or saponin solution, the microbial seeds need that long to oxidize the carbon compounds, which may be due to the high toxicity in the tested samples. As shown in curve A, Figure 1, the BOD values started from 220 mg/l and ended up at the final value of 850–950 mg/l for saponin solution with Rhododcoccus (R) and oily wastewater with R, respectively. Comparing curves B and A, in the same Figure 1, curve B is actually curve A, since all the samples used contained equal amounts of organic material. It seems that the sample of saponin solution and sample of oily wastewater contained

<table>
<thead>
<tr>
<th>Type of solution</th>
<th>Rhododcoccus (R) ml</th>
<th>Bacillus mycoidesis &amp; Bacillus subtilis (M) ml</th>
<th>Distilled water (D) ml</th>
<th>CODi mg/l</th>
<th>BOD28 mg/l</th>
<th>BOD28/CODi</th>
<th>Ultimate BOD mg/l</th>
<th>k</th>
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<td>1.25</td>
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equal amount of organic material (BOD), as the rates can differ quite significantly. Although, curve B in Figure 1 shows that the biodegradation process was slightly improved by the mixture of Bacillus mycoides and Bacillus subtilis M. Curve A and curve B in Figure 1 identified that the BOD values of the oily wastewater or saponin solution increased over time. The concentrations of degradable organics and removal rate $k$ enhanced when a mixture of R and M was added together with the saponin solution or oily wastewaters, as shown in curve C, Figure 1. It has been seen in Figure 1, that the BOD values of the saponin solution or oily wastewaters were slightly increased after 18 days. Refer to curve D in Figure 1, the initial decline of microbial growth curves occurred after 20 days of the incubation period. During this phase the growth rate begins to slow, therefore this phase was identified as the death phase. The reduction value of the BOD curves may be attributed to the following reasons: (a) sample did not incubate with sufficient bacteria; (b) bottle leaks from caps; (c) highly toxic sample; (d) amendment substance was not used in this test. Actually, not all of the samples have the same BOD values. This relates back to the biodegradation activity of the microbial population and the concentration levels of hydrocarbons present in the sample. Again, different microbial seeds were used for the BOD test, the samples of oily wastewater and saponin solution were seeded with PSS, LPF and mixture of PSS and LPF. The $\text{BOD}_{28}$, $\text{CODi}$, ratio of $\text{BOD}_{28}/\text{CODi}$, ultimate BOD and reaction removal rate $k$ values are listed in Table 3.

Table 3 shows that the mean values of $\text{BOD}_{28}$ for the saponin solution using D and PSS were 339 mg/l and 438 mg/l, respectively. While the mean values of $\text{BOD}_{28}$ for the oily wastewater using D and mixture of LPF and PSS were 254 and 480 mg/l by, respectively. Further, $\text{CODi}$ of the saponin solution using D and mixture of LPF and PSS were 1,663 mg/l and 2,625 mg/l by, respectively. Furthermore, the COD of oily wastewater using D and mixture of LPF and PSS were 3,125 and 3,478 mg/l, respectively. Additionally, higher values of $\text{BOD}_{28}$ were registered when adding the mixture of LPF and PSS together with the saponin solution or oily wastewaters. The $\text{BOD}_{28}$ values for oily wastewater and saponin solution indicated that PSS and LPF were also not contributing to the biodegradation of the oil residue in oily wastewater. The maximum removal rate is 0.27 day for saponin solution, which is higher than the maximum value of 0.19 day for oily wastewater. The outcome proposed that the easily degradable organic compounds can be more completely removed than the less degradable organics during wastewater treatment. Therefore, in the oily wastewater samples, relative proportions of the less
Biodegradable organic compounds were higher, giving lower BOD rate constant than the saponin solution. Also, maximum values of ultimate BOD for saponin solution and oily wastewater were 733 and 728 mg/l, respectively. Moreover, the minimum UBOD were obtained with no microbial seed. This study demonstrated that the level of toxicity in the oily wastewater was high enough to destroy microbial activity. Based on curves A, B and C in Figure 2, the rates of BOD28 test through PSS or LPF reactions were slightly similar. Referring to curve D, as expected there is difference for the BOD samples tested with varying seed.

Based on Curves A and B in Figure 2, the rates of BOD28 test through PSS or LPF reactions were slightly similar. The maximum generated values of BOD28 for oily wastewater with LPF and saponin solution with LPF were 480 and 452 mg/l, respectively. Also, the maximum generated values of BOD28 for oily wastewater with PSS and saponin solution with PSS were 254 and 339 mg/l, respectively. However, the BOD values were increased when adding the mixture of PSS and LPF together with the saponin solution or oily wastewaters, as shown in curve (C) Figure 2. The result of this study suggested that the BOD28 values of the saponin solution or oily wastewaters were slightly increased after 20 days. This point indicated that the degradability process only occurred on the saponin, which is well known as a biodegradable surfactant. The microbial seed may not have been able to consume enough oxygen to degrade the oily wastewater, since the concentration of toxicity in the oily wastewater was high, and could be capable of destroying the microorganisms. Bioassay for oily wastewater without seeding exhibited the highest reduction in terms of BOD. During the period of incubation, time lags in the BOD degradation curves for oily wastewater were noticed as presented in curve D Figure 2, while the maximum values of the BOD curves for oily wastewater and saponin solution without seeding were 254 and 339 mg/l, respectively. The present results suggest that the BOD test may need an appropriate bacteria during the incubation period. Therefore, bacteria are considered as vital to enhance the biodegradation rate. There seems to be a significant reduction in k that affects the overall BOD magnitude, as shown in Table 3. The mean removal rate k for oily wastewater without seeding and for oily wastewater with a mixture of PSS and LPF were 0.15 and 0.16, respectively. The reduction rate of k differs from one sample to the other, resulted from slow biodegradation rate of the organic compounds or due to the transfer of oxygen at the air-water interface (Zainudin et al. 2013). The experiment suggested that the bacteria were unable to (or could barely) adapt to the environmental conditions of the oily wastewater. The result of BOD28 seems to be resulted from biodegrading of saponin. Based on Tables 2 and 3, the ratios of BOD to CODi for saponin solution and oily wastewater by (PSS) and (LPF) were between 0.070 to 0.300, while the ratios of BOD28 to CODi for saponin solution and oily wastewater by Rhodococcus, Bacillus mycoides and Bacillus subtilis were between 0.001 to 0.300, which indicate that the biodegradation process is extremely slow (Gilbert 1987). As suggested by Gilbert (1987), diluents with a ratio of 0.4 could be regarded as non-biodegradable compounds. The dissolved organic compounds can be completely oxidized when

<table>
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<tr>
<th>Type of solution</th>
<th>LPF ml</th>
<th>PSS ml</th>
<th>Distilled water (D) ml</th>
<th>CODi mg/l</th>
<th>BOD28 mg/l</th>
<th>BOD28/CODi</th>
<th>Ultimate BOD mg/l</th>
<th>k</th>
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<td>254</td>
<td>0.080</td>
<td>279</td>
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# Missing sample.
compared to less particulate organic compounds. Wang et al. (2016) reported that the presence of particulate matter such as oil residue may cause depletion of dissolved oxygen, which has an adverse effect on the biodegradation rate. In this work, the total COD measurement is presumably the sum of the COD of dissolved and particulate organic and inorganic compounds in the system. Therefore, BOD to COD ratios were very low. Furthermore, the concentration of toxicity in the sample was significantly high, which could slow the biodegradation rate. It may prove beneficial to undertake longer BOD experiments to monitor the long term biodegradability. The outcome of the experiment demonstrated that the long term biodegradability was maintained, however with slower rates and with a slightly enhancement as was observed in the ratio of $\text{BOD}_{28}/\text{COD}_{i}$.

**DISCUSSION**

There is a real need for a good oily wastewater treatment to address the present issues in the subject of environmental engineering. Some of the oily wastewater treatments offered presently consist of vacuum evaporation, flocculation, adsorbents using magnetic core-shell adsorbent such as Halloysite nanotubes (HNTs), titanium dioxide, centrifugal devices, ultrafiltration and deep bed filtration, flotation, membrane separation technology, combined technologies, advanced oxidation processes, etc (Tatarchuk et al. 2020). There are advantages and disadvantages in each treatment, all subject to the difficulty of operations, capital and operational expenditures. No technology method which is satisfactory in dealing with all conditions or contaminations is presently available. The applicability of these treatments has a great potential to remove toxic contamination and heavy metals from wastewater (Anastopoulos et al. 2020). Oily wastewater samples often contain toxic materials and need special conditions when running BOD tests. Therefore, the sample was required to be diluted to minimize the toxic substance and their impacts. The main factor influencing the rate of hydrocarbon biodegradation in solid or liquid phase is the presence of microorganisms (Azimi et al. 2019). If microbial populations are present, then optimal growth rates and biodegradation rate of petroleum hydrocarbon can be controlled by providing the optimum conditions such as enough nutrients and oxygen, temperature 15–20 °C in marine environments and a pH value of 6–9 (Ma et al. 2020). The main purpose of the samples’ seeding is to make certain that the existence of microorganisms is sufficient. A number of samples contain

![Figure 2](http://iwaponline.com/wst/article-pdf/81/12/2650/732249/wst081122650.pdf)
components that are usually not degradable by the microorganisms present in wastewater. In order to achieve the required microbiology for oil degradation, the wastewater samples were seeded with the bacteria, which were isolated already from Kuwaiti oil contaminated sand, such as *Bacillus mycoides* and *Bacillus subtilis*. The biodegradation of oily wastewater and saponin solution were recorded indirectly by measuring the oxygen consumption rate of the microbial seed. The concentration of saponin for oily wastewater and saponin solution was 0.5 wt %. The mean values of BOD$_{28}$ for the saponin solution and oily wastewater using RM were 1,653 and 1,792 mg/l, respectively.

In addition, the maximum generated values of BOD$_{28}$ for saponin solution and oily wastewater using LPF were 452 and 480 mg/l, respectively. Generally, it has not been successful to view any major differences in the rates of oil degradation among the microorganisms. The outcome shows that the ultimate BOD values were slightly increased after 28 days for most of the seeded samples. These results suggest that the variance in the rates of biodegradation between various microorganisms could not be much higher if the entire experiment duration is longer than 28 days. According to Nasirpour et al. (2015), adaptation of microbial communities is considered vital to increase the effectiveness of the hydrocarbon degradation rates, particularly when utilizing hydrocarbon-degrading bacteria. As terrestrial petroleum hydrocarbon-degrading bacteria typically use various substrates, the majority are extremely specialized, utilizing hydrocarbons as the only source of carbon (Yakimov et al. 2007). Crude oil comprises a significant range of petroleum hydrocarbons with various chemical properties. One of the typical characteristics of bacteria are their ability to metabolize only certain hydrocarbons such as VOC, SVOC and LMW. According to Al-Saleh et al. (2009), a host of different bacteria are required to degrade a wide range of hydrocarbon compounds. In addition, for the degrading communities of petroleum hydrocarbon, it has been found that surfactants make the oil more accessible to the degraders (Head et al. 2006). For a number of samples, the BOD of oily wastewater samples was found to less than the BOD of saponin solution with seed. This is probably due to the presence of saponin, which is well-known to increase BOD as it is readily biodegradable. It was noted that the highest ratio of BOD$_{28}$/CODi for oily wastewater samples was 0.56, while the highest ratio of BOD$_{28}$/CODi for saponin solution was 0.55. This provides an indication that the constituents in the wastewater samples were non-biodegradable, which could affect the microbial degradation rate. Further, these recorded levels of BOD$_{28}$ and CODi signal potential pollution problems and may lead to harmful bodies. Based on the Introduction, salt concentration above 3.0% reduced the performance of aerobic activated sludge in wastewater treatment plants; however, the present work found that the population of microbial seed can tolerate salinity levels 45,000 mg/l (4.5%), which is the concentration of salinity for artificial Kuwait seawater. This study matched results found by Salvadó et al. (2001), where it can be noted that even though NaCl concentration of 0.5–5% would reduce the performance of the biological process in wastewater treatment plants, the microbes could tolerate the salinity. It appears that the microbial cells used in the experiments were capable of keeping an osmotic balance with their micro environment, thus preventing high salt concentrations from impeding their important metabolic processes. In view of the lessening of microbial activity during the oily wastewater treatment, it has been found that the high salt concentration (NaCl) is the main reason for the decrease of the COD removal rate, affecting the biodegradation rate of organic compounds such as VOC, SVOC and LMW (Pendashteh et al. 2012). It can be suggested that the low rate of biodegradation of the hydrocarbons in the oily wastewater could be due to the high concentration of petroleum hydrocarbon compounds and high percentage of salt. Given the distinctive current scenarios in the oily wastewater and the dissimilar bacteria in charge of petroleum hydrocarbon degradation, different approaches and techniques ought to be employed for the development of biological oil spill response in oily wastewater. However, because of the highly impermeable outer membrane of Gram-negative bacteria, it is generally accepted that this type of bacteria is more tolerant to hydrocarbons than Gram-positive bacteria (Aono & Kobayashi 1997; Trombetta et al. 2005; Badawy et al. 2019). The microorganism used for seeding played a significant role in reduction of the reaction rate $k$ of the oily wastewater samples and the ultimate BOD. This case study has clearly indicated that aerobic bio-treatment alone is not a suitable method for treating the oily wastewaters. Based on literature search, it is also noted that studies on rate constant of BOD consumption are very limited and hence this experiment adds novelty to this research.

**CONCLUSIONS**

This research concludes the existence of bacteria in the oily wastewater were not capable of degrading petroleum hydrocarbon. The results of this research can be valuable in the estimation of the bio-degradation capacity of the oily
wastewater. Given the distinctive current scenarios in the oily wastewater and the dissimilar bacteria in charge of petroleum hydrocarbon degradation, different approaches and techniques ought to be employed for the development of biological oil spill response in oily wastewater. It was noted that the BOD/COD ratios for all samples were 0.4 or lower. This provides an indication that the constituents in the sample effluents were somewhat non-biodegradable. This will probably cause high toxicity in the samples that could affect aquatic life. The microorganism used for seeding plays a significant role in the reaction rates \( k \) of the oily wastewater samples and the ultimate BOD (UBOD). This case study has clearly indicated that aerobic bio-treatment alone is not a suitable method for treating the oily wastewaters.

ACKNOWLEDGEMENT

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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