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# Growth Dynamics of *Pseudomonas fluorescens* and *Vibrio fluvialis* Exposed to Various Concentrations of Nickel

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**Abstract:- Two hydrocarbon utilizers *Pseudomonas fluorescens* and *Vibrio fluvialis* isolated from crude oil impacted mesotidal water and sediment samples and identified using Microgen ID Kit (Microgen Bioproduct Limited, UK), and 16s rRNA were exposed to different concentrations of nickel (Ni) (0.00, 1.17, 2.34, 4.68, 9.375 and 18.75mM). Following exposure to the various Ni concentrations, the isolates were monitored for effect on growth every six hours for 48 hours, percentage survability, and LC50 determined. The generation times for *P. fluorescens and V. fluvialis* increased from 1.55 to 3.73 and 1.56 to 3.75 (h/gen) as Ni concentration increased from 0.00 to 1.17Mm, respectively. Furthermore, growth rates decreased from 0.447 to 0.186 and 0.443 to 0.185 ($h^{-1}$) for *P. fluorescens and V. fluvialis*, respectively. Without the toxicant, the peak numbers of cells were 21.77 and 21.68 log number of cells for *P. fluorescens and V. fluvialis*, respectively. However, in the presence of Ni, the peak log numbers of viable cells were 15.52 and 15.477 after 24 hours for *P. fluorescens and V. fluvialis*, respectively. Comparism of the number of surviving cells at 1.17mM showed that *V. fluvialis* had 76.69% of surviving cells against 9.32 % for *P. fluorescens*. Linear regression line fit plots revealed $R^2$ values of 0.5372 for *P. fluorescens* and 0.9341 for *V. fluvialis*. The LC50 for *V. fluvialis* was 5.77 mM while that of *P. fluorescens* could not be determined from the model. Generally, pH values above 6.8 favoured both isolates even in Ni concentrations above 1.17mM. The findings in our study indicate that Ni in concentrations above 1.17mM has the potential to reduce the growth of hydrocarbonoclastic isolates.**

***Keywords:-*** *Niger Delta, Hydrocarbon Utilizers, Heavy Metals, Bacteria, Toxicity.*

## I. INTRODUCTION

Crude oil spills are common place in the Niger Delta region of Nigeria [1]. Thus, hydrocarbon pollution in the region is one of the major environmental issues [2-3]. One of the major drawbacks of bioremediation is the slow rate at which it occurs [4]. As a process, it is affected by many factors and the most important biotic and abiotic factors include the presence of hydrocarbonoclastic microbes and toxic metals in above trace concentrations [5].

Heavy metals are electronegative metals that are chemically inert and have densities of 5g/cm3, and display various categories of toxicity depending on their concentration [6]. Furthermore, they are classified into essential or non-essential heavy metals depending on whether or not they have a definite biological role. Cobalt, nickel and copper are examples of essential heavy metals while cadmium, mercury and lead are examples of non-essential heavy metals [7]. However, even essential heavy metals like nickel (Ni) are toxic or limiting at higher concentration but stimulating at lower concentrations [8-9]. Its geochemical properties are similar to those iron and cobalt. In soil, they appear in the second and third degrees of oxidation [10]. Heavy metals possess strong binding affinity to metal-sensitive functional groups on surfaces such as amides, thiol or histidyl moieties at minute concentrations [11]. Some bacteria have adapted and developed resistance to heavy metals in the environment [8].

The effects of heavy metals on biodegradation process or microbial isolates are not extensively studied. However, a few studies have shown that they can inhibit biodegradation process [5][12]. Ekpenyong and Antai (2007) [5] showed that total viable count was more reliable than optical density in the evaluation of heavy metal toxicity. Furthermore, they also showed that *Bacillus* was more resistance to cadmium and was a better candidate for bioremediation of co-contaminated environments with crude oil.

It has been suggested that manipulation of pH is a possible way to reduce toxicity of heavy metals to crude oil degraders [12-13]. In line with this, a recent study reported pure and mixed culture of the isolates (*Candida tropicalis*

and *Aspergillus clavatus*) at pH 5 showing great promise for effective bioremediation of spent engine oil polluted soil co-contaminated with cadmium. The aim of this study was to determine the effects of different concentrations of nickel on the growth dynamics of crude oil degraders (*Pseudomonas fluorescens* and *Vibrio fluvialis*).

## II. MATERIALS AND METHODS

➢ *Site Description and Sample Collection*

The study site was the Qua Iboe Estuary of the Niger Delta region of Nigeria. Crude oil impacted water and sediment samples were collected in triplicates from Okposo and Utan Iyatah communities. The coordinates of the sampling points were $4^0$35' 14.9'N, $008^0$ 17' 0.16E and $4^0$40' 49.6'N, $008^o$17 45.4'E for Okposo and Utan Iyatah sampling points, respectively. The collected water and sediment samples were pooled into 4 composite samples as previously reported [4]. Samples were transported immediately to the microbiology laboratory for further analyses.

➢ *Sample Preparation and Microbiological Analyses*

Sample preparation and microbiological analyses were all done as previously reported [4,14]. The composite samples (water and sediment) were each subjected to ten-fold serial dilutions using 10 g and 10 ml of the sediment and water samples, respectively that were dissolved in 90ml of sterile distilled water. From three sets of dilutions for each of the samples, 1ml each was plated onto freshly plated tryptic soy agar (TSA) and incubated at $30^0$C for 24 - 36 hours. Resulting discrete colonies were then plated out repeatedly onto freshly prepared agar and then stored in tryptic soy agar slopes at $4^0$C in a refrigerator for further investigations.

➢ *Screening of Pure Bacterial Isolates for Ability to Utilize Crude Oil*

The resulting purified isolates were screened for ability to degrade crude oil using the vapour phase method of Thijsee and Van der Linden (1961)[15] recently reported by Asitok *et al* (2017)[4]. Briefly, 0.4 ml of the collected Qua Iboe light crude oil was absorbed onto oven sterilized Whatman No 1 filter paper and gently placed on the inside of the lid of a petri dish that was previously seeded with pure culture of the test isolates on mineral salt medium supplemented with streptomycin and nystatin (250mg/ml) to inhibit fungal growth. The plates were then incubated for 48 hours; resulting colonies were regarded as hydrocarbonoclastic bacteria.

➢ *Morphological, Biochemical and Molecular Characterization of the Isolates*

Pure culture of two bacterial isolates were selected for molecular characterization after morphological and biochemical characterization using Microgen ID Kit (Microgen Bioproduct Limited, UK) together with their identification software. Furthermore, molecular characterization was done as previously reported [4]. The selected and identified isolates were *Pseudomonas fluorescens* and *Vibrio fluvialis.*

➢ *Determination of the Effect of Nickel on the Growth of the Test Bacterial Isolates*

The method of Dutton *et al* (1990)[16] also adopted by Odokuma and Okpokwasili (2003) [17] was employed but with slight modification. The general toxicity assay protocols included the following basic steps in common namely: cell growth, cell washing or preparation and exposure of washed cells to various concentrations of nickel. The selected bacterial isolates were cultured in two nutrient broth tubes for 18 h at room temperature. After growing the cells for about 18hours in nutrient broth, the cells were diluted with fresh nutrient broth until absorbance at 550nm ($A_{550nm}$) was 0.2. Then the cells were washed twice in sterile deionized distilled water and standardized using the McFarland method of inoculum preparation as described by Chapin and Lauderdale (2003)[18]. Washed cell suspension was further diluted with sterile deionized water when it was visually more turbid than the McFarland 0.5 standard in front of a Wickerham card. More cells from a reserved washed cell tube were added to the test suspension tube when the test cell suspension was lighter than the McFarland 0.5 standard. Deionized distilled water was used as blank. One millilitre (1ml) of the washed bacterial cells was exposed to nine millilitre (9ml) of the various concentrations of nickel (1.17, 2.34, 4.68, 9.375 and 18.75mM) and control (0.00 mM) contained 9 ml of deionized distilled water without addition of any nickel. All treatments and controls were conducted in triplicate for each bacterial isolate. Immediately after exposing the washed cells to the various concentrations of the various toxicants, one millilitre (1 ml) of the cell-toxicant mixture was withdrawn for zero hour determinations of viable count and pH. The flasks were incubated at room temperature on a rotary shaker at 220 rpm for 1 hour, for the exposed cells to interact with the various nickel concentrations.

## III. RESULTS

Table 1 shows the effect of the various nickel concentrations on the growth of *Pseudomonas fluorescens and Vibrio fluvialis.* The result shows the survival rate of the isolate after one hour (%), the growth rate ($h^{-1}$) and generation time (h/gen). The generation times for *P. fluorescens and V. fluvialis* increased from 1.55 to 3.73 and 1.56 to 3.75 (h/gen) as the concentration increased from 0.00 to 1.17mM and this correlated negatively with growth rates that decreased from 0.447 to 0.186 and 0.443 to 0.185 ($h^{-1}$) for *P. fluorescens and V. fluvialis,* respectively. Furthermore, the survival of the isolates (%) decreased as the concentration of the toxicant increased.

Figure 1 shows that only nickel concentration of 1.17 mM permitted growth of *P. fluorescens* in olive oil minimal medium. The figure shows that initial exposure of the

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bacterium to the nickel concentration brought about a drastic reduction in the log-transformed number of survived cells of 8.688 up until the first 1 h. An acclimation was maintained from 1 h to 6 h after which an exponential growth period was reached where the bacterial log-transformed number increased to 15.520 at 24 h. The figure shows no stationary growth phase. Analysis of variance of data revealed that time and toxicant concentrations made significant ($p < 0.05$) contributions to the responses of the bacterium to nickel intoxication.

Figure 2 is a presentation of the effects of nickel concentrations on log-transformed number of survived cells of *Vibrio fluvialis*. The figure shows that peak log-transformed number of survived cells of *Vibrio fluvialis* was reached at 24 h in both the control and nickel concentration of 1.17 mM with values of 21.683 and 15.477, respectively. Higher nickel concentrations did not permit growth of the bacterium under the study conditions.

Figure 3 shows a comparism of the percent number of surviving cells of *Pseudomonas fluorescens* and *Vibrio fluvialis* under the influence of various concentrations of nickel. The figure reveals that nickel concentration as low as 1.17 mM reduced number of surviving cells to 9.32 % in *Pseudomonas fluorescens* whereas percent survival of *Vibrio fluvialis* under similar treatment yielded 79.69 % of surviving cells.

Figures 4 and 5 are quadratic and linear regression line fit plots respectively of toxicant concentrations against percent number of survived cells of *Pseudomonas fluorescens*. The quadratic function relationship gave an $R^2$ value of 0.8694. The linear regression model gave an $R^2$ value of 0.5372 and was unable to determine the median lethal concentration (LC50) of the toxicant.

Figures 6 and 7 are quadratic and linear regression line fit plots respectively of toxicant concentrations against percent number of survived cells of *Vibrio fluvialis* to various levels of nickel concentration with a significant probability ($p < 0.05$) and an $R^2$ value of 0.9839. The linear regression model presents an equally significant probability ($p < 0.05$) and an $R^2$ value of 0.9431. Unlike the linear regression model for *Pseudomonas fluorescens* that was unable to determine the median lethal concentration (LC50) of the toxicant that of *Vibrio fluvialis* gave a median lethal concentration (LC50) of 5.77 mM.

Figure 8 shows a very interesting result of nickel on medium pH changes when inoculated with *P. fluorescens*. Nickel concentrations at time 0 h decreased the pH of their media slightly below neutrality. Prolonged incubation reduced the pH to 6.61 at 48 h in the control, but the figure reveals that pH of test media increased slightly above neutral with increasing metal concentration. Analysis of variance revealed that exposure time effect on the pH changes was not significant ($p > 0.05$) but the nickel concentration effects were very significant ($p = 0.001$). Figure 9 shows that nickel concentrations of 1.17 and 2.34 mM caused the pH of their respective media to change from neutrality to 7.75 and 7.22, respectively. Higher concentrations placed the pH of their media in the acidic region or very close to neutrality. Analysis of variance of data revealed that time of exposure and metal concentrations had significant ($p = 0.000$) influences on the pH changes.

| Nickel conc. (mM) | Percent survival at 1 h (%) | | Growth rate ($h^{-1}$) | | Generation time (h/gen) | |
|---|---|---|---|---|---|---|
| | *Pseudomonas fluorescens* | *Vibrio fluvialis* | *Pseudomonas fluorescens* | *Vibrio fluvialis* | *Pseudomonas fluorescens* | *Vibrio fluvialis* |
| 0 | 0 | 0 | 0.447 | 0.443 | 1.55 | 1.56 |
| 1.17 | 9.32 | 79.69 | 0.186 | 0.185 | 3.73 | 3.75 |
| 2.34 | 6.75 | 62.5 | 0 | 0 | 0 | 0 |
| 4.68 | 0.57 | 47.4 | 0 | 0 | 0 | 0 |
| 9.375 | 0.41 | 29.17 | 0 | 0 | 0 | 0 |
| 18.75 | 0 | 0 | 0 | 0 | 0 | 0 |

Table 1:- Effect of nickel on growth of bacteria

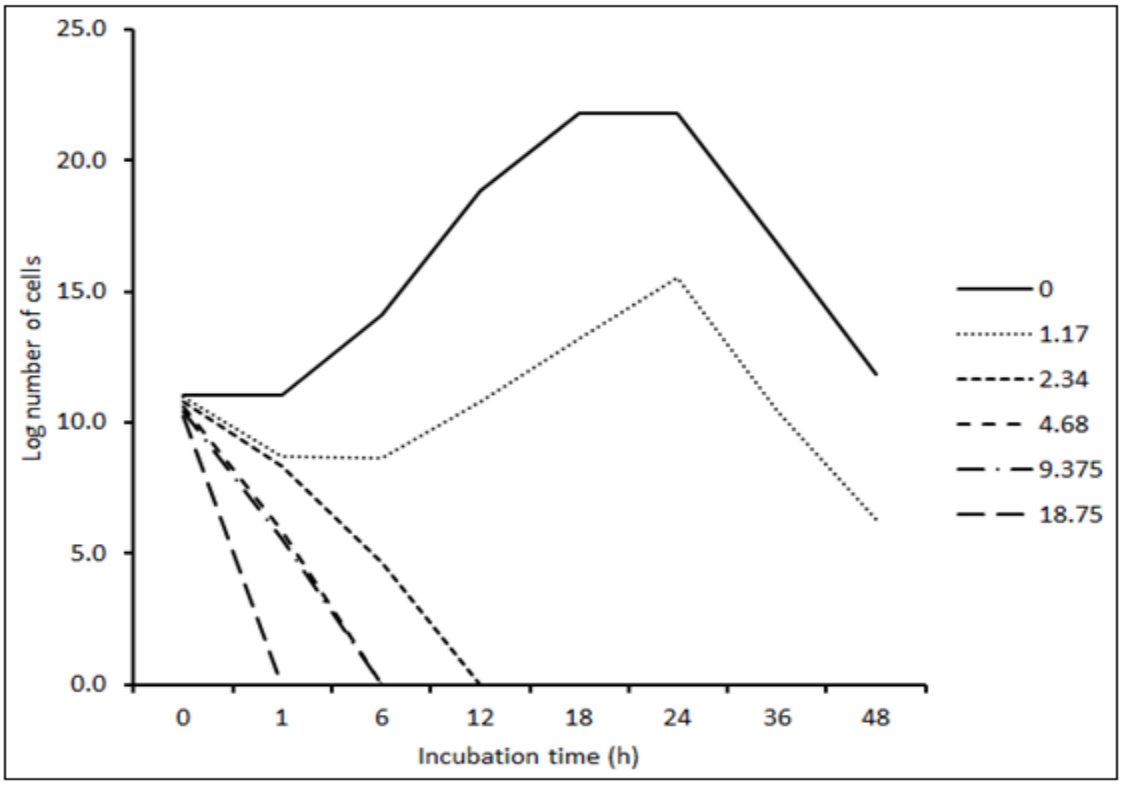


Fig 1:- Effect of nickel on growth of *Pseudomonas fluorescens*.
Legend indicates nickel concentrations [mM].

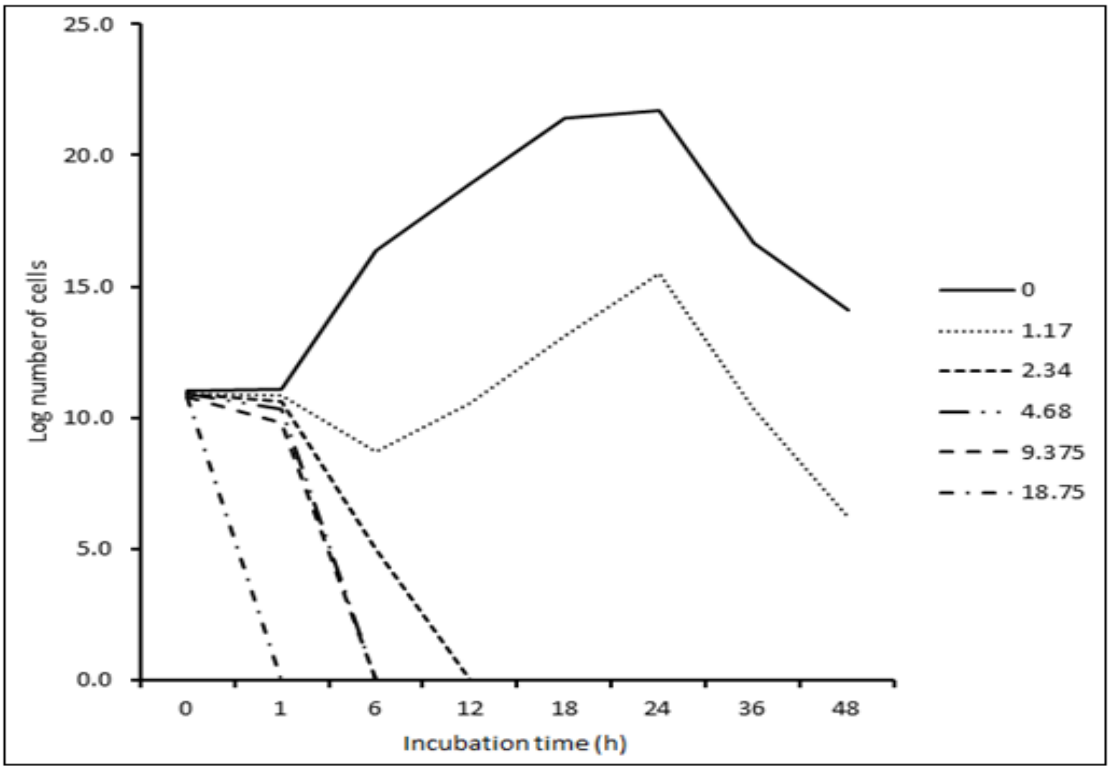


Fig 2:- Effect of nickel on growth of *Vibrio fluvialis.*
Legend indicates nickel concentrations [mM]

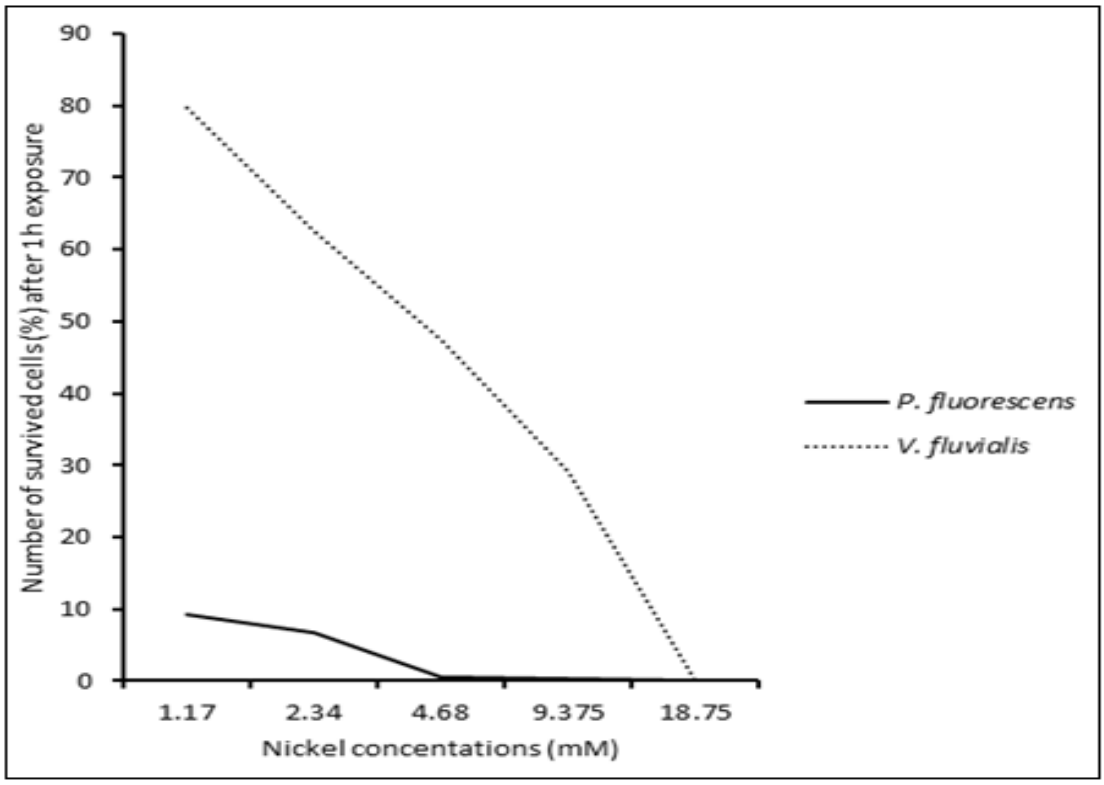


Fig 3:- Comparative percent survivabilities of *Pseudomonas fluorescens* and *Vibrio fluvialis* over a 1-h exposure to varying concentrations of nickel.

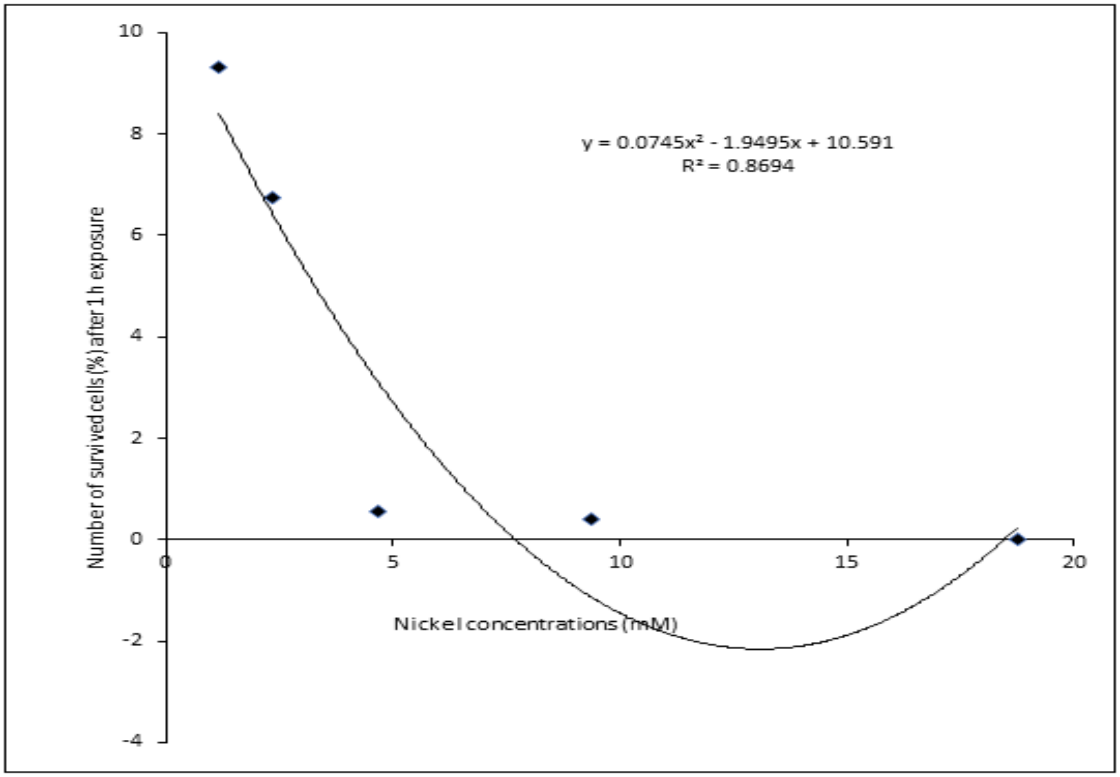


Fig 4:- Quadratic regression line fit plot of percent survivability of *Pseudomonas fluorescens* after a 1-h exposure to various concentrations of nickel

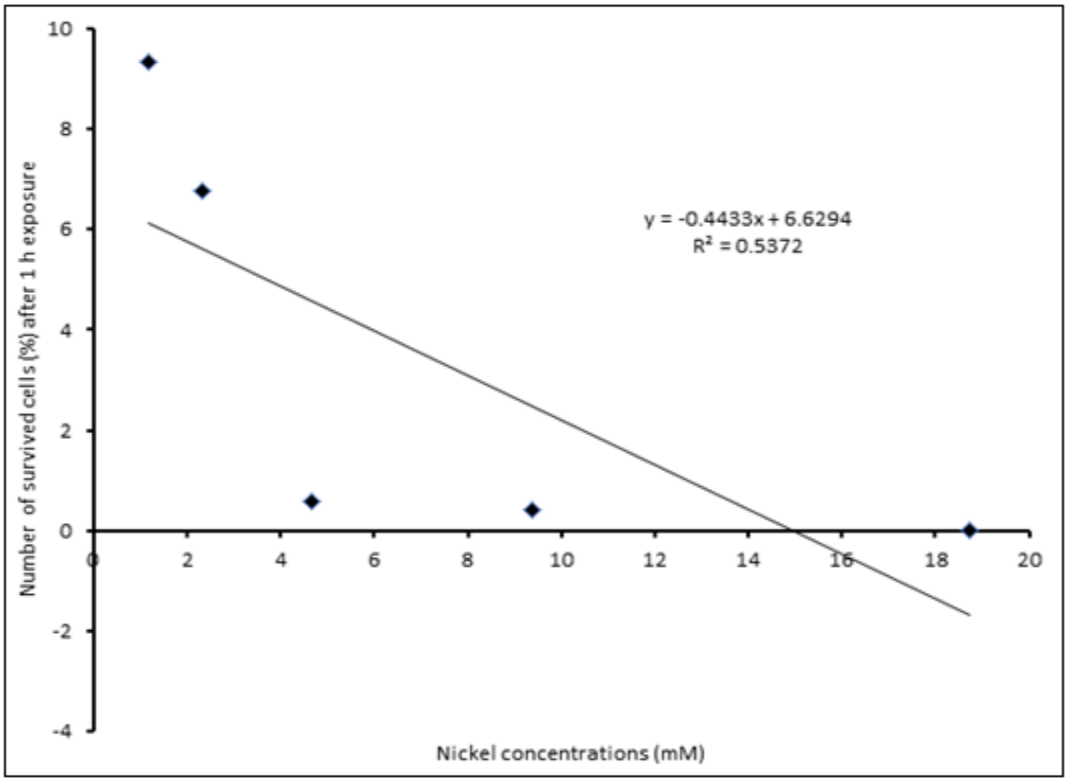


Fig 5:- Linear regression line fit plot showing undeterminable lethal concentration 50 ($LC_{50}$) of nickel to *Pseudomonas fluorescens* after 1 h exposure.

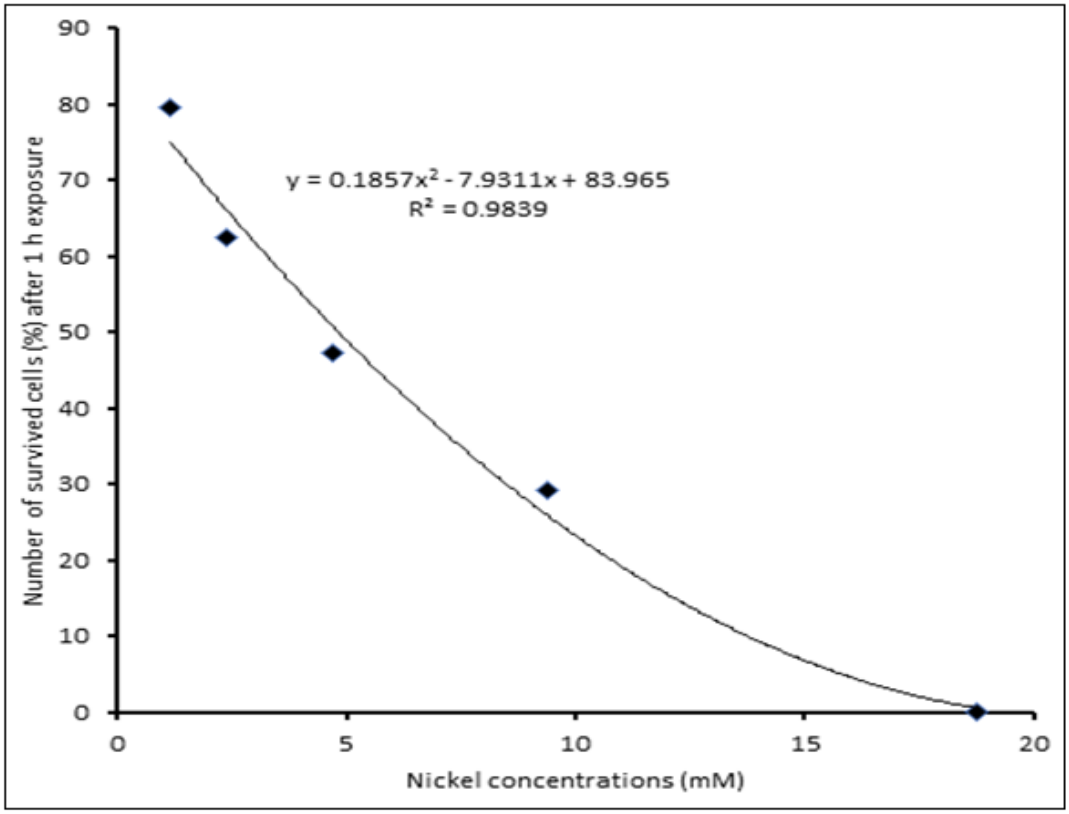


Fig 6:- Quadratic regression line fit plot of percent survivability of *Vibrio fluvialis* after a 1-h exposure to various concentrations of nickel

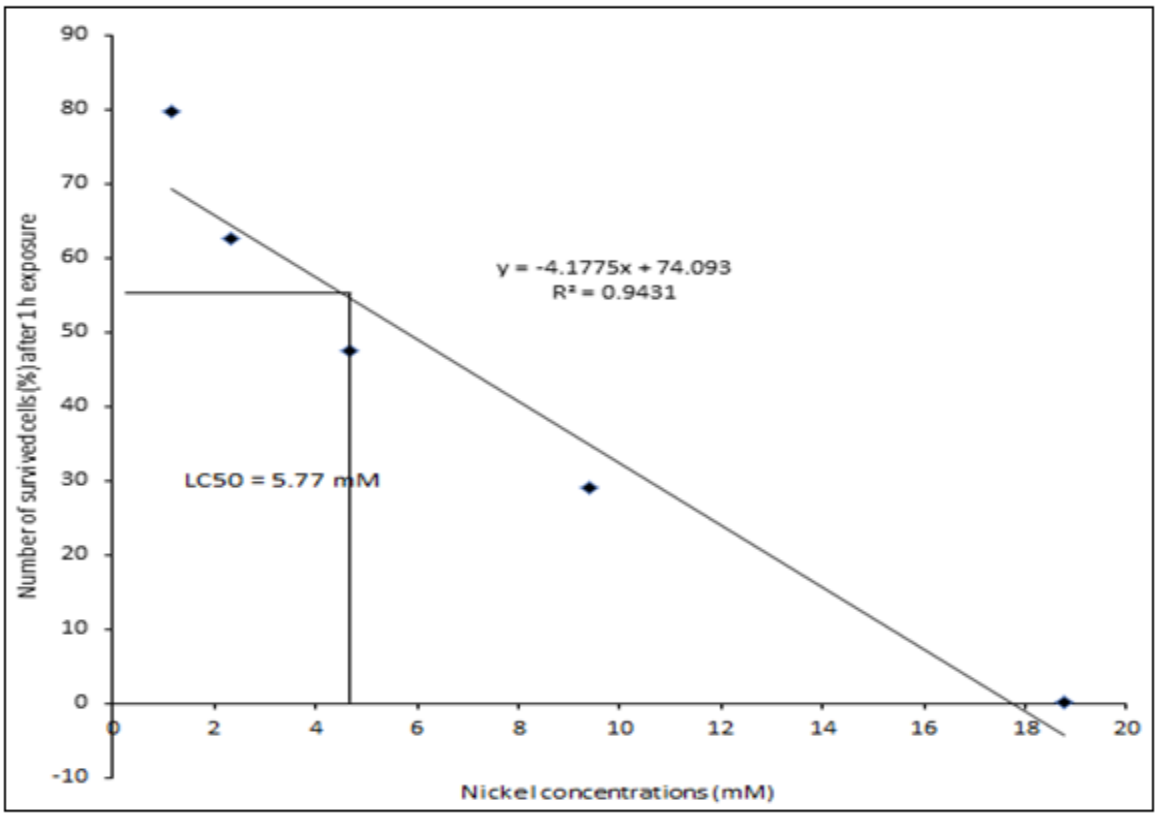


Fig 7:- Linear regression line fit plot showing lethal concentration 50 ($LC_{50}$) of nickel to *Vibrio fluvialis* after 1 h exposure

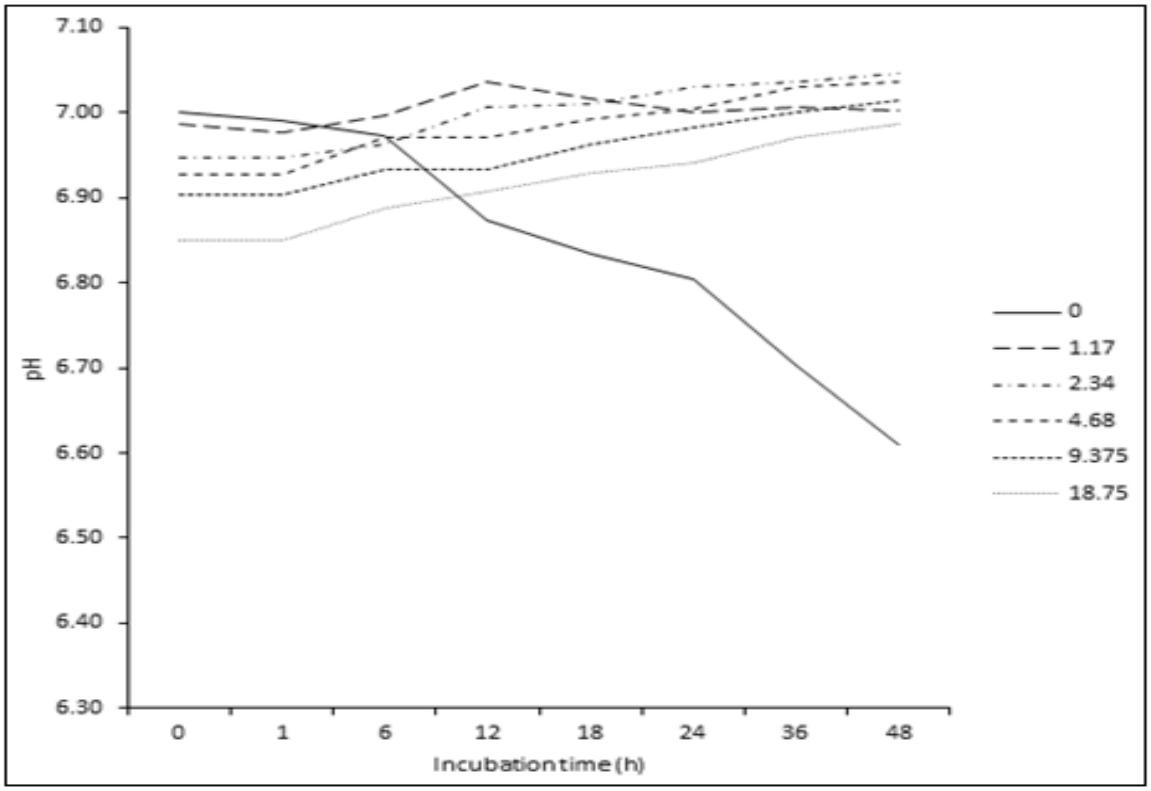


Fig 8:- Effect of nickel on pH dynamics during growth of *Pseudomonas fluorescens* in olive oil minimal medium. Legend indicates nickel concentrations [mM]

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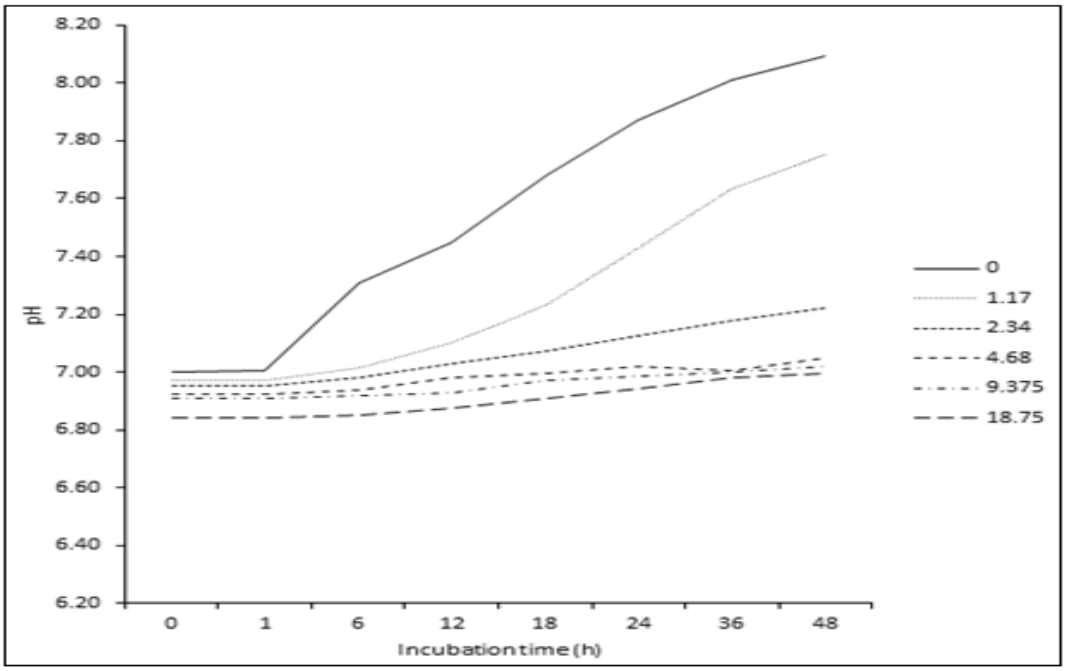


Fig 9:- Effect of nickel on pH dynamics during growth of *Vibrio fluvialis* casein minimal medium.

## IV. DISCUSSION

*P. aeruginosa* is a ubiquitous microorganism that possesses extensive metabolic diversity which enables it to survive in a variety of environments and even hydrocarbon impacted environments [19]. On the other hand, *Vibrio* species on the whole have developed adaptive features that enable them to predominantly thrive in aquatic environment [20]. It is therefore not surprising that both isolates were amongst the crude oil degrader isolated in this study. Bioremediation remains the method of choice in degradation of crude oil hydrocarbons; however, its slow rate is one of its main drawbacks [4]. In addition, the presence of hydrocarbonoclastic microbes, toxic metals in above trace concentrations is also known to affect biodegradation [5]. Several studies have shown that heavy metals in above stimulatory concentrations affect the growth dynamics of bacteria [7,12, 21-24].

Doubling times for bacteria varies in the wild and the laboratory [19, 25]. Gibson *et al* (2017)[25] showed that laboratory doubling time (DT) for *Escherichia coli, P. aeruginosa, Salmonella enteric* and *Vibrio cholera* using accumulation and mutation rates to be 0.33, 0.5, 0.5 and 0.66 hours (20, 30, 30 and 40 minutes), respectively. However, in the presence of Ni in our study, the generation times were 1.55 and 1.56 (h/gen) for *P. fluorescens* and *V. fluvialis* in the absence of nickel. However, as the concentration of Ni increased to 1.17mM, the generation times for both isolates reduced by a factor of atleast two. Other concentrations were toxic to the *Pseudomonas fluorescens* and *Vibrio fluvialis* and their survivability rates decreased with increasing concentrations. In the absence of the metal, the bacterial isolates assume a regular normal growth curve. However, at 1.17mM, the lag phase was longer and there was no stationary phase for both *Pseudomonas fluorescens* and *Vibrio fluvialis.* In an earlier study, nickel, cobalt and zinc induced decrease in the growth of *Pseudomonas* species and mixed microbiota from a waste water treatment plant [24].

The higher observable toxicity of nickel is related to the lower dissociation constant of its complexes [23]. This toxicity was expressed in the non-significant ($p > 0.05$) influence of exposure time on its enzyme biosynthesis and pH fluxes. The metal is a component of many important enzymes especially hydrogenases commonly involved in biological oxidation-reduction reactions [21]. Attempt by cells to take up this metal by the non-specific open gate system described by Nies (1999)[23] results in its intracellular build-up above tolerable limits and a consequent toxicity. Minimum inhibitory concentration (MIC) of nickel to *Escherichia coli* is about 1.0 mM [22], and inhibition concentrations 50 (IC50) for both bacteria could not be determined as they were below the least exposure concentration in the study.

Quadratic regression line fit plot of the percentage survivability gave an $R^2$ value of 0.869 for *Pseudomonas fluorescens* while it was 0.983 for *Vibrio fluvialis.* The LC50 for *Vibrio fluvialis* was 5.77mM but could not be obtained for *P. fluvialis.* Higher pH values (above 6.80) favoured the growth of both isolates even as the concentration of nickel increased. However, in the absence of nickel, *V. fluvialis* grew better at higher pH values and this was in complete contrast to that of *P. fluorescens.*

## V. CONCLUSION

The results of this study suggest that nickel have varying levels of toxicity on the selected hydrocarbonoclastic bacteria in the study. The survival rate for *V. fluvialis* was comparatively higher than that of *P. fluorescens* at all the concentrations of nickel employed. As the concentration increased, there was a gradual reduction in microbial activities as observed in the growth rate reduction and extended lag and death phases by both isolates. The LC50 for *V. fluvialis* was 5.77mM while that of *P. fluorescens* could not be determined. Interestingly, pH values around and above neutral favoured growth of both isolates. Generally, there was marked toxicity displayed by nickel at concentrations ≥ 2.34mM towards both *P. fluorescens* and *V. fluvialis.*

## REFERENCES

[1]. Antai SP. Biodegradation of Bonny Light crude oil by Bacillus sp and Pseudomonas sp. Waste Management. 1990;10:61-64.
[2]. Antai SP, Mgbomo E. Distribution of hydrocarbon utilizing bacteria in the Ahoada Oil-spilled areas. Microbios Letters. 1989; 40:137-143.
[3]. UNEP (2011). *Environmental Assessment of Ogoniland*. *International Standard Book Number* 978-92-807-3130-9 (pp. 1–25).
[4]. Asitok AD, Antai SP, Ekpenyong MG. Water soluble fraction of crude oil uncouples protease biosynthesis and activity in hydrocarbonoclastic bacteria; implications for natural attenuation. International Journal of Sciences. 2017; 6(7).
[5]. Ekpenyong MG, Antai SP. Cadmium toxicity on species of *Bacillus* and *Pseudomonas* during growth on crude oil. Trends in Applied Sciences Research. 2007; 2(2): 115-123.
[6]. Hawkes SJ. What is a 'Heavy Metal', Journal of Chemistry Education. 1997; 74(11): 1374.
[7]. Duffus JH. "Heavy metals" - A meaningless term? (IUPAC technical report). Pure Appl Chem. 2002; 74:793-807.
[8]. Bruins MR, Kapil S, Oehme FW. Microbial resistance to metals in the environment. Ecotoxicol. Environ. Saf. 2000; 45:198-207.
[9]. Gikas P, Sengor SS, Ginn T, Peyton B. The effects of heavy metals and temperature on microbial growth and lag. Global NEST. 2009; 11(3): 325-332.
[10]. Wszkowska J, Boros E, Kucharski J. Effect of interactions between nickel and other heavy metals on the soil microbiological properties. Plant Soil Environ. 2007; 53(12): 544–552.
[11]. David M, Krishna PM, Sangeetha J. Elucidation of impact of heavy metal pollution on soil bacterial growth and extracellular polymeric substances flexibility. 3 Biotech. 2016; 6:172.
[12]. Mbachu AE, Mbachu NA, Chukwura EI. pH-dependent heavy metal toxicity differentials in fungal isolates during biodegradation of spent engine oil. American Journal of Current Microbiology. 2019; 7(1): 1-11.
[13]. Sandri TR, Maier RM. Impact of Metals on the Biodegradation of Organic Pollutants, Environmental Health Perspectives.2003; 111(8).
[14]. Edet UO, Antai SP. Correlation and Distribution of Xenobiotics Genes and Metabolic Activities with level of Total petroleum hydrocarbon in soil, sediment, and estuary water in the Niger Delta. Asian Journal of Biotechnology and Genetic Engineering. 2018; 1(1):1-10.
[15]. Thijsee GJE, van der L. Iso-alkaline oxidation by a Pseudomonas. Antonnie van Leeuwenhoek, 1961; 27:171-179.
[16]. Dutton RJ, Bitton G, Koopman B, Agami O. Effect of environmental toxicants on enzyme biosynthesis: A comparison of β-galactosidase, α-glucosidase and tryptophanase. Archives of Environmental Contamination and Toxicology. 1990; 19:395-398.
[17]. Odokuma LO, Okpokwasili GC. Response of microbial enzymes synthesis to toxicity of weathered and biodegraded oils. Global Journal of Pure and Applied Science. 2003; *9*:465-474.
[18]. Chapin KC, Lauderdale T. Reagents, stains and media: bacteriology. In P. R. Murray, E. J. Baron , J. H. Jorgensen, M. A. Pfaller and R. H. Yolken (Eds.) *Manual of clinical microbiology*. Washington, D.C: ASM Press. 2003; 358.
[19]. Labauve AE, Wargo MJ. Growth and laboratory maintenance of *Pseudomonas aeruginosa*. Current Protocol Microbiology. 2012; doi:10.1002/9780471729259.mc06e01s25.
[20]. Osunla CA, Okoh AI. *Vibrio* Pathogens: A Public Health Concern in Rural Water Resources in Sub-Saharan Africa, International Journal Research and Public Health. 2017; 14(10): 1188.
[21]. Maier T, Jacobi A, Sauter M, Bock A. The product of the *hypB* gene, which is required for nickel incorporation into hydrogenases, is a novel guanine nucleotide-binding protein. Journal of Bacteriology. 1993; 175: 630-635.
[22]. Mergeay, M., Nies, D., Schlegel, H. G., Gertis, J., Charles, P. & van Gijsegam, F. (1985). *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. Journal of Bacteriology. 1985;162, 328-334.
[23]. Nies, D. H. Microbial heavy metal resistance. Applied Microbiology and Biotechnology. 1999; 51:730-750.
[24]. Şengör SS, Barua S, Gikas P, Ginn TR, Peyton B, Sani RK, Spycher NF. Influence of heavy metals on microbial growth kinetics including lag time: Mathematical modeling and 544 experimental verification. Environ. Toxicol. Chem. 2009; 28:2020-2029
[25]. Gibson B, Wilson D, Feil E, Eyre-Walker A. The Distribution of Bacterial Doubling Times in the Wild. 2017; doi: http://dx.doi.org/10.1101/214783.