Kinetics of petroleum oil biodegradation by a consortium of three protozoan isolates (Aspidisca sp., Trachelophyllum sp. and Peranema sp.)

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ABSTRACT

Petroleum oil is a complex mixture of substances, the majority of which are hydrocarbons: the latter represent an extremely important and heterogeneous group of compounds that find their way into water resources by anthropogenic or natural ways. The majority of toxic hydrocarbon components of petroleum are biodegradable, where bioremediation using microbial species has become an integral process for the restoration of oil-polluted areas. In this study, three bioremediation processes, namely natural attenuation, nutrient supplementation by adding glucose and biostimulation by adding Tween® 80, were carried out in various petroleum hydrocarbon concentrations in polluted water media using a consortium of three protozoan isolates (Aspidisca sp., Trachelophyllum sp. and Peranema sp.). A first-order kinetics model was fitted to the biodegradation data to evaluate the biodegradation rate and to determine the corresponding half-life time. First-order kinetics satisfactorily described the biodegradation of the petroleum-based contaminants under abiotic conditions. The results showed an increase in the percentage removal of petroleum oil at the lower petroleum concentrations and a gradual percentage decrease in removing petroleum oil residues occurred when there was an increase in the initial concentrations of the petroleum oil: 39%, 27%, 22%, 12%, 10% for various petroleum oil concentrations of 50, 100, 150, 200, 250 mg/L, respectively. A similar trend was also observed in the glucose-supplemented culture media where the reduction was 45% and 78% for petroleum concentrations of 250 mg/L and 50 mg/L, respectively. Biodegradation of between 33 and 90% was achieved at a Tween® 80 concentration of between 50 mg/L and 250 mg/L. The degradation rate constants for the natural attenuation process ranged between 0 to ≤0.50, ≥0 to ≤0.35, ≥0 to ≤0.25, ≥0 to ≤0.14 and ≥0 to ≤0.11 for petroleum oil concentrations varying from 50, 100, 150, 200 and 250 mg/L, respectively, during the study after 30 days. In the presence of glucose as a nutrient supplement, the degradation rate constants increased from 0 day−1 to 0.8 day−1 when exposed to the lowest oil concentration of 50 mg/L, while the lowest rate constants (from 0 day−1 to 0.25 day−1) were observed at the highest petroleum oil concentration of 250 mg/L for the same period. Overall enhancement of the degradation rates was achieved when adding the Tween® 80 surfactant compared to the first two biodegradation processes. The half-life of 2017 days was achieved over 217 days during the natural attenuation process for samples with an oil concentration of 250 mg/L and this was reduced to 85 days using the glucose-supplemented process. There was a further decrease to 45 days when Tween® 80 surfactant was added during the biostimulation process. The highest efficiency of ≥20% of Tween® 80 was observed between 6 and 18 days and thereafter it decreased slightly to ≤20%.

1. Introduction

The release of hydrocarbons into the environment whether accidentally during production, transportation, tanker accidents and war-related incidents or due to other anthropogenic activities has been reported as the main cause of water and soil pollution [1,2], which has major detrimental effects on both flora and fauna of the affected ecosystems [3]. Several treatment options such as incineration, solvent extraction, pump and by burial in secure landfills used for the remediation of contaminated aquatic and terrestrial ecosystems can be exorbitantly expensive when large amounts of contaminants are involved [4,2]. Mechanical and chemical methods generally used to clean up hydrocarbons from contaminated sites have been proved to be ineffective in addition to the high cost. Physical methods for example have an oil recovery of less than 20%. However, chemical methods (such as dispersants, demulsifiers, biosurfactants and cleaners) that...
have been applied in the remediation processes for over three decades and have also been reported as being inadequate in removing all oil after a spill [5,2] and have potentially harmful environmental impacts. Biological techniques such as bioremediation offer reduced environmental risks as it is a natural process that relies on microorganisms to degrade the contaminants in water sources; the success of bioremediation technology for cleaning up large-scale oil spills has been shown. Many indigenous microorganisms in water and soil are capable of degrading hydrocarbon contaminants [6]. Bioremediation is currently a very promising technology for the treatment of sites contaminated with petroleum hydrocarbons. This method is typically more cost-effective compared to other physicochemical options. Bioremediation relies on biodegradation, which generally refers to the complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds and cell protein, or transformation of complex organic contaminants to other simpler organic compounds by microorganisms (Das and Chandran, 2011). It has been reported that several hydrocarbon constituents of petroleum can be removed completely or mineralized further to carbon dioxide and water through microbial activity [7]. According to Ayotamuno et al. [8]; Hazen et al. [9], a large influx of petroleum hydrocarbons results in an environment where biodegradation of the carbon compounds is limited by nutrient availability. Hence, biostimulation through nutrient supplementation and dispersants may be necessary.

Various hydrocarbon compounds that exhibit high toxicity are polycyclic aromatic hydrocarbons (PAHs) which include benzene, toluene, ethylbenzene, and xylene (BTEX components). The assessment of their contents is based on the aggregated constituents and specific pollutants. According to Watkinson [10], biodegradation of petroleum hydrocarbon components usually yields the formation of polar metabolites that can still have a high proportion of hydrocarbon structures and their determination can provide better insight into the degree of degradation. Due to many adverse factors and the complexity of the process it is not possible to predict the duration of bioremediation process. Numerous studies on the biodegradation process have been focused on various factors that directly or indirectly influence the amount of hydrocarbon compounds transformed over time and provide the rate of degradation as well as curves that describe the transformation (referred to as kinetics) of any given compounds by the various bacterial microbes but, studies using protozoan isolates are not well documented [5]. Dispersant application to accelerate crude oil bioavailability has been extensively studied and numerous findings are currently available [11,12]. Dispersants contain surfactants which are agents with molecules composed of groups of opposing polarity and solubility. They usually contain an oil-soluble hydrocarbon chain and a water-soluble group. Surfactant mixtures often include other chemical agents, such as solvents, which enhance the dispersing capability of the surfactant [13]. Enhanced solubility facilitates the increased degradation of compounds. Tween® 80 (Polysorbate 80) is a hydrophilic non-ionic surfactant, which is one of the most commonly used surfactants and it has been shown to enhance the solubility of various hydrocarbon compounds [14].

Strategies that are employed in studying the kinetics of a process have been based on the theory that the reaction rate constant in the rate equation quantifies the rate of the reaction; the reaction rate constant (k) is an average of values calculated at various experimental kinetic data points, e.g. reactant concentrations at various reaction times. A smaller rate constant indicates a slower reaction, while a larger rate indicates a faster reaction. As the biodegradation of petroleum hydrocarbons is influenced by a complex array of different factors, it is important to note that a simple kinetic model cannot realistically provide precise and accurate descriptions of various concentrations at different time lines in different areas by a single oil spill [5]. These authors further stated that most of the short-term bioremediation studies are usually presented with an implicit assumption of zero-order kinetics. Short-term biodegradation experiments may not be adequate in appropriately articulating the biodegradation kinetics of a chemical contaminant by various microorganisms used [15]. However, first-order kinetics (exponential decay) often describes biodegradation when the initial substrate concentration is low. Based on the nature of the substrate and experimental conditions, different values of rate constants have been obtained in various studies on hydrocarbon biodegradation. For example, Holder et al. [16] reported that normal alkanes were obtained at a rate constant of between 0.14 and 0.61 day⁻¹, while Seabara et al. [17] indicated that the rate constant for crude oil was between 0.0051 and 0.0074 day⁻¹, and finally, the rate constants for PAHs were found to range between 0.01 and 0.14 day⁻¹ [18,16,19]. Hinga [18] further reported that the selection of the appropriate kinetics and rate constants is essential for accurate predictions of various hydrocarbon concentrations with time in the affected water source after each spill. Therefore, the main aim of this study was to evaluate, model and analyze the kinetics of biodegradation of petroleum oil by selected protozoan species through processes such as natural attenuation, nutrient supplementation by adding glucose and biostimulation by adding Tween® 80 and to evaluate the effectiveness of the dispersant (Tween® 80) for a period of 30 days.

2. Materials and Methods

2.1. Sampling and preparation of protozoan species

The study was carried out between September and December 2015 and the petroleum-contaminated water samples were collected from petroleum-polluted water aquifers in Gezina, a suburb of Pretoria, South Africa. The 2.5 L samples with a known oil concentration of 250 mg/L were subjected to preparation as described by Kachieng’a and Momba [20] [6]. The study was performed using a consortium of three protozoan species, namely Aspidisa sp., Trachelophyllum sp. and Peranema sp., which were obtained from the laboratory stock cultures of Tshwane University of Technology Water Research Group. The laboratory stock cultures of protozoan organisms were earlier isolated from wastewater-mixed liquors collected from the aeration tanks of the Daspoo Poytreatment tank was fitted with a thermometer and a constant temperature of 30°C
was maintained using a heater with assistance of the thermo-couple [22]. An approximate protozoan consortium biomass of 1.0 × 10^4 cells/mL was added to each bioreactor tank. In addition, an experimental control in the aquarium tank was done with petroleum oil sample media without microorganisms. The electric stirrer was fitted to the bioreactor to assist in creating a homogenous solution of water-oil without forming a two or more distinct layers during bioremediation studies. The tank reactors were shaken continuously at a speed of 100 r/min for a period of 30 days. Samples were taken after every 6 days for analysis throughout the study period.

2.3. Determination of biodegraded petroleum concentration in contaminated water

The determination of the petroleum concentration was done using the partition-gravimetric technique which involves the extraction of dissolved and emulsified oil from petroleum-contaminated wastewater using an extraction solvent such as n-hexane and methyl-tert-butyl ether (MTBE). The experimental procedure was performed according to that given in the previous study by [6]. The calculation of the concentration of recovered petroleum content was done according to the procedure described by Baig et al. [23]. The percentage of oil biodegraded \( D \) was calculated by Eq. (1):

\[
D = \frac{C_0 - C_r}{C_0} \times 100
\]

where \( C_0 \) is the initial oil concentration and \( C_r \) is the residual oil concentration.

The biodegradation of petroleum oil is described by first-order kinetics [5,24] and the values were calculated using Eq. (2):

\[
C = C_0 e^{-kt}
\]

where \( C \) is the concentration of hydrocarbon (g/kg) at a certain time, \( C_0 \) is the initial concentration of hydrocarbon (g/kg), \( k \) is the rate constant of the change in the oil content (day^{-1}) and \( t \) is the study period (day).

The bioavailability or effectiveness of Tween® 80 (BE) in the study was analyzed by Equation 3 [12]:

\[
BE = \frac{RDHC0 - RHC0}{RDHC0} \times 100
\]

where RDHC0 is the percentage removal of oil in the presence of surfactant Tween® 80, RHC0 is the percentage removal of oil in the absence of dispersant.

Biodegradation half-life times were calculated using Eq. (4):

\[
t_{1/2} = \frac{\ln(2)}{k}
\]

where \( k \) is the rate constant which is calculated according to Eq. (2).

2.4. Statistical analysis

The data were statistically analyzed using the Stata computer software. A two-way analysis of variance (ANOVA) was used to compare the biodegradation activities and their respective reaction kinetics (assuming first-order kinetics) of the consortium of protozoan isolates together with the efficiency of the surfactant. The interpretation was performed at \( \alpha = 0.05 \) (two-sided).

3. Results

3.1. Biodegradation strategies

Figs. 2–4 illustrate the average biodegradation values obtained by a consortium of protozoan isolates of different concentrations of petroleum oil in oil-polluted water samples when using the following biodegradation processes: natural attenuation, the use of nutrient supplementation with glucose, and biostimulation with Tween® 80 surfactant. Fig. 2 clearly demonstrates the natural attenuation process whereby a gradual percentage decrease in petroleum oil residues occurred when there was an increase in the initial concentrations of the oil: 39%, 27%, 22%, 12%, 10% for various petroleum oil concentrations of 50, 100, 150, 200, 250 mg/L, respectively. A similar trend was also observed in the process where glucose was added as a nutrient and where there was a decrease of 45% and 78% for petroleum oil concentrations of 250 mg/L and 50 mg/L, respectively (Fig. 3). The addition of Tween® 80 surfactant for biostimulation yielded a further reduction of 33 and 90% for petroleum oil concentrations of between 250 mg/L and 50 mg/L, respectively (Fig. 4). The highest removal efficiency was noted in the samples contaminated with lower concentrations of petroleum oil while the lowest removal efficiency was recorded in samples containing higher petroleum oil concentrations (90% and 45% biodegradation for lowest and highest petroleum oil concentrations, respectively) (Figs. 3 and 4).

3.2. Protozoan biodegradation of petroleum oil and its kinetics

Figs. 5–7 depict the results obtained during the studies on the kinetics of various petroleum oil biodegradation processes by a consortium of three protozoan isolates. In general, the biodegradation...
rates of the protozoan consortium for the various biodegradation processes employed were found to be higher when the consortium was exposed to lower petroleum oil concentrations in the substrate and lower when exposed to higher petroleum oil concentrations.

The rate constants ($k$) of the isolates during the natural attenuation biodegradation process ranged from $≥0$ to $≤0.50$, $≥0$ to $≤0.35$, $≥0$ to $≤0.25$, $≥0$ to $≤0.14$ and $≥0$ to $≤0.11$ day$^{-1}$ for petroleum oil concentrations of 50, 100, 150, 200 and 300 mg/L used during the study after 30 days, respectively (Fig. 5). In the presence the glucose as an added substrate, the rate constants of the protozoan consortium increased from $0$ day$^{-1}$ to $0.80$ day$^{-1}$ when exposed to the lowest petroleum oil concentration of 50 mg/L, while the highest concentration of 250 mg/L promoted the lowest rate constants ($k = 0$ day$^{-1}$ to $k = 0.25$ day$^{-1}$) at the end of the study period of 30 days (Fig. 6). A similar trend was also noted during biostimulation with Tween 80 although there was an overall enhancement of biodegradation rates when adding the surfactant compared to the first two biodegradation processes. In this case the consortium of the protozoan isolates exposed to the lowest petroleum oil concentration of 50 mg/L exhibited the highest rate constants which ranged between $0$ day$^{-1}$ and $1.11$ day$^{-1}$ after 30 days (Fig. 7).

3.3. Estimation of the biodegradation process half-life times by the protozoan isolates

The results of the half-life times and first-order degradation curves of the three biodegradation processes explored in this study are presented in Table 1. The rate constants derived in Eq. (2) and depicted in Figs. 5–7 can then be used to derive the half-life times ($t_{1/2}$) which are shown in Table 1. The half-life time ($t_{1/2}$) values were obtained from Eq. (4). The highest half-life was achieved after 44 and 217 days during the natural attenuation process for petroleum oil concentrations of 50 and 250 mg/L respectively, and this was reduced to between 34 and 85 days during the nutrient supplementation process using glucose. There was a further decrease in half-life times to 16 and 45 days during the biostimulation process when Tween 80 surfactant was added. Linear regression analysis was used to generate the correlation coefficient ($R^2$) values shown in Table 1 and obtained from the linear plots depicted in Figs. 5–7. The high correlation coefficient ($R^2$) values of between 0.85 and 1 indicated a good fit of the biodegradation data to the first-order kinetic model. The results of the study revealed that there was a statistically significant difference ($p < 0.05$) between the biodegradation processes used (natural attenuation, nutrient supplementation with added glucose, and biostimulation with added Tween 80 surfactant) and the degradation rates of the protozoan isolates.

3.4. The effectiveness of Tween 80 during biodegradation by isolates

The effectiveness of Tween 80 as a dispersant in the biodegradation of petroleum oil was tested and results are presented in Fig. 8. The effectiveness of the surfactant was obtained from results illustrated in Fig. 3 and revealed the best fit line of $y = −0.0595 x^2 + 1.7714x + 1.5714$ (Fig. 8). The highest efficiency of $≥20\%$ by Tween 80 was observed to be between 6 and 18 days, and thereafter it was reduced slightly to $≤20\%$ (Fig. 8).

4. Discussion

According to US-EPA [25], the natural biodegradation (attenuation) by the microorganisms usually acts to reduce the mass, mobility, volume and the pollutant concentration to a less toxic level in any given
Fig. 4. The efficiency of biostimulation (with addition of Tween® 80) as a biodegradation process for removing petroleum oil by a consortium of three protozoan isolates (Aspidisca sp., Trachelophyllum sp. and Peranema sp.).

Fig. 5. Degradation of different concentrations of petroleum oil through natural attenuation by the consortium of the protozoan isolates.

Fig. 6. Degradation of different concentrations of petroleum oil by the consortium of the protozoan isolates with nutrient (glucose) supplementation of the substrate.

Fig. 7. Degradation of different concentrations of petroleum oil by the consortium of the protozoan isolates with biostimulation of the substrate (addition of Tween® 80 as a dispersant).
environment. In Fig. 2, the average percentage of the oil residue removed by protozoan isolates was 39% at a concentration of 50 mg/L petroleum oil and the trend was downward as the concentration of oil increased. Similar observations were noted in the case of the nutrient supplementation process when adding glucose as a substrate (Fig. 3).

With regard to biostimulation with the addition of Tween® 80 dispersant during the petroleum oil biodegradation (Fig. 4), the most notable hydrocarbon degradation was achieved within 24 days. A window period of approximately three weeks of bioremediation has been recommended to reduce the expenses of clean-up for this type of crude oil [5]. Good petroleum oil degradation was achieved at medium to low concentrations of hydrocarbons (200 mg/L and below) while higher concentrations inhibited the protozoan biodegradation activities. This might be due to their toxic effects and inhibitory aspects which are affected by the fact that the molecular composition of crude oil varies widely in affected areas [12]. According to Zahed et al. [5], the efficiency of crude oil bioremediation in an aquatic environment is directly affected by the concentration of the oil and the application of dispersant. There was a statistically significant difference (p < 0.05) between the results obtained during the natural attenuation of petroleum oil by the protozoan isolates and those obtained during the nutrient supplementation process using glucose and biostimulation process (addition of Tween® 80 surfactant) during the study.

The kinetics of biodegradation processes is solely dependent on the speed and development of the efficient removal of oil in any contaminated water bodies or normal environment. According to [26], for the evaluation of the biodegradation activity, the following factors are taken into account: type of oil, surfactant formulation, effect of surfactants on microbial attachments towards oil droplets, dilution effects of oil, differences in uptakes of the various hydrocarbon constituents, and dispersant efficiency in biodegradation processes. While all these factors are considered to be important, the present study showed an increase in biodegradation rates when Tween® 80 dispersant was supplemented and the highest rate constant of 1.11 day⁻¹ was observed, especially with the lowest petroleum oil concentration of 50 mg/L (Fig. 7).

A half-life of a biological biodegradation is the time taken for a substance to lose half of its amount and therefore, biodegradation half-lives are vital tools for many applications such as chemical screening, environmental modelling fate and description of pollutants transformation as specified by a number of investigators [27–29]. The longest half-life was achieved after 217 days during the natural attenuation process for a petroleum concentration of 250 mg/L and this was reduced to 85 days during the nutrient (glucose) supplementation process. While there was a further decrease to 45 days when Tween® 80 surfactant was added to the substrate, therefore showing the positive effects

| Biodegradation process | Concentration of crude oil (mg/L) | First-order linear regression analysis | R² (coefficient of determination) | Half-life (t₁/₂) in days |
|------------------------|----------------------------------|--------------------------------------|-----------------------------------|-------------------------|
| Natural attenuation by protozoan isolates | 50 | $y = 0.0157x$ | R² = 0.985 | 44 |
| | 100 | $y = 0.01x$ | R² = 0.9798 | 69 |
| | 150 | $y = 0.0075x$ | R² = 0.9596 | 92 |
| | 200 | $y = 0.004x$ | R² = 0.9662 | 173 |
| | 250 | $y = 0.0032x$ | R² = 0.9636 | 217 |
| | 50 | $y = 0.0269x$ | R² = 0.9875 | 26 |
| Nutrient supplementation with glucose | 100 | $y = 0.0203x$ | R² = 0.9819 | 34 |
| | 150 | $y = 0.0168x$ | R² = 0.9635 | 41 |
| | 200 | $y = 0.0128x$ | R² = 0.9523 | 54 |
| | 250 | $y = 0.0082x$ | R² = 0.9659 | 85 |
| | 50 | $y = 0.0423x$ | R² = 0.8865 | 16 |
| Biostimulation by supplementing Tween® 80 dispersant | 100 | $y = 0.0317x$ | R² = 0.8227 | 23 |
| | 150 | $y = 0.0221x$ | R² = 0.8678 | 31 |
| | 200 | $y = 0.0179x$ | R² = 0.8724 | 39 |
| | 250 | $y = 0.0153x$ | R² = 0.9146 | 45 |

$Y = \ln(C_0/C)\), x = t$ (days).
of nutrient supplementation and surfactant addition on petroleum biodegradation by protozoa (Table 1). The addition of the Tween® 80 dispersant played a major role in the decrease of half-life for the biodegradation of high concentrations of oil ≥200 mg/L. A half-life of 40 days recorded during the biostimulation process has been highlighted to be the approximate number of days which is the criterion used to classify compounds as potentially persistent in various national and international schemes for identifying persistent bioaccumulative compounds [27]. These findings clearly showed that the application of Tween® 80 surfactant enhanced the biodegradation activities of the consortium of protozoan species to biodegrade petroleum oil through an increase in the bioavailability of petroleum hydrocarbons of oily wastewater. The highest efficiency of ≥20% by Tween® 80 was reported between the 6th and the 18th day, and thereafter decreased slightly to ≤20% (Fig. 8). Surfactants can act as emulsifying agents that produce micro-droplets of hydrocarbons, thus enhancing the biodegradation of dispersed oil by providing a digestible substrate that stimulates the growth of organisms and finally, makes hydrocarbons available for uptake and use as source of energy [30]. Bioavailability may be the critical limiting factor controlling biodegradation rates for many organic compounds with low water solubility. According to Prince et al. [30], the use of dispersants has been thought to stimulate the natural process of biodegradation, due to the formation of an oil-water interface and the dispersion of the oil, which will dramatically increase the surface area for microbial attack. The results of this study also confirmed the effectiveness of a dispersant in improving bioavailability of hydrocarbons and showed the biodegradation capability of a consortium of Aspidisca sp., Trachelophyllum sp. and Peranema sp.

5. Conclusion

Overall, the first two processes of bioremediation assessed during this study, which are natural attenuation and nutrient supplementation with glucose as a substrate, have the capability to reduce the level of petroleum pollutants in wastewater using a consortium of Aspidisca sp., Trachelophyllum sp. and Peranema. The most effective biodegradation of these pollutants by these protozoan species was found to occur during the biostimulation process with the addition of Tween® 80 dispersant, especially at low petroleum oil concentrations. According to the results of this study, biostimulation by a surfactant is highly recommended as this enhances the protozoan biodegradation rates and will thus reduce the time it takes to biodegrade the petroleum oil, especially for both pilot and large-scale applications in aquatic or environmental sites. This study on the kinetics of petroleum oil biodegradation by target protozoan species is a first of its kind; to date, most studies have focused on microbial (bacterial) biodegradation kinetics. This brings a novel approach to petroleum hydrocarbon biodegradation, especially in this century where there are numerous petroleum oil spills causing water and environmental pollution.

Conflict of interest

None.

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