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Effect of microbial load of *Aspergillus niger* and *Pseudomonas aeruginosa* on the bioremediation of crude oil polluted water

K.O. Obahiagbon, E.O. Agbonghae, N.A. Amenaghawon*
Department of Chemical Engineering, Faculty of Engineering, University of Benin, P.M.B. 1154, Benin City, Edo State, Nigeria.

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**Corresponding Author. E-mail: andrew.amenaghawon@uniben.edu; Tel: (+2348069275563)*

Abstract
The effect of microbial load of *Aspergillus niger* and *Pseudomonas aeruginosa* on the bioremediation of crude oil polluted water was investigated in this study. Sixteen samples, each having a crude oil to water ratio of 1:9 were divided into three sets of 5 samples while the last sample was taken as control. The three sets of samples were each inoculated with varying microbial load (0.2×10^7, 0.4×10^7, 0.8×10^7, 1×10^7 and 2×10^7 cfu/g) of *Aspergillus niger*, *Pseudomonas aeruginosa*, and a microbial consortium made up of 50% of both. All samples, including the control, were monitored for a total period of seven weeks for physicochemical parameters such as pH, Biochemical Oxygen Demand (BOD), turbidity, and Residual Hydrocarbon Content (RHC). There was a general decrease in BOD, turbidity and RHC with remediation time for all the samples. Increasing the microbial loading resulted in the enhancement of the remediation process. Maximum reductions in BOD, turbidity and RHC were obtained when the microbial consortium was used at a loading of 2×10^7 cfu/g and these were recorded to be 88.09, 83.33 and 91.94% respectively.

Keywords: Bioremediation, Crude Oil, Water, Aspergillus Niger, Pseudomonas Aeruginosa

Introduction
Oil pollution problems are increasingly becoming a common theme in the world today and this has resulted in the degradation of the environment particularly in the oil producing areas of the world [1]. Environmental contamination resulting from crude oil pollution typically occurs through accidental release of crude oil and from the large quantities of oil sludge produced in refineries during the separation of oil from water as well as the oily materials present at the bottom of crude oil storage tanks [2]. Crude oil is a complex organic compound made up of a large variety of hydrocarbons [3]. The effect of crude oil pollution on the environment depends on the type and quantity of crude oil involved. The water soluble fraction of the oil has been reported to reduce the growth of biomass in the contaminated environment as a result of the reduction in dissolved oxygen, increase in turbidity and toxicity of the crude oil components [4]. This problem has been amplified by the fact that a lot of the conventional treatment methods employed for the decontamination of crude oil polluted sites are often limited in terms of application and they are not economically viable and may be only partially effective in cleaning up the contaminated site [5].
Bioremediation which involves the microbial degradation of the pollutant has emerged as the most significant natural mechanism for the removal of non-volatile hydrocarbon pollutants from the environment [6]. It is a relatively low cost treatment option that has low technology requirement and most of the pollutants are degraded into less toxic forms within a relatively short period of time [7]. Bioremediation is a complex process whose qualitative and quantitative aspects depend on the nature and the amount of the pollutant present, the ambient and seasonal environmental conditions, and the constitution of the indigenous microbial community. In its simplest form of operation, crude oil degrading microorganisms are employed for the purpose of mineralising the crude oil pollutant into simpler and less toxic forms such as carbon dioxide (CO_2) and water (H_2O) [8]. Many crude oil degrading microorganisms have been isolated. These include bacteria species such as *Pseudomonas, Escherichia coli, Clostridium, Candida* and fungal species such as *Aspergillus niger, Yeasts, Penicillium* etc [9-11]. Bioremediation of polluted sites can be implemented through either of biostimulation or bioaugmentation. Biostimulation involves the stimulation of indigenous microorganisms to degrade the contaminants by the addition of nutrients to the water or soil to enhance the growth of the microbial population already present in the

water or soil [12]. Bioaugmentation involves the controlled addition of specially formulated bio-culture to assist those found naturally in the polluted soil or water [13]. Bioaugmentation ensures that the proper sets of microorganisms are present in the soil in sufficient type, number and compatibility to effectively attack the pollutant, which results in it being broken down into its most stable forms [14]. However, no single microbial species has the enzymatic ability to metabolise more than two or three classes of compounds typically found in crude oil; thus a consortium composed of many different microbial species is often required to degrade a crude oil spill significantly [11]. Numerous studies have shown that isolates of *Aspergillus niger* and *Pseudomonas aeruginosa* possess the capacity to degrade petroleum hydrocarbons and the isolates can be applied individually or combined to form a microbial consortium [6,11,14,15].

This study focuses on the effect of microbial load of *Aspergillus niger* and *Pseudomonas aeruginosa* on the bioremediation of crude oil polluted water. Both microorganisms were first applied individually to the bioremediation process and subsequently together as a microbial consortium. The bioremediation process was monitored by measuring physicochemical parameters such as pH, Biochemical Oxygen Demand (BOD), turbidity and Residual Hydrocarbon Content (RHC).

2. Materials and methods

2.1. Microorganisms

Aspergillus niger and *Pseudomona aeruginosa* were the crude oil degrading microorganisms used in this study. The microorganisms were obtained from the biotechnology division of the Federal Institute of Industrial Research Oshodi (FIIRO), Lagos, Nigeria. *Aspergillus niger* was maintained on Potato Dextrose Agar (PDA) slants and stored in a refrigerator at 4^oC until it was needed. *Pseudomonas aerugenosa* was grown in flasks of 500 mL with aeration by mechanical mixing. The separation of bacterial suspension from the liquid medium was achieved by centrifugation. The concentration of bacterial consortium (numbers of cells in 1 mL of a suspension) was determined using the Thom's chamber [7].

2.2. Sample preparation and bioremediation studies

Escravos light crude oil was used in simulating crude oil polluted water. It was obtained from an Oil Producing Company located in the Niger Delta region of Southern Nigeria. The properties of the crude oil sample were as follows: API gravity (35.3 API^o), specific gravity (0.85), sulphur content (0.15 wt%), viscosity at 40^oC (3.28 cSt). Crude oil polluted water was simulated in sixteen vessels by adding 200 mL of Escravous light crude oil to 1800 mL of water. The simulated wastewater samples were stored in sixteen black plastic vessels and they were allowed to stand for one week to allow the indigenous microorganisms to adapt to their new environment. One set of five samples was inoculated, each with *Aspergillus niger* in varying concentrations of 0.2×10^7, 0.4×10^7, 0.8×10^7, 1×10^7 and 2×10^7 cfu/g. Another set of five samples was inoculated, each with *Pseudomonas aeruginosa* in varying concentrations of 0.2×10^7, 0.4×10^7, 0.8×10^7, 1×10^7 and 2×10^7 cfu/g and a third set of five samples was inoculated, each with a consortium of 50% *Aspergillus niger* and 50% *Pseudomonas aeruginosa* in varying concentrations of 0.2×10^7, 0.4×10^7, 0.8×10^7, 1×10^7 and 2×10^7 cfu/g. The last (sixteenth) sample was taken as control without any microbial inoculation. Bioremediation indicating parameters of the polluted water were monitored in the course of the remediation process. The following parameters; pH, Biochemical Oxygen Demand (BOD), turbidity and Residual Hydrocarbon Content (RHC) were monitored in the course of bioremediation. Sampling was done on day zero (before inoculation) and subsequently at intervals of seven days (one week) for a total of 49 days (seven weeks).

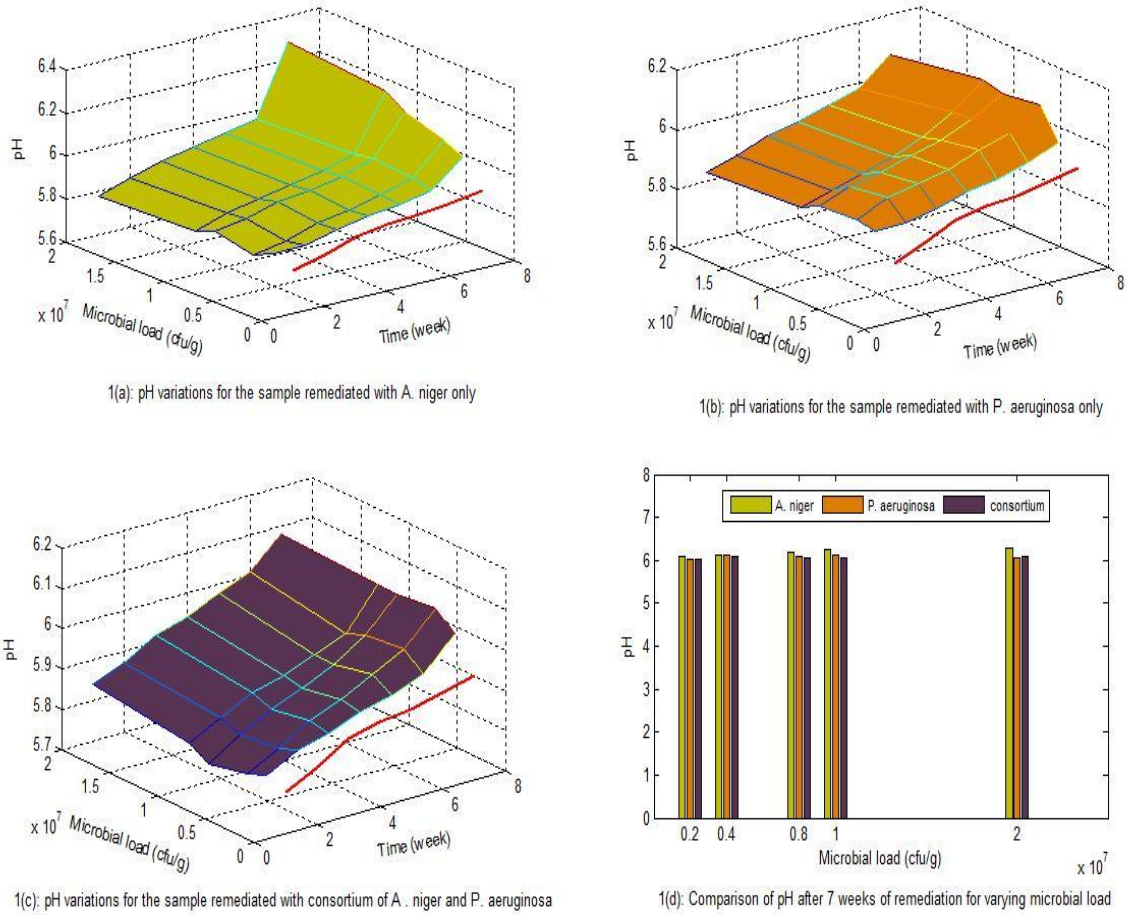
2.3. Analytical methods

An electronic pH meter (Fisher Accruement pH meter) was used to measure the pH of the samples. The BOD of the wastewater samples was determined using the Winkler method [16]. The residual hydrocarbon content of the water was determined by shaking 5 g of a representative wastewater sample with 10 mL of carbon tetrachloride and the oil extracted was determined by the absorbance of the extract at 450 nm using a spectronic 70 spectrophotometer [1]. The turbidity of the sample was measured at 450 nm using a spectronic 70 spectrophotometer.

3. Results and discussion

Figure 1 shows the pH variations with respect to remediation time and microbial load for the three sets of samples as well as the control. Figure 1(a) is a three-dimensional (3-D) plot showing the effect of microbial load and remediation time on pH for the set of samples inoculated with *Aspergillus niger*. Similarly, Figures 1(b) and (c) are 3-D plots for the set of samples inoculated with *Pseudomonas aeruginosa* and a 50%-50% consortium of both microorganisms respectively. Figure 1(d) shows the comparison of pH values after 7 weeks of remediation time for the three sets of samples at various microbial loadings.

The thick red line in Figures 1(a) to (c) shows the variation in the pH of the control sample with respect to time. It was observed that the pH of the control sample displayed a trend similar to those of the other samples.



1(a): pH variations for the sample remediated with A. niger only

1(b): pH variations for the sample remediated with P. aeruginosa only

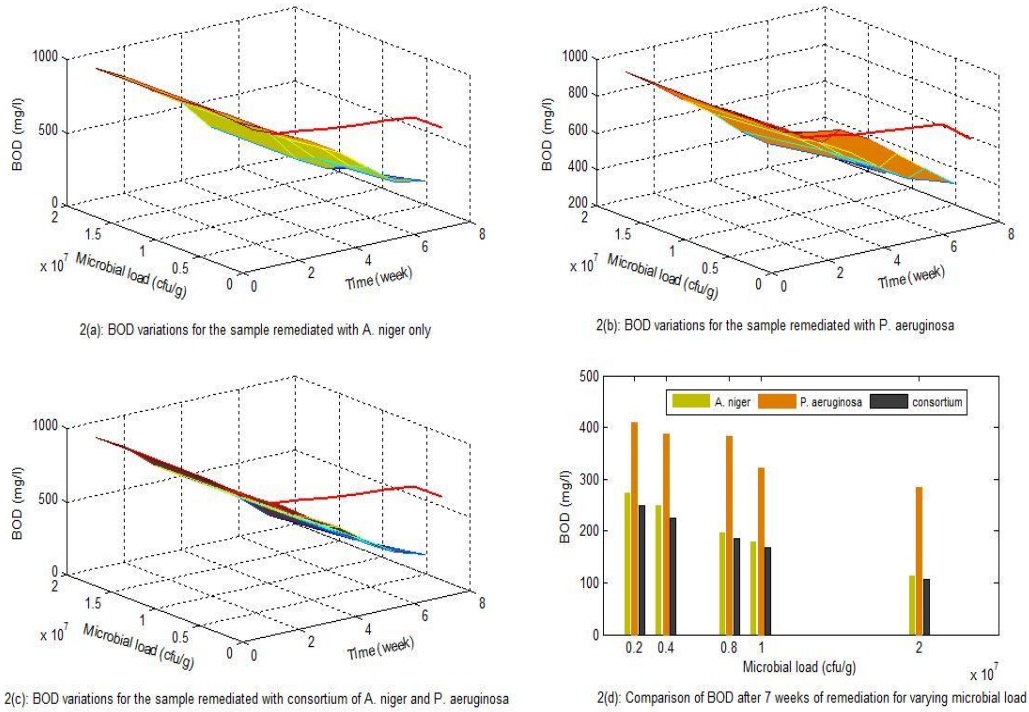
1(c): pH variations for the sample remediated with consortium of A . niger and P. aeruginosa

1(d): Comparison of pH after 7 weeks of remediation for varying microbial load

Figure 1: pH variations with remediation time and microbial load

The change in the pH of the control sample could be attributed to some level of biodegradation of the crude oil which must have resulted from the action of the indigenous microorganisms present in the crude oil polluted water. There was a general increase in the pH of the inoculated samples with respect to remediation time as seen from the 3-D plots. The pH measurements taken at the end of every week revealed that the pH of the inoculated samples did not vary significantly with respect to microbial load for all the three sets of samples except for some slight fluctuations as seen in the Figures. The variation in the pH of the inoculated samples is an indication of bioremediation. The increase in the pH values observed suggests that the crude oil pollutant in the wastewater samples was being degraded to less toxic and less acidic products [17]. Obahiagbon et al. [1] reported that the degradation of crude oil into intermediate products might actually have an effect on the pH of the remediation medium while Amenaghawon et al. [18] reported that the pH of the contaminated wastewater undergoing remediation could increase with time if the population of the remediating microbial population is allowed to grow and thrive. Amenaghawon et al. [18] investigated the effect of nutrient supplementation, aeration and mechanical agitation on the bioremediation of crude oil contaminated water. They reported a similar increase in the pH of the samples in the course of bioremediation. According to them, the increase in pH might have resulted from the conversion of the crude oil pollutant into less acidic products.

The effect of microbial load and remediation time on the BOD of the inoculated samples as well as the control is presented in Figure 2. Figure 2(a) is a 3-D plot showing the effect of microbial load and remediation time on the BOD for the set of samples inoculated with *Aspergillus niger*. Similarly, Figures 2(b) and (c) are 3-D plots for the set of samples inoculated with *Pseudomonas aeruginosa* and a 50%-50% consortium of both microorganisms respectively. Figure 2(d) shows the comparison of BOD values after 7 weeks of remediation time for the three sets of samples at various microbial loadings. The thick red line in Figures 2(a) to (c) shows the variation in the BOD value of the control sample with respect to time. The BOD of the control sample decreased by 23.95% indicating that there was an observable level of bioremediation albeit not very significant.



2(a): BOD variations for the sample remediated with A. niger only

2(b): BOD variations for the sample remediated with P. aeruginosa

2(c): BOD variations for the sample remediated with consortium of A. niger and P. aeruginosa

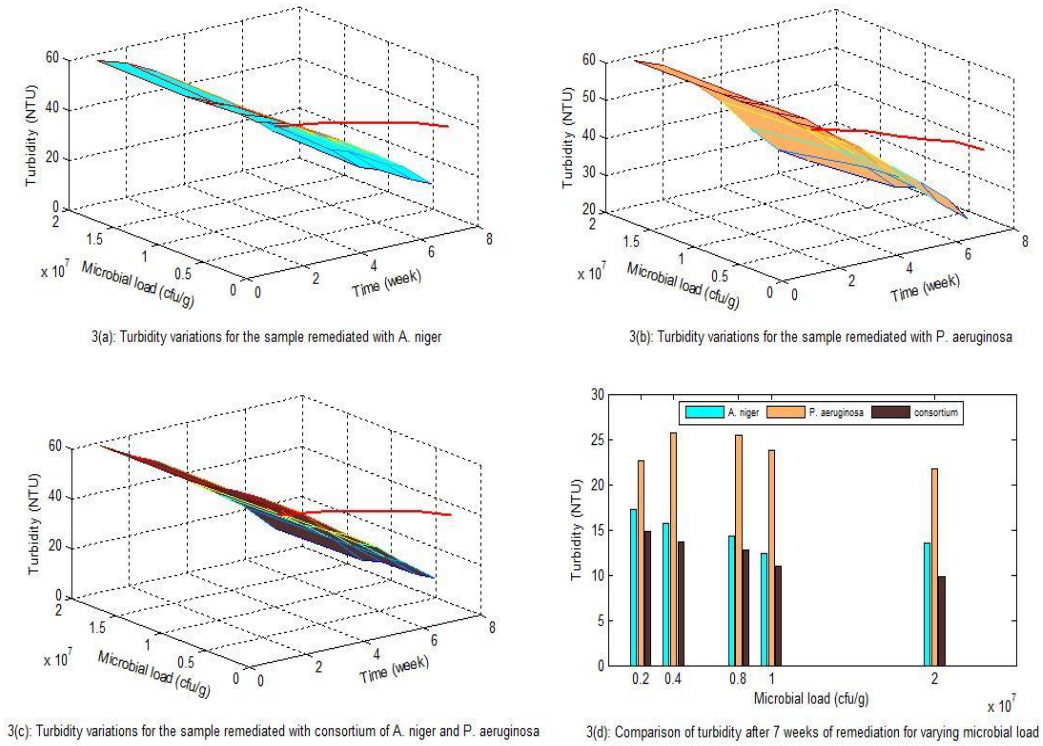
2(d): Comparison of BOD after 7 weeks of remediation for varying microbial load

Figure 2: Biochemical Oxygen Demand (BOD) variations with remediation time and microbial load

The reduction in BOD could be attributed to the activities of the indigenous microbes present in the wastewater which converts the crude oil into less toxic substances such as CO_2, H_2O and many intermediates like organic acids, lipids, esters, complex alcohols and microbial proteins in form of enzymes [8]. For the inoculated samples, a trend similar to that of the control was observed in that the BOD of the samples showed a general and progressive decrease with respect to time from the start to the end of the bioremediation process. Figure 2(d) shows that BOD also decreased with increase in microbial load. The greatest reduction in BOD (88.09%) at the end of the bioremediation process was recorded when a 50%-50% consortium of *Pseudomonas aeruginosa* and *Aspergillus niger* was used at a loading of 2×10^7 cfu/g as shown in Figure 2(d). The BOD is an indication of the oxygen requirement of microorganisms during the biodegradation of organic matter. Hence a reduction in BOD is indicative of a reduction in the organic matter present in the wastewater which in this case is crude oil [7]. The decrease in BOD of the samples could be attributed to the metabolic activities of the microbial population present in the remediation medium. These organisms are able to degrade crude oil by converting it to simpler and less toxic products [1]. Amenaghawon et al. [18] reported similar reductions in BOD of crude oil contaminated water in the course of bioremediation and they attributed the trend they observed to the action of the microorganisms in the samples both those indigenous and those exogenously added. Similar results were also obtained by Satyawali and Balakrishnan [19] for the treatment of wastewater from molasses-based alcohol distilleries. Obahiagbon and Aluyor [15] also reported a similar trend for the bioremediation of crude oil contaminated water supplemented with nitrates.

Figure 3 shows the variation in the turbidity of the three sets of samples as well as the control with respect to remediation time and microbial load. Figure 3(a) is a 3-D plot showing the effect of microbial load and remediation time on turbidity for the set of samples inoculated with *Aspergillus niger*. Similarly, Figures 3(b) and (c) are 3-D plots for the set of samples inoculated with *Pseudomonas aeruginosa* and a 50%-50% consortium of both microorganisms respectively. Figure 3(d) gives the comparison of turbidity values after 7 weeks of remediation time for the three sets of samples at various microbial loadings. The thick red line in Figures 3(a) to (c) shows the variation in the turbidity value of the control sample with respect to time. The control sample showed a decrease in turbidity with remediation time as a result of biodegradation caused by the indigenous microorganisms present in the crude oil polluted water. The 3-D plots clearly showed a decrease in turbidity with remediation time for all the three sets of samples. Similarly, the turbidity decreased generally with increase in microbial load for all the three sets of samples throughout the remediation period, except for one or two fluctuations that were observed. Figure 3(d) shows that turbidity also decreased with

microbial load. The greatest reduction in turbidity (83.33%) at the end of the bioremediation process was recorded when a 50%-50% consortium of *Pseudomonas aeruginosa* and *Aspergillus niger* was used at a loading of 2×10^7 cfu/g.



3(a): Turbidity variations for the sample remediated with A. niger

3(b): Turbidity variations for the sample remediated with P. aeruginosa

3(c): Turbidity variations for the sample remediated with consortium of A. niger and P. aeruginosa

3(d): Comparison of turbidity after 7 weeks of remediation for varying microbial load

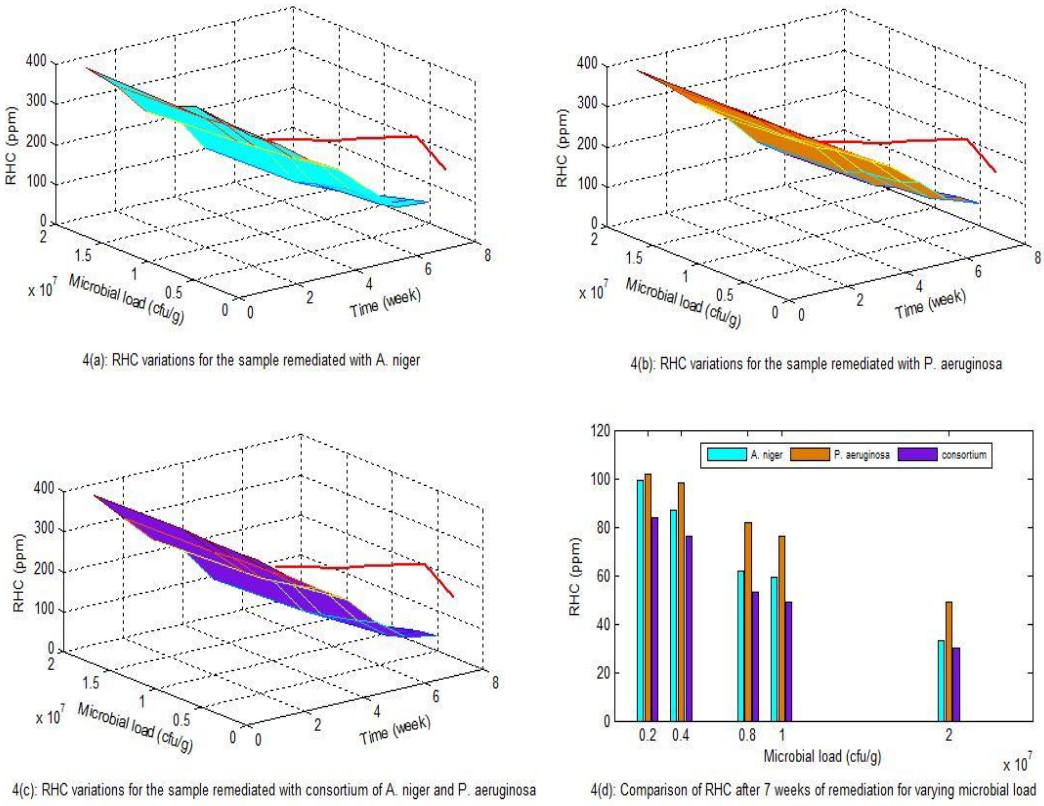
Figure 3: Turbidity variations with remediation time and microbial load

The effect of microbial load and remediation time on the RHC of the inoculated samples as well as the control is presented in Figure 4. Figure 4(a) is a 3-D plot showing the effect of microbial load and remediation time on the RHC for the set of samples inoculated with *Aspergillus niger*. Similarly, Figures 4(b) and (c) are 3-D plots for the set of samples inoculated with *Pseudomonas aeruginosa* and a 50%-50% consortium of both microorganisms respectively. Figure 4(d) gives the comparison of RHC values after 7 weeks of remediation time for the three sets of samples at various microbial loadings. The thick red line in Figures 4(a) to (c) shows the variation in the RHC value of the control sample with respect to time. The control sample showed a decrease in RHC in the course of bioremediation although this was not very significant. The RHC of the inoculated samples was observed to decrease with increase in remediation time. Similarly, the RHC of the samples decreased generally with increase in microbial load for all the three sample sets throughout the remediation period. The values of RHC of the remediated samples recorded at the end of the bioremediation process were observed to decrease with increase in microbial loading as shown in Figure 4(d). Figure 4(d) also shows that the greatest reduction in turbidity (91.94%) at the end of the bioremediation process was recorded when a 50%-50% consortium of *Pseudomonas aeruginosa* and *Aspergillus niger* was used at a loading of 2×10^7 cfu/g.

Similar reductions in RHC of crude oil polluted wastewater have been reported by previous researchers [8,20,21]. These researchers all attributed the reductions observed to the biodegrading activity of the microorganisms both those indigenous to the wastewater and those exogenously added.

Conclusion

The effect of microbial load of *Aspergillus niger* and *Pseudomonas Aeruginosa* on the bioremediation of crude oil polluted water was investigated. The use of a microbial consortium made up of *Aspergillus niger* and *Pseudomonas Aeruginosa* enhanced the bioremediation process as against the use of the microorganisms individually. Increasing the microbial loading of the microbial consortium enhanced the rate of bioremediation of crude oil polluted water. The highest remediation capacity was observed when the microbial consortium was used. The samples inoculated with *Pseudomonas aeruginosa* showed the least remediation capacity. A microbial loading of 2.0 x 10^7 cfu/g resulted in the highest remediation capacity.



4(a): RHC variations for the sample remediated with A. niger

4(b): RHC variations for the sample remediated with P. aeruginosa

4(c): RHC variations for the sample remediated with consortium of A. niger and P. aeruginosa

4(d): Comparison of RHC after 7 weeks of remediation for varying microbial load

Figure 4: Residual Hydrocarbon Content (RHC) variations with remediation time and microbial load

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