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Biodegradation of Hydrocarbon Chains of Crude Oil By-products by Selected Protozoan Isolates in Polluted Wastewaters

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Abstract The demand of crude oil or any other oil by-products across the world and various activities are such as human and industrial activities has led to continuous pollution of various existing fresh water. The study was conducted using three protozoan isolates (Aspidisca sp., Trachelophyllum sp. and Peranema sp) where their growth and biodegradation abilities were screened using crude oil spill rich polluted wastewater in shaking flasks at pH 8 and incubated at 30 ℃ with a speed of 100 rpm for 5 days. Chemical Oxygen Demand, Dissolved Oxygen and growth of protozoan isolates were determined using standard methods. Crude oil concentrations in the samples were analysed using partition gravimetric method. The study revealed that high amount of COD released and over 80% DO were removed from wastewater. The protozoan biomass of 1.00×10^3 cells/mL was able to degrade crude oil (initial concentration:<50 mg/L) from wastewater at average rates ranging from 10.41% to 55.5%, from 11.0% to 64.5%, from 11.66% to 44.21% and from 21.2% to 61.1% for Aspidisca sp.,Trachelophyllum sp., Peranema sp., and a consortium of the three isolates, respectively.An increase in protozoan biomasses of 1.00×10^5 cells/mL and 1.00×10^6 cells/mL resulted in oil biodegradation rates ranging from 15.4% to 71.0%, 15.0% to 68.9%, 13.0% to 56.0% and 28.0% to 85.2%, respectively. However, an increase in oil concentrations in wastewater mixed liquor (>50 mg/L) decreased their biodegradation capacity to >60%. Statistically, the microbes were found to have a significant difference in their biodegradation ability in different various oily wastewater concentrations (p<0.001).
Keywords biodegradation, crude (petroleum) oil, protozoan biomass, wastewater
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Introduction

The global demand for oil as the only form of energy has increased tremendously where in 2008 alone 85.6 million barrels per day (OPEC 2009). The global activities such as transport sector and other use for both petroleum and its derivatives have contributed in both prevalence and quantity of pollution of existing fresh water around the world. Fresh water is one of the natural resources essential for human, animal and plant life. Semrany et al. (2012) most of the focus have been based on the availability and quantity of water for all. is the hydrocarbon contamination resulting from various activities such as oil refineries, accidental releases or spills and other natural phenomena which are beyond our control are the current environmental challenges facing scarce existing freshwater across the globe whether groundwater or surface-water sources, (Hamed et al. 2010, Jain and Bajpai 2012). World water sources are increasingly polluted with oily wastewater discharges to the extent that the effects cannot be easily reversed with far-reaching consequences for the ecosystem and for any aquatic living organisms.

The bioremediation technologies commonly used to enhance biodegradation processes of oil spills or leaks in water sources include mechanical processes, evaporation, dispersion and washing (Das and Chandran 2011). Bako et al. (2008) reported that most of these techniques are not effective enough removing ever increasing hydrocarbon contaminants from receiving water bodies and entire ecosystems in order to protect end-users.
In the beginning of 21st century bioremediation technology has been strongly advocated technique for treating crude oil (petroleum) contaminated sites due to low cost, non-invasive

and environmentally friendly (Jain and Bajpai 2012). This technique depends on the complete biodegradation of organic (oily) contaminants into carbon dioxide, energy and water which are harmless the environment or any water resources. Jain and Bajpai (2012) further stipulated that biological organisms such as bacteria and other plants species have been extensively used in reducing these toxic organic pollutants from existing freshwater and environmentally polluted site to acceptable levels.

The most interesting phenomena regarding bioremediation studies conducted have extensively focused on bacteria-based remediation have been on the treatment of oil spills or domestic oily wastewater, however, there is no single been done on the capability of protozoan species in biodegradation of oily rich wastewater. Protozoa are currently known for their role in the removal of pathogens and as biological indicators of wastewater quality, as well as being indispensable in the reduction of chemical oxygen demand (COD) of wastewaters (Curds and Cockburn 1970). According to they are very useful biological indicators of the condition of an activated sludge (Madoni et al. 1996). Protozoa are also able to diversify and to evolve into many different species, subspecies or morphotypes which assist them in adjusting to different prey sizes and to develop mechanisms of prey recognition through specific cell surface epitopes (Dini and Nyberg 1999, Martel 2008, Wooton et al. 2007).

Currently, there is an extensive work done regarding the role of protozoan species, especially Aspidisca sp., Trachelophyllum sp. and Peranema sp. in the bioremediation/biodegradation of oil-polluted wastewater in our lab and the articles have been submitted for scientific publication. Their role again, are well-defined and has been reported in previous work done (Akpor et al. 2008) in the removal of phosphates and nitrates from polluted wastewater systems. The study of Kamika and Momba (2011) has also reported their role in vanadium and nickel removal from wastewater. Based on this knowledge these protozoan isolates were selected for this study on the biodegradation of crude oil by-products in domestic oil-polluted wastewater. Therefore, the aim of this study was first to determine the effect of increasing biomass of protozoan isolates (Aspidisca sp., Trachelophyllum sp. and Peranema sp.) as well as a consortium of these three isolates in biodegradation of oily-polluted wastewater.

Materials and methods

Preparation of protozoan species

The three protozoan species (Aspidisca sp., Trachelophyllum sp. and Peranema sp.) were obtained from Tshwane University of Technology (TUT) laboratory stock cultures. These organisms were previously isolated from wastewater mixed liquors collected from the aeration tanks of the Daspoort Wastewater Treatment Plant (Pretoria, South Africa). They have been shown to be proficient in removing nitrates and phosphates successfully, as reported in previous studies conducted by Akpor et al. (2008) and Akpor (2009), as well as removing nickel and vanadium as observed in a study done by Kamika and Momba (2011). During the study period, each type of protozoan species was transferred from the stock culture to the microtitre plates where they were visualised using an inverted microscope (Axiovert S100, Carl Zeiss (Pty) Limited, SA) at 100× or 400× magnifications. They were counted and directly picked with a handmade glass capillary which was used to aseptically transfer the isolates to sterile 250 mL Erlenmeyer flasks containing 100 mL fresh sterile proteose-peptone-glucose (PPG) medium (PPG). To inhibit bacterial contamination, the medium was supplemented with 50 μg/mL streptomycin (Merck, SA). Heat-killed Escherichia coli WG 4 were aseptically added as a source of nutrient (Akpor et al. 2008). To

allow growth at acceptable concentrations for the experimental bioremediation studies, the protozoan isolates were incubated at room temperature in the dark for 10 d.

Determination of the biomass/growth of the protozoan isolates

Protozoan growth was checked on a daily basis using the inverted microscope and their cell densities were determined according to the procedure described by Akpor et al. (2008), where they were determined by spectrophotometer (Spekol 1300, Analtikjena, Germany) at 600 nm. The approximate growth of each isolate was calculated with reference to the standard curve derived from previously prepared various concentrations of the isolate cultured in protease peptone gelatine and the following standard equation:

$$Y = mx + C \quad (1)$$

where:
Y = the absorbance of the isolate concentration
X = the approximate number of isolate cells
m = the constant value of the gradient of the curve (0.0005)
C = the y-intercept of the curve (approximately - 0.0001)

Sample collection and preparation of the oil-polluted wastewater

The oil-contaminated wastewater samples used during the study period were obtained from 10 different British Petroleum (BP) petrol service stations (between June and April 2012) around Arcadia in Pretoria, South Africa. The method previously described by Baig et al (2003) was used to simulate these conditions. After filtration to eliminate any solid impurities, the oily ridden wastewater sample (2.5 L) was thoroughly mixed using an electric mixer to create turbulent flow conditions to mix and obtain a homogeneous oily wastewater sample; this sample formed the initial concentration. After vigorous shaking, the oily wastewater medium was divided into 10 portions of 100 mL, each of which was transferred to a 250 mL Erlenmeyer shake flask.

The profile of the filtered samples was determined in terms of COD, DO and pH. The COD concentration was determined using closed reflux methods as described in standard methods (APHA 2001). Other parameters, such as pH and DO were analysed using a pH probe (Model: PHC101, HACH) and DO probe (Model: LDO, HACH), respectively. The culture medium was autoclaved and cooled to room temperature before use. Sterile D-glucose monohydrate/anhydrous (2.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L) and KNO_3 solutions (0.18 g/L) (all from Merck, SA) were added to the culture medium to serve as a carbon source and nutrient supplement for the growth of organisms (Momba and Cloete 1996, Akpor et al. 2008). The sterility of this medium was checked by plating a 1 mL aliquot on the sterile bacteriological agar, and then incubating the plate at 37 ℃ for 24 h. The concentrations of the oils and fats in wastewater samples were determined before, after and during each experimental study, which was conducted in triplicate.

Bioremediation experiments with single organism and a consortium of three isolates

For each test protozoan isolate and each series of the experimental study, 10 flasks each containing 150 mL of initial petroleum oily wastewater samples were used to test the biodegradation efficiency of individual organisms in removing oils and fats. The flasks were inoculated with varying concentrations of protozoan isolates ($\pm 1.00 \times 10^3$ cells/mL, 1.00×10^5 cells/mL and 1.00×10^6 cells/mL). In addition, experimental control flasks were made up, i.e. positive control flasks [protozoan isolates were inoculated into sterile deionised saline water (8.5% NaCl)], and negative control flasks [no protozoan isolates were inoculated in oily rich wastewater media]. The experimental study was conducted at 30 ℃ and pH 8 in a shaking

incubator at a speed of 100 r/min. Only one aliquot from every incubated flask was used each day to determine the oil and fat content over a period of 5 d. The same experimental procedure was followed to test the biodegradation capability of the consortium of three isolates and the culture media were inoculated with the consortium protozoa.

Determination of crude oil content and COD and DO concentrations

Fat and oil concentrations in wastewater were determined using the partition-gravimetric method which involves extraction or emulsifying oils and fats in oily wastewater using an extraction solvent such as n-hexane and methyl-tert-butyl ether (MTBE). The experimental procedure was performed by taking approximately 100 mL of oily wastewater, followed by acidification with 5 mL of concentrated sulphuric acid and then transferring the mixture to a separatory funnel. The extraction solvent (30 mL) was then added to the wastewater and thoroughly mixed by shaking the sample for 2 min. The upper layer of the solvent containing the hydrophobic oily content was extracted and transferred to a clean sterile flask while the lower aqueous layer was further extracted; the extraction procedure was repeated with an extraction solvent until no further visible layers were noted. All the oil extracts were then pooled together in one conical flask and a drying agent (10 g anhydrous sodium sulphate) (Merck, SA) was added to absorb any remaining traces of water impurities. The dried extract (no water molecule is present) was thereafter placed in a tared distillation flask and distilled using distillate recovery apparatus immersed in a water bath at a temperature ranging between 60 ℃ and 70 ℃. The air was drawn through the flask for 1 min using a vacuum pump. The oily extracts were cooled in a desiccator for at least 30 min and weighed. The calculation of the concentration of recovered oils and fats was done according to Baig and co-workers (2003).

The concentrations of the COD and DO were measured as mentioned above and the following formula was used to determine the COD concentration of the samples:

$$\text{mg COD / L} = \frac{\text{mg in final volume} \times 1000}{\text{sample volume}} \quad (1)$$

Statistical analysis

The data were statistically analysed using the Stata computer software. ANOVA was used to compare the average growth means between cells and the biodegradation ability of each tested organism and the consortium of isolates. The interpretation was performed at $\alpha = 0.05$ (two-sided).

Results

The biomass increase of protozoan isolates at various concentrations of crude (petroleum) oil in wastewater

The average biomass increase of Aspidisca sp., Trachelophyllum sp., Peranema sp. and a consortium of the three isolates at different cell densities were ascertained at various concentrations of crude (petroleum) oil as indicated in fig. 1-3. For various cell densities of individual protozoan isolates, a similar growth profile was observed when isolates were exposed to various ranges (22 mg/L, 44 mg/L, 50 mg/L and 66 mg/L) of crude oil concentrations at 30 ℃ and pH8. Overall, results showed that regardless of the organism/consortium biomass and the crude oil concentrations, the maximum biomass increase was shown to had a marked by a significant increase ($p<0.001$) which occurred at 72 h; thereafter there was a continuous decrease for the later part of the study period.

The biomass increase of Aspidisca sp. was approximately 1.52×10^3 cells/mL and 1.40×10^3 cells/mL from (an initial of 1.00×10^3 cells/mL) when exposed to 22 mg/L and 44 mg/L of crude (petroleum) oil respectively. However, as the oil concentration was increased to ≥50 mg/L, the biomass response further decreased to less than 1.37×10^3 cells/mL during the study period (fig. 1). For Trachelophyllum sp. the average increase in biomass was 1.60×10^3 cells/mL when exposed to ≤50 mg/L of crude (petroleum) oil. However, as the concentration of oil in the media was increased to ≥50 mg/L, the biomass response further decreased to 1.30×10^3 cells/mL (fig. 1). The average biomass increase for Peranema sp. was 1.20×10^3 cells/mL from an initial biomass of 1.00×10^3 cells/mL after exposure to 22 mg/L and 44 mg/L of crude (petroleum) oil. A further exposure of cells to a crude oil concentration of ≥50 mg/L resulted in a decrease in biomass of less than 1.10×10^3 cells/mL during the study period (fig. 1). For the consortium of three isolates, the average increase in biomass was 1.90×10^3 cells/mL and 1.80×10^3 cells/mL from an initial cell density of 1.00×10^3 cells/mL when exposed to 22 mg/L and 44 mg/L of oil respectively. The continuous exposure of the consortium to a crude oil concentration of ≥50 mg/L triggered a further decrease in the biomass to less than 1.50×10^3 cells/mL (fig. 1).

With an initial biomass of 1.00×10^5 cells/mL, the average maximum increase in biomass of Aspidisca sp. was 1.37×10^5 cells/mL and 1.37×10^5 cells/mL when exposed to 22 mg/L and 44 mg/L of crude (petroleum) oil respectively. However, with an exposure to a crude (petroleum) oil concentration of ≥50 mg/L, the biomass further decreased less than 1.30×10^5 cells/mL (fig. 2). For Trachelophyllum sp. the average biomass increase was 1.50×10^5 cells/mL and 1.40×10^5 cells/mL when exposed to oil concentrations of 22 mg/L and 44 mg/L respectively and a biomass decrease occurred with a continuous exposure of ≥50 mg/L crude (petroleum) oil concentrations, which led to a continuous decrease to less than 1.31×10^5 cells/mL (fig. 2). Peranema sp. demonstrated an average biomass increase of approximately 1.28×10^5 cells/mL and 1.25×10^5 cells/mL from an initial cell density of 1.00×10^5 cells/mL when exposed to crude (petroleum) oil concentrations of 22 mg/L and 44 mg/L respectively. The exposure to crude (petroleum) oil concentrations of ≥50 mg/L triggered a decrease in biomass to less than 1.10×10^5 cells/mL (fig. 2). With an exposure to ≤50 mg/L of crude oil, the consortium of three isolates resulted in an average biomass of 1.75×10^5 cells/mL from an initial density of 1.00×10^5 cells/mL, while in the presence of crude (petroleum) oil concentration of ≥50 mg/L they further decreased to less than 1.50×10^5 cells/mL.

The exposure to crude (petroleum) oil concentrations of ≤60 mg/L, Aspidisca sp. demonstrated an average increase of biomass of 1.35×10^6 cells/mL from an initial cell density of 1.00×10^6 cells/m with an exception 66 mg/L where the average increase was 1.20×10^6 cells/mL. In the presence of a crude oil concentration of 22 mg/L and 44 mg/L, Trachelophyllum sp. demonstrated an average an increase of 1.53×10^6 cells/mL and 1.41×10^6 cells/mL respectively. However, with a continuous exposure in crude (petroleum) oil concentration to ≥50 mg/L resulted in a decrease in the average biomass of less than 1.32×10^6 cells/mL (fig. 3). The average biomass for Peranema sp. was approximately 1.17×10^6 cells/mL when exposed to ≤50 mg/L of crude (petroleum) oil. However, with an exposure to crude (petroleum) oil concentrations of ≥50 mg/L to a further decrease in biomass to 1.12×10^6 cells/mL. The pattern also was demonstrated by a consortium of three isolates shown similar increase of biomass when exposed to crude (petroleum) oil concentrations of ≤50 mg/L or ≥50 mg/L. Based on these the biomass increase there was significant differences shown between the cell densities of pure protozoan isolates exposed to various concentrations of oily wastewater and those of protozoan isolates exposed to the control positive media that contained sterile saline solution (fig. 1-3). The growth rates for this protozoan biomass were determined in the previous studies and the articles have been submitted for publication.

Statistical analysis further revealed that with the initial cell densities of 1.00×10^3 cells/mL, 1.00×10^5 cells/mL and 1.00×10^6 cells/mL, the biomass were found to differ between exposures to various crude oil concentrations in wastewater medium ($p<0.001$).

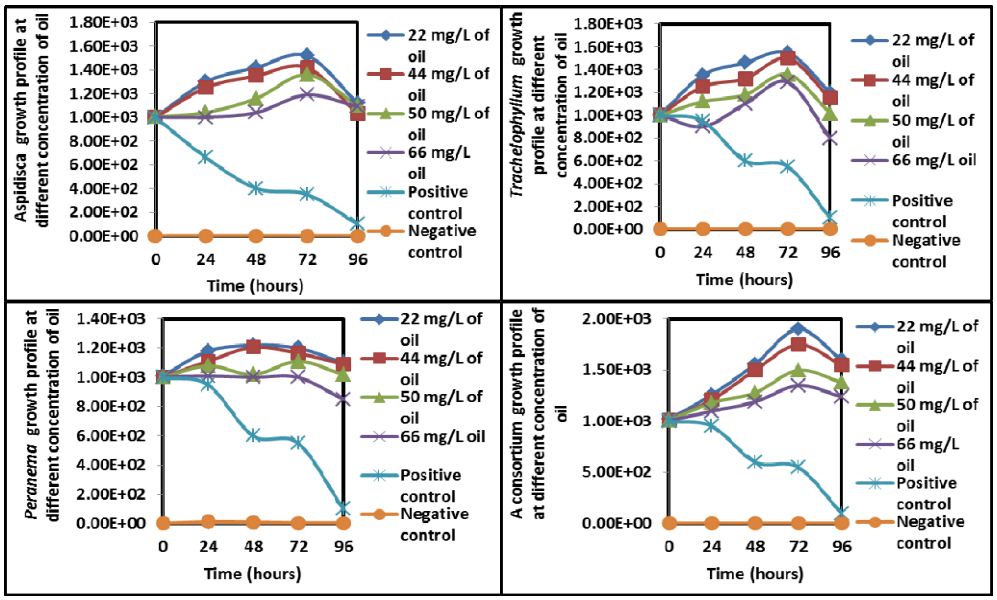

Fig.1 *Biomass of protozoan isolates from an initial of 1.00×10^3 cells/mL of Aspidisca, Trachelophyllum, Peranema sp. and a consortium of three isolates exposed to various concentrations of crude (petroleum) oil in wastewater during the study*

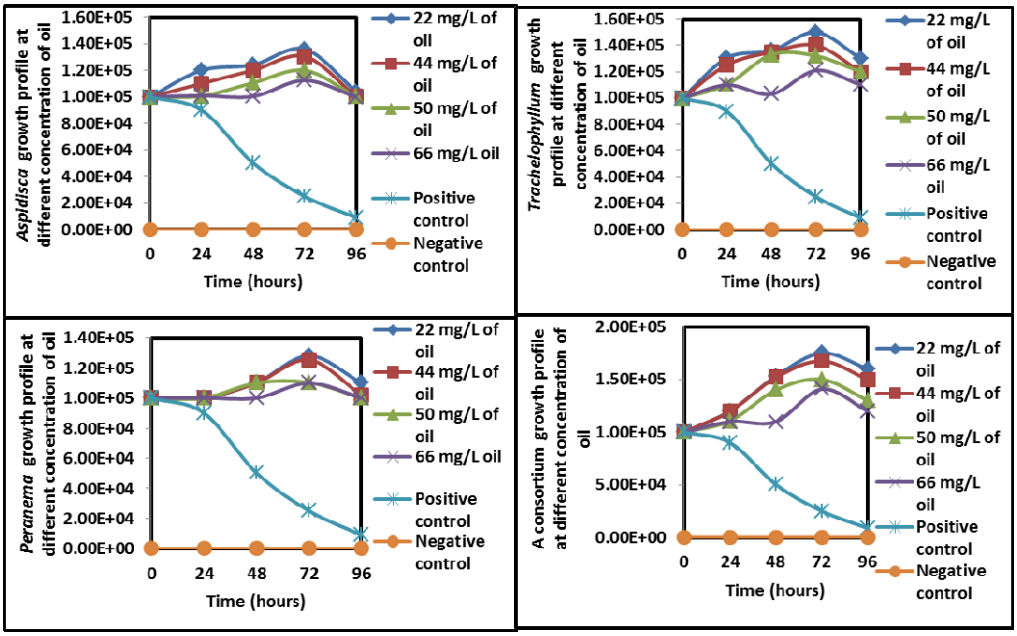

Fig.2 *Biomass of protozoan isolates from an initial of 1.00×10^5 cells/mL of Aspidisca, Trachelophyllum, Peranema sp. and a consortium of three isolates exposed to various concentrations of crude (petroleum) oil in wastewater during the study*

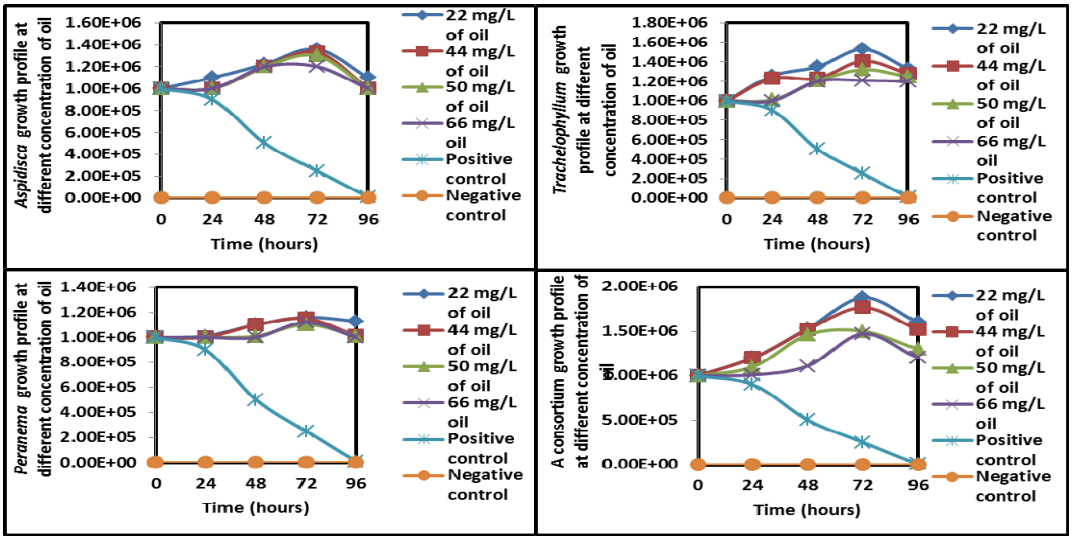

Fig.3 *Biomass of protozoan isolates from an initial of 1.00×10^6 cells/mL of Aspidisca, Trachelophyllum, Peranema sp. and a consortium of three isolates exposed to various concentrations of crude (petroleum) oil in wastewater during the study*

Biodegradation of various concentrations of crude (petroleum) oil in wastewater by protozoa at different biomass

An initial protozoan cell density of 1.00×10^3 cells/mL when exposed to 22 mg/L of crude oil in the polluted wastewater, the biodegradability of Aspidisca had a minimum rate of 15.3% to a maximum rate of 55.5%. There was a decreased of between 10.4% and 43.12% after exposed to 44 mg/L of crude oil for the incubation period of 5 d. further increase of oil concentrations were increased to between 50 mg/L and 66 mg/L, the biodegradation ability of the specific isolate decreased to less than 33.2% at the end of the incubation period of the study (96 h) and this was similar to the decrease of biomass response of the isolate (fig. 1 and 4). The percentage of the crude oil biodegraded by Trachelophyllum sp. was in between 13.95% to 64.5% and from 11% to 46.06%, after exposure to 22 mg/L and to 44 mg/L of oil in the polluted wastewater, respectively (fig.4). However, as the oil concentrations the wastewater media increased to ≥50 mg/L, the biodegradation rates decreased from 33.9% to ≤ 25%. These biodegradation patterns were also noted for other protozoan isolates. For *Peranema*, the biodegradation rates ranged between 13.22% and 50.1%, and between 11.66% and 44.21% when exposed to 22 mg/L and 44 mg/L crude oil concentrations, respectively. For concentration of ≥50 mg/L further, led to a decrease of biodegradation ability of the isolate to ± 35% at the end of the incubation period of 96 h (fig.4). The biodegradation ability of the consortium of three isolates was observed that increased from the range of 23.01% to 69.12% and 21.21% to 64.12% when incubated in crude oil concentrations of 22 mg/L and 44 mg/L, respectively. The biodegradation ability of the consortium organisms significantly decreased to less than 50% with increasing concentrations of crude oil in the wastewater medium of ≥50 mg/L (fig. 4).

With protozoa of 1.00×10^5 cells/mL exposed to various concentrations (22 mg/L, 44 mg/L and 50 mg/L and 66 mg/L) of crude oil in an oil-polluted wastewater medium, similar outcomes from the protozoan isolates was noted, as compared to the previous of an initial biomass (1.00×10^3 cells/mL). The percentage of oil degraded by Aspidisca demonstrated that it was in a range from 20.32% to 60.5%; 15.41% to 54.32%; 10.71% to 41.23% and from 8.39% to 26.5%, when exposed to various concentrations (22 mg/L, 44 mg/L and 50 mg/L

and 66 mg/L) of crude oil, respectively. For Trachelophyllum sp. exhibited average biodegradation rates was ranging from 17.32% to 59.54% and from 15.02% to 52.35%, when exposed to 22 mg/L and 44 mg/L of crude oil, respectively. However, as the concentrations of the oil content in wastewater were increased ≥ 50 mg/L, the biodegradation capacity decreased to ≤45% (fig.5). The Peranema isolate again exhibited biodegradation rates ranging from 16.22% to 50.1% when exposed to polluted oily wastewaters containing 22 mg/L of oil, and from 13.7% to almost 35.21% when exposed to 44 mg/L of oil. However, as the concentrations of the oil content in wastewater were increased ≥ 50 mg/L, the biodegradation capacity decreased to approximately ≤30% (fig. 5). The biodegradation rates by the consortium of the three isolates ranged from 33.03% to 75.5% and from 28% to 66.2% when exposed to wastewater media containing 22 mg/L and 44 mg/L of crude oil, respectively (fig.5). However, a further increase in crude oil concentrations in the wastewater up to 66 mg/L resulted in a considerable decrease in the biodegradation capability of the consortium to less than 60% (fig.5). The group of a consortium of three isolates was shown to exhibited higher biodegradation abilities compared to those of the pure protozoan isolates.

The general trend of biodegradation was observed again when biomass of an initial 1.00×10^6 cells/mL when they were exposed to oily wastewaters containing the similar concentrations of crude oil (fig.6). The biodegradation rates of the protozoan isolates were exhibited with: Aspidisca isolate -ranging from 25.3% to 71.0% when exposed to 22 mg/L of crude oil; from 19.4% to 66.3% when exposed to 44 mg/L of crude oil; from 13.7% to 62.2% and finally, from 8.40 to 56% when exposed to concentrations of 50 mg/L and 66 mg/L of crude oil during the of 5 d; Trachelophyllum sp. - from 26.3% to 68.9% and 18.9% to 52.3% when exposed to 22 mg/L and 44 mg/L of crude oil, respectively; However, when exposed to 50 mg/L and 66 mg/L of crude oil the biodegradation from 18.9% to 52.1% and from 3.5% to 43.2%, respectively; Peranema isolate - from 21.2% to 56.03% when exposed 22 mg/L of oil; from 17.66% to 50.12% in the presence of 44 mg/L of oil, from 11.01% to 44.12% in the presence 50 mg/L of crude oil; and the amount of oil biodegraded decreased further to less than 30% in the as the concentration further increased to ≥ 50 mg/L of crude oil; and a consortium of three isolates - at crude oil concentrations of 22 mg/L and 44 mg/L the biodegradation rates ranged from 45% to 85.2% and from 39% to 75.2%, respectively, at the end of the incubation period. However, exposure to concentrations of 50 mg/L and 66 mg/L resulted in less than 65% degradation of crude oils (fig. 6).

Statistically, there was a significant difference in biodegradation activities between various protozoan biomass (1.00×10^3 cells/mL to 1.00×10^5 cells/mL) ($p<0.05$). Significant differences were also noted between the concentrations of the oily wastewater medium and the biodegradation activities of the protozoan isolates ($p<0.05$).

Average percentage of the COD released and DO uptake by different protozoan biomass during biodegradation studies of crude (petroleum) oil

At an initial protozoan biomass of 1.00×10^3 cells/mL, 1.00×10^5 cells/mL and 1.00×10^6 cells/mL exposed to oil concentrations (22 mg/L, 44 mg/L, 50 mg/L and 66 mg/L) all the test organisms and a consortium of the three isolates were able to remove the DO in oil polluted wastewater at minimum average rates of ≥60% and maximum rates of ≤100%. An increase in crude oil concentrations in the polluted wastewater stimulated faster uptake of DO in the media and the quantity of the biomass also contributed significantly to higher uptake of the available oxygen in the media. In general, there was no uptake of available oxygen in the negative control regardless of the crude oil concentrations in the polluted wastewater (table 1). The average percentage of COD released in the media was consistence all through the entire protozoan isolates with a progressive increase as the concentration of oil in the media was

increased (table 1). Statistically there was a significant difference in the amount of DO uptake by the isolates in terms of the concentration of oil in the medium ($p<0.05$). However, there was no significant difference in DO uptake noted between protozoan cell densities of 1.00×10^3 cells/mL, 1.00×10^5 cells/mL and 1.00×10^6 cells/mL ($p<0.05$). For the amount of COD released there was a significant difference ($p<0.05$) between the various protozoan biomasses of 1.00×10^3 cells/mL, 1.00×10^5 cells/mL and 1.00×10^6 cells/mL (table 1).

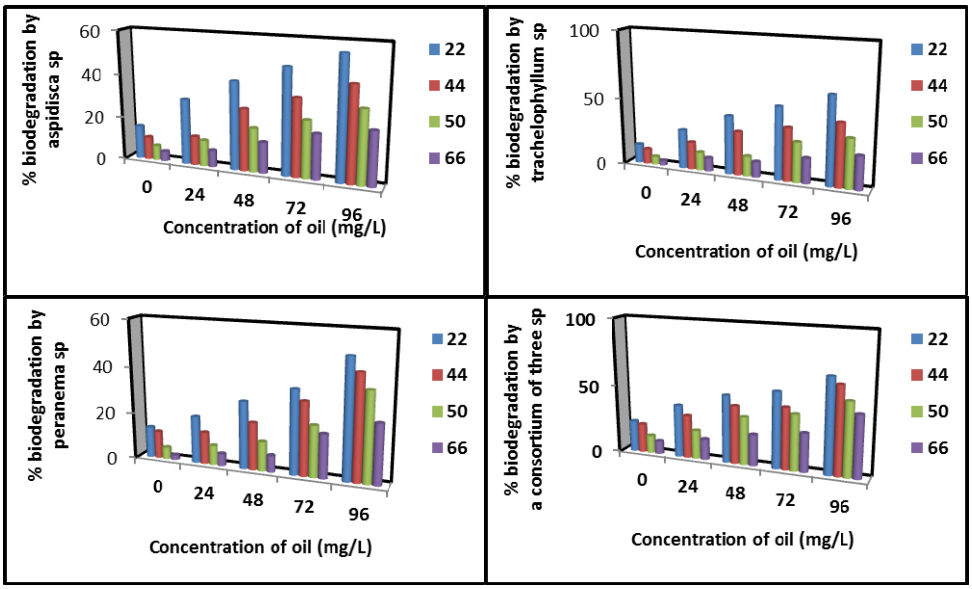

Fig.4 *Biodegradation of various concentrations of crude (petroleum) oil in wastewater by the protozoan biomass of 1.00×10^3 cells/mL (Aspidisca sp., Trachelophyllum sp., Peranema sp. and a consortium of three isolates)*

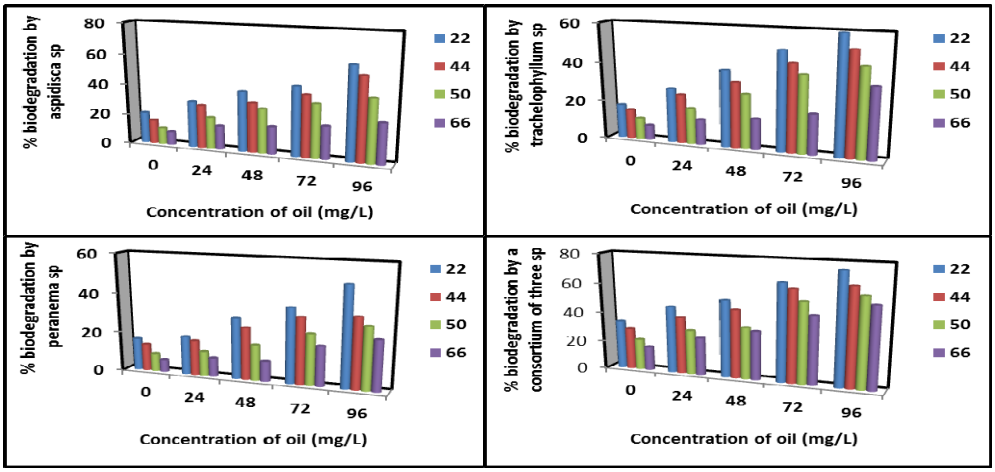

Fig.5 *Biodegradation of various concentrations of crude (petroleum) oil in wastewater by the protozoan biomass of 1.00×10^5 cells/mL (Aspidisca sp., Trachelophyllum sp., Peranema sp. and a consortium of three isolates)*

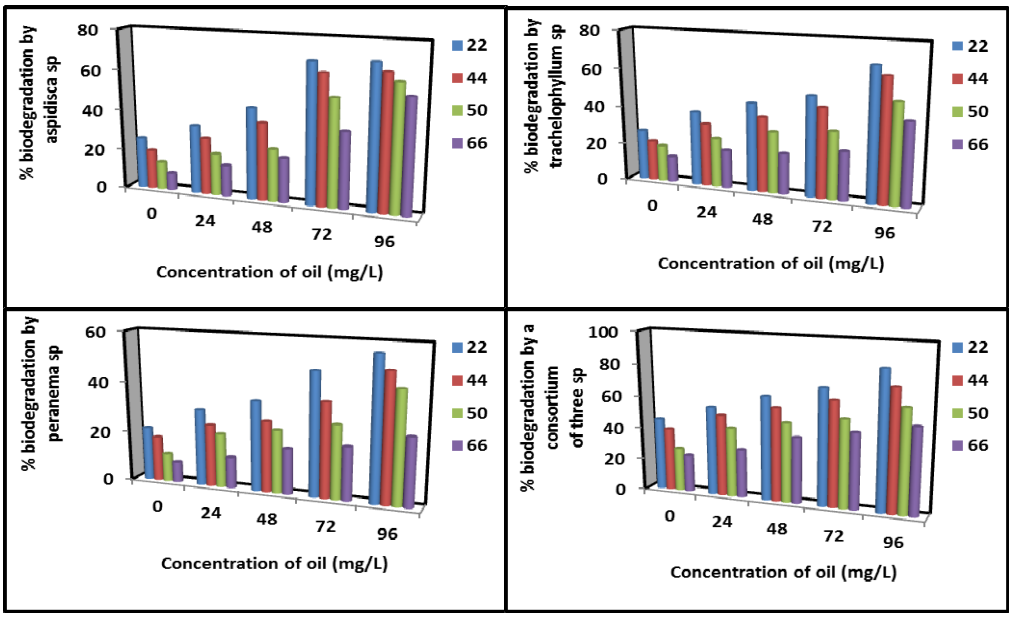

Fig.6 *Biodegradation of various concentrations of crude (petroleum) oil in wastewater by the protozoan biomass of 1.00×10^6 cells/mL (Aspidisca sp., Trachelophyllum sp., Peranema sp. and a consortium of three isolates)*

Table 1 *The average percentage of DO uptake and COD removed/released by protozoan isolates during biodegradation studies of crude oil (petroleum) wastewater*

Protozoan isolates	Protozoan biomass (cells/mL)					
	1.00×10^3		1.00×10^5		1.00×10^6	
	% DO average uptake	%COD average removed/ released	% DO average uptake	%COD average removed/ released	% DO average uptake	%COD average removed/ released
Aspidisca	60.5	-8.31	81.5	-15.7	95.0	-35.3
Trachelophyllum	84.3	-3.90	82.9	-33.3	82.4	-43.2
Peranema	68.4	-10.9	71.2	-25.1	76.1	-54.1
A consortium of three isolates	98.8	-48.0	98	-68.8	99.8	-77.2
Positive control	0	0	0	0	0	0
Negative control	0	5.80*	0	6.10*	0	9.10*

Negative control [crude oil in the media with no microbes included],
Positive control [protozoan isolates were inoculated into sterile saline solution (8.5% NaCl)]
**%COD Removed*
-%COD Released

Discussion

Toxic hydrocarbons that are released in the existing water resources whether deliberately or by accidentals are major cause of water pollution. These hydrocarbons pose critical health challenges to both human and various diverse species that are forming part of aquatic habitants. The problems associated with this type of pollution by these toxic hydrocarbons are stimulated by various activities such as crude oil processing, metal processing, transportation, car washing, petroleum refineries and other oily substances released in water (Lan et al. 2009). Hydrocarbons are a broad family of hundreds of chemical compounds which contain toxic substances such as phenol, poly-aromatic which are either directly or indirectly carcinogenic, mutagenic or cytotoxic to human populations and aquatic species (Peixoto et al.

2011). According to Das and co-worker (2011) the biodegradation of crude oil spills in any water or other affected site is a complex process that depends entirely on the nature and the concentration of different other hydrocarbon chain or structures present. Bioremediation technique is widely used in biodegradation contaminants into less harmful substances in the affected ecosystem (Jain and Bajpai 2012). While microorganisms such as protozoa have been utilised in the removal of domestic wastewater nutrients (Akpor et al. 2008, Akpor 2009) and heavy metals in industrial wastewaters (Kamika and Momba 2011) owing to their dynamic nature of their population, however, no tangible study has been done on their biodegradation capacity reducing or eliminating ever increasing hydrocarbons in wastewater, unlike their bacterial counterparts that have been widely used for a long time in oil biodegradation studies (Das and Chandran 2011, Kamika and Momba 2011). This gap has prompted critically investigate the effect of their biomass of these protozoan species in the bioremediation of hydrocarbon contamination and prevention of water-source pollution by crude oils.

The outcome of this study demonstrated the capability each tested microbes to grow independently or in a consortium in polluted wastewaters containing crude (petroleum) oil at various concentrations. Based on the study of the biomass of the test organism/consortium (1.00×10^3 cells/mL; 1.00×10^5 cells/mL; 1.00×10^6 cell/mL) when exposed to various crude oil concentrations (22 mg/L, 44 mg/L, 50 mg/L, 66 mg/L), the maximum growth of these protozoan species occurred at 72 h (fig. 1-3). Their growth response was entirely depended on individual pure culture of protozoa or in their consortium and the concentrations of crude oil in the wastewater In spite of their similarity in the growth pattern (fig. 1-3). Statistically, there was a significant difference in the individual growth rates of the isolates in terms of various biomasses ($p<0.001$) with the exception of Peranema isolates which showed no significant difference. The biomass response in the media were also found to differ significantly ($p<0.001$) when exposed to $\leq$50 mg/L and $\geq$50 mg/L oil concentrations in wastewater medium. The interactions between protozoan isolates at various concentrations of oil were shown to be significantly different ($p<0.001$). the downward trajectory of the various protozoan biomass decrease as the concentration of oil increased in the media was due to higher toxicity of the crude (petroleum) oil, irrespective of whether protozoan isolates were in pure culture or in a consortium of three isolates (fig. 1-6).

The high increase exhibited by a consortium of the three isolates, compared to those of protozoan species in pure culture, were due to a synergistic mechanism; because of their ubiquity and numerous diverse enzymatic activities under these extreme conditions, they were able to utilise different substrates within the oily wastewater media. Most of individual protozoan isolates within the consortium of isolates were able to convert single substrates (component of oil) into intermediate products while others used these as their substrate to further transform these into end-products which are harmLess to individual isolates (Peixoto et al. 2011).

The high growth rate demonstrated by the protozoan isolates at low petroleum oil concentrations in the wastewater medium (table 1, fig. 1-3) could be associated with the low toxicity of the hydrocarbons. These hydrocarbons were serving as a source of carbon for their nutrient and energy requirements by the protozoan microbes according to Wang et al. (2011). The presence of different isolates in one sphere enabled various members to adapt very easily especially during in times of insufficient nutrient and energy sources. The combined action in a consortium facilitates biodegradation of oily compounds crude oil is a complex mixture of many substances such as aromatics, poly-aromatics amongst others hence individual species are not able biodegrade these oily components fully on their own (Ghazali et al. 2004). Vina et al. (2005) further stated that high hydrocarbon biodegradation capacity exhibited by a

consortium was due to their diverse enzymatic repertoires which are generally more efficiently adapted in biodegrading hydrocarbons complex. The study reported that exhaustive growth of one strain of the microorganisms in the crude oil medium as a source of carbon energy, the residual component of the oil will always support the growth of a second and third strain of the microbes used in the consortium for the study (Horowitz et al. 1975). William (1986) also reported that the toxicity of highly unsaturated fat and oil components of crude oil could result in the inhibition of the ability of these protozoan isolates to metabolise them fully or partially. This condition also led to higher energy requirements for their biodegradation processes (William 1986). It has also been reported that an inadequate supply of oxygen or vital nutrients affects the steady growth of the microbes due to high concentrations of toxic volatile hydrocarbons which are as a result of the un-dispersed hydrocarbons in the medium (Sathishkumar et al. 2008).

According to Atlas (1975) the viscosity of the oil always increases with a decrease in temperature, thus directly affecting the volatility and toxicity of low-molecular-weight hydrocarbons and halting or reducing the onset of the biodegradation of the oil in wastewater, especially at low temperatures. This clearly explains the positive impact of the temperature of 30°C and the pH of 8 on the biodegradation ability of the target protozoan species during the study period. According to, extreme pH conditions usually have a negative effect on the ability of microbial populations to degrade most of the hydrocarbon components of crude oil (Atlas 1981). There is currently general consensus that higher biodegradation can be obtained by a consortium of microorganisms and that any individual or a single species of microorganism will not completely biodegrade any particular petroleum oil (Westlake 1982).

Low DO concentration observed at the end of the incubation period has also been reported by Sedlak (1991). During the present study, the amount of DO in the media was observed to be directly proportional to biodegradation of crude oil by the protozoan isolates. High uptake of DO by the isolates during the biodegradation of crude oil in polluted oily wastewater was due to an active biomass and the increased concentration of oil. The level of DO in water is directly proportional to the temperature and the activity of the test isolates at certain concentrations (Herman and Maier 2000).

The study revealed that significant amounts of COD were released as the target protozoan species were exposed to high concentrations of crude oil in the wastewater media, unlike removal of COD at low oil concentrations in the media. The high amounts of organic matter of high concentration of high hydrocarbon chains that are broken further to shorter chain or biodegradable colloidal matter contribute to COD release. These results confirmed the findings by previous investigators who also reported an increase in COD when they assessed the abilities of the three protozoan isolates (Aspidisca sp., Trachelophyllum sp. and Peranema sp.) in removing nitrates and phosphates (Akpor et al. 2008) and heavy metals (Kamika and Momba 2011) in wastewater mixed liquor at 30°C and at a pH of 8.

Conclusion

The present study revealed the biodegradation capabilities and limitations of Aspidisca, Trachelophyllum and Peranema exposed to crude oil (petroleum) at various concentrations in oily wastewater. Aspidisca, Trachelophyllum, Peranema and a consortium of these three isolates achieved maximum hydrocarbon (petroleum) oil degradation of 71.0%, 68.94%, 56.03% and 85.2%, respectively. The average amount of DO uptake by test isolates was found to be over 80% during the biodegradation studies. Moreover, the amount of COD released by the above isolates was found to be high as the concentration of oil was increased in the wastewater media. The overall clean-up efforts require long time of hard work with lots of investments to restore the hydrocarbon-contaminated water to its original value. Further

studies are needed to identify more protozoan species that are able to biodegrade crude oil in both oil-polluted industrial effluents and domestic oily wastewater as the increase of these specific three protozoan biomass have seen tremendous increase of biodegradation capacity of crude oil in polluted wastewater.

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