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Growth Responses of *Pseudomonas fluorescens* and *Vibrio fluvialis* to Trivalent Chromium Toxicity

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Abstract:- Two marine hydrocarbonoclastic bacterial isolates, *Pseudomonas fluorescens* and *Vibrio fluvialis*, obtained from water and sediment samples from Qua Iboe Estuary, Niger Delta, Nigeria were exposed to different concentrations of trivalent chromium (Cr) (0.00, 6.25, 12.50, 25.00, 50.00, 100.00 and 200.00 mM) and a control (0.00mM). After exposure, the isolates were monitored for effect of Cr on growth and LC50 also determined using a linear regression line fit plot. The generation times for *P. fluorescens and V. fluvialis* increased from 1.55 to 6.24 and 1.56 to 6.93 (h/gen), respectively as Cr concentration increased from 0.00 to 12.50mM. In contrast, the growth rates decreased from 0.447 to 0.111 and 0.443 to 0.100 (h^{-1}) for *P. fluorescens and V. fluvialis*, respectively. At concentrations ≥ 25mM, the generation times and growth rates for both isolates dropped to zero. Control assay showed a short lag phase and extended exponential phase, however, Cr concentrations of 6.25 and 12.50mM showed a longer acclimatization and short exponential phases of growth for both isolates. Percentage survivability after one 1h of exposure showed 0% of survived cells for both isolates at concentrations of 100 and 200 mM. Quadratic regression line fit plot of survivability for both isolates gave R^2 values of 0.763 and 0.905, respectively for *P. fluorescens and V. fluvialis.* Linear regression line fit plot gave LC50 values of 80.70 and 45.85 for *P. fluorescens and V. fluvialis*, respectively. Cr concentrations 100mM and above were very toxic to the test isolates in this study.

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

I. INTRODUCTION

The Niger Delta region of Nigeria is one of the most fragile ecosystems in the world due to high levels hydrocarbon pollution [1-7]. Crude oil pollution is often associated with heavy metal pollution [8]. Given the ability of hydrocarbonoclastic microorganisms to utilize crude oil hydrocarbons as a source of carbon, bioremediation remain the method of choice in cleaning up crude oil spillages [9-10]. Several studies have been carried that examined the effect of various factors on biodegradation of crude oil hydrocarbon [9-12]. The process of biodegradation is negatively affected by the presence of heavy metals in toxic or above stimulatory concentrations [13-15].

Chromium (Cr) is a toxic non-essential metallic element found in the transition series of the periodic table [15-16]. As a metal, it occurs in the environment in oxidation states of zero (as a metal), +3 (trivalent chromium), and +6 (hexavalent chromium) and possess different chemical properties [17-18]. The trivalent state is usually associated with the formation of stable complexes with organic and inorganic ligands while the hexavalent form exists as strongly oxidizing species (CrO_4^{2-}) [16]. Cr^{6+} is well known for its toxicity to humans [17], plants [19] and microorganisms with consequent alteration of microbial composition and function [17]. In humans, it causes cancer and even irritation [20-21]. The toxicity of Cr^{6+} is well studied and is due to its ability to induce oxidative stress in both eukaryotic and prokaryotic cells [22-23]. Several studies have shown that bacteria isolates such as species of *Pseudomonas*, *Microbacterium*, and *Bacillus* are capable of reducing Cr^{6+} to Cr^{3+} [18][25-27].

On the other hand, it is generally believed that Cr^{3+} is not toxic due to its less solubility and impermeability to cells [17]. However, it is toxic to plants [19], and *Euglena gracilis* [27]. Furthermore, it is capable of being transformed back to Cr^{6+} in presence of excess oxygen [19]. Given that Cr^{3+} is the end product of Cr^{6+} microbial reduction, there is a potential for Cr^{3+} to build up in the environment in concentrations that is above stimulatory. The primary aim of this study was to evaluate the toxicity of trivalent chromium on the growth response of marine hydrocarbonoclastic isolates.

II. MATERIALS AND METHODS

➢ *Site Description and Sample Collection*

Crude oil hydrocarbon impacted water and sediment were collected from Okposo and Utan Iyatah estuaries as previously described (Asitok et al., 2017; Edet et al., 2018a; Edet et al., 2018b). These communities are located on Qua Iboe Estuary of the Niger Delta region of Nigeria. The coordinates of the sampling point were $4^0$35' 14.9'N, 008^0 17' 0.16E and $4^0$40' 49.6'N, 008^o17 45.4'E for Okposo and Utan Iyatah sampling points, respectively. The collected samples were then pooled into a total of four composite samples as reported previously (Asitok *et al.*, 2017). The pooled samples were then transported to the laboratory within one hour of collection for further analyses.

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➢ *Sample Preparation and Microbiological Analyses*

Preparation of samples and all microbiological analyses were done using previously reported standard methodologies [4]. Briefly, the pooled samples were then used to carry out ten-fold serial dilution. For the sediment samples, 10g of each sample were dissolved in 90ml of sterile distilled water. For the water sample, 10ml was measured and dissolved in 90ml of sterile distilled water. From each of the samples, 3 sets of dilutions were selected and plated onto freshly plated tryptic soy agar (TSA) and incubated at 30^0C for 24 - 36 hours. Resulting discrete colonies were then purified via repeated sub-culturing and then stored using agar slopes at 4^0C for further use.

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

The purified isolates were further screened for their ability to degrade crude oil. This was done using the vapour phase method pioneered by Thijsee and Van der Linden (1961)[28] and recently reported by Asitok et al (2017)[4]. Approximately, 0.4 ml of the collected Qua Iboe light crude oil was used to uniformly soak one Whatman No 1 filter paper. After soaking, the paper was gently placed on the inside of a petri dish lid and the lid placed gently on the petri dish previously seeded with pure culture of the test isolates on mineral salt medium. The medium was then supplemented with streptomycin and nystatin (250mg/ml) to inhibit fungal growth and then incubated for 48 hours. Resulting colonies resulting colonies were regarded as hydrocarbonoclastic bacteria.

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

Two isolates were selected for morphological and biochemical characterization. This was done usng Microgen ID Kit (Microgen Bioproduct Limited, UK) together with their identification software. In addition, molecular characterization was done using 16s rRNA previously reported [4].. The selected isolates were identified as *Pseudomonas fluorescens* and *Vibrio fluvialis.*

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

The method of Dutton *et al.* (1990)[29] also adopted by Odokuma and Okpokwasili (2003)[30] was used 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 first cultured in separate nutrient broth tubes for 18 h at room temperature. After growing the cells for 18 hrs, in nutrient broth the cells were then diluted out until an absorbance reading of 0.2 at wavelength of 550nm (A_{550nm}) was obtained. The resulting cells were then washed twice using sterile deionized water and standardized using McFarland [31]. One millilitre (1ml) of the washed bacterial cells was exposed to nine millilitre (9ml) of the various concentrations of chromium (6.250, 12.50, 25.00, 50.00, 100.00 and 200mM), and control (0.00) contained 9 ml of deionized distilled water without addition of any chromium. All treatments and controls were conducted in triplicate for each bacteria isolate. Immediately after exposing the washed cells to various concentrations of Cr, 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 chromium concentrations.

III. DATA ANALYSIS

Effect of chromium on growth was analysed by plotting log number of cells against incubation time using a semi-log plot and analysis of variance (ANOVA). The replicate data obtained for changes in pH as the concentration of chromium increased was subjected to x – y graph and also ANOVA. For the estimation of the LC50 and R^2 values for both isolates, a linear regression line fit plot was plotted using the number of survived cells after one hour of exposure against chromium concentration. P values < 0.05 were considered significant.

IV. RESULTS

Results of the experiments investigating the effects of various concentrations of trivalent chromium on the growth of *Pseudomonas fluorescens* OWLB1 and *Vibrio fluvialis* OWPB63 are reported in Table 1. The table shows that growth rates of the bacteria decreased with increasing concentrations of the metal. Analysis of variance of the data in the table reveals no significant ($p > 0.05$) difference in the toxicant effects between the two bacteria.

Figure 1 reveals that the acclimation period of *Pseudomonas fluorescens* OWLB1 in olive oil minimal medium was extended from 1 h in the control medium to 6 h and 12 h in media containing chromium concentrations of 6.25 and 12.5 mM. Chromium concentrations above 25 mM did not permit growth of the bacterium. The figure reveals that stationary phase of growth was reached between 18 and 24 h in both control and the test media that permitted growth of the bacterium, but while log-transformed number of survived cells in the control was as high as 21.776, those in the test media were 16.101 and 13.710 for chromium concentrations of 6.25 and 12.5 mM respectively. Analysis of variance revealed that both time and toxicant concentrations significantly ($p < 0.05$) influenced the responses of *Pseudomonas fluorescens* to chromium exposure. Results of periodic exposure of *Vibrio fluvialis* to various concentrations of chromium are presented in Figure 2. The results are very similar to the one presented for *Pseudomonas fluorescens* above in terms of acclimation periods, time to reach stationary phase and the concentrations of the toxicant not permitting growth. Analysis of variance result also revealed that time and toxicant concentrations significantly ($p < 0.05$) influenced the responses of the bacterial population to the toxicant. An appropriate comparison of the response patterns of the two bacteria to chromium intoxication is presented in Figure 3. The figure shows that chromium concentration of 50 mM

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supported growth of *Pseudomonas fluorescens* with a percent number of survived cells of 98.95 % at 1 h. the corresponding response of *Vibrio fluvialis* as presented in the figure was 8.89 %.

The results of regression analyses of influence of chromium concentrations on percent number of survived cells are presented in Figures 4, 5, 6 and 7. Figure 4 is a quadratic regression line fit plot of chromium concentrations against percent number of survived cells of *Pseudomonas fluorescens* OWLB1 and presents an R^2 value of 0.7638. This value differs from the one obtained by the linear regression plot of 0.724 in Figure 5. This figure also shows that the lethal concentration 50 (LC50) of chromium is 80.70 mM. Figures 6 and 7 are respectively the quadratic and linear regression line fit plots of chromium concentrations against percent number of survived cells of *Vibro fluvialis* OWPB63. Figure 6 shows an R^2 value of 0.9059 while Figure 7 presents an R^2 value of 0.6281 with an LC50 value of 45.85 mM.

Figure 8 presents the pH dynamics of medium containing *Pseudomonas fluorescens* under the influence of various concentrations of chromium. The figure shows that initial exposure of a population of the bacterium to chromium concentration of 200 mM reduced the pH from 7.00 in the control to 4.05. The pH reductions in media with chromium concentrations lower than 200 mM were not as significant. While the pH reductions in the control and lower concentrations of chromium were near neutrality at the end of the study period (48 h), however, at 200 mM, the pH of the test medium dropped to 3.18. Analysis of variance revealed that both time and concentration made significant ($p = 0.000$) contributions to the pH responses. Figure 9 shows that initial exposure of *Vibrio fluvialis* cells to chromium concentrations less than 200 mM slightly reduced medium pH from 7.00. Incubation of bacteria for 48 h resulted in the increase of the pH to slightly alkaline values with chromium concentration 6.25 mM producing a pH of 7.77. Quite significantly medium pH with chromium concentration of 200 mM which was initially (0 h) 4.00 only increased to 4.35 at the end of the study. Similarly, analysis of variance revealed that exposure time and toxicant concentrations were significantly ($p = 0.000$) responsible for pH dynamics in the test media with chromium concentration.

	Percent survival at 1 h (%)		Growth rate (h^{-1})		Generation time (h/gen)	
Chromium conc. (mM)	*Pseudomonas fluorescens*	*Vibrio fluvialis*	*Pseudomonas fluorescens*	*Vibrio fluvialis*	*Pseudomonas fluorescens*	*Vibrio fluvialis*
0	0	0	0.447	0.443	1.55	1.56
6.25	91.1	82.1	0.21	0.21	3.3	3.3
12.5	81.67	78.95	0.111	0.1	6.24	6.93
25	75.53	76.84	0	0	0	0
50	98.95	8.89	0	0	0	0
100	0	0	0	0	0	0
200	0	0	0	0	0	0

Table 1:- Effect of Chromium on Growth of Bacteria

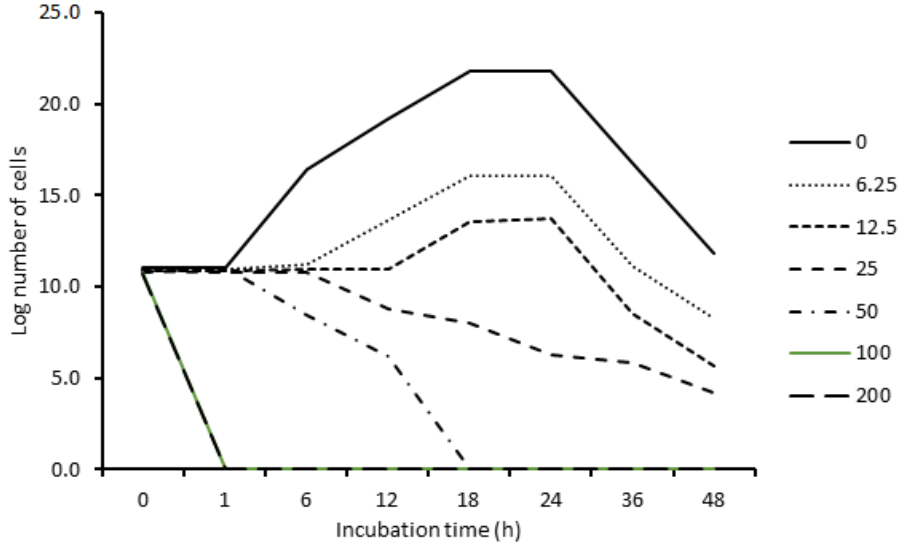

Fig 1:- Effect of Chromium on Growth of *Pseudomonas fluorescens* OWLB1 (Legend Indicates Chromium Concentrations [%])

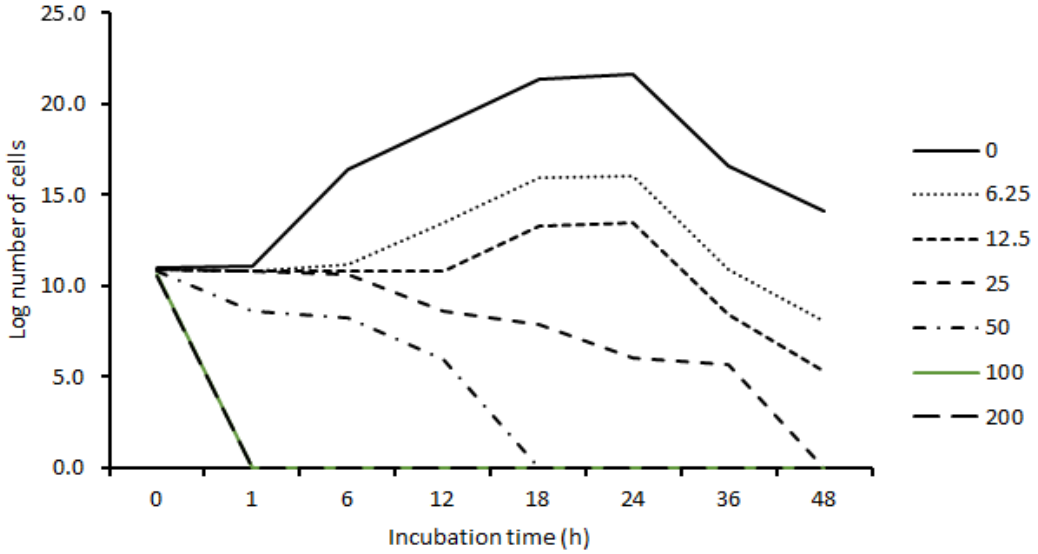

Fig 2:- Effect of Chromium on Growth of *Vibrio fluvialis* OWPB63

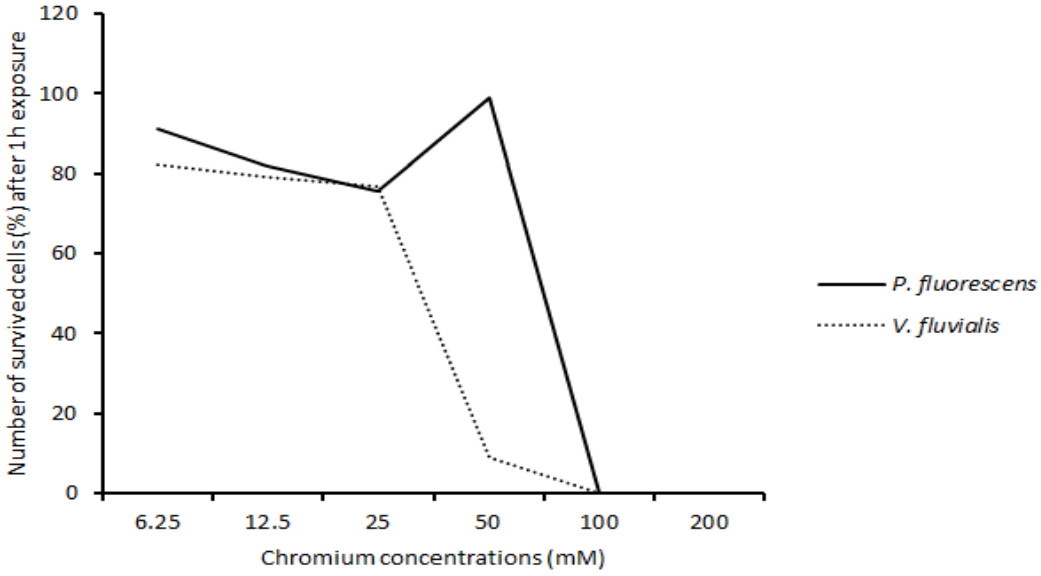

Fig 3:- Comparative Percent Survivabilities of *Pseudomonas fluorescens* and *Vibrio fluvialis* over a 1-h Exposure to Varying Concentrations of Chromium.

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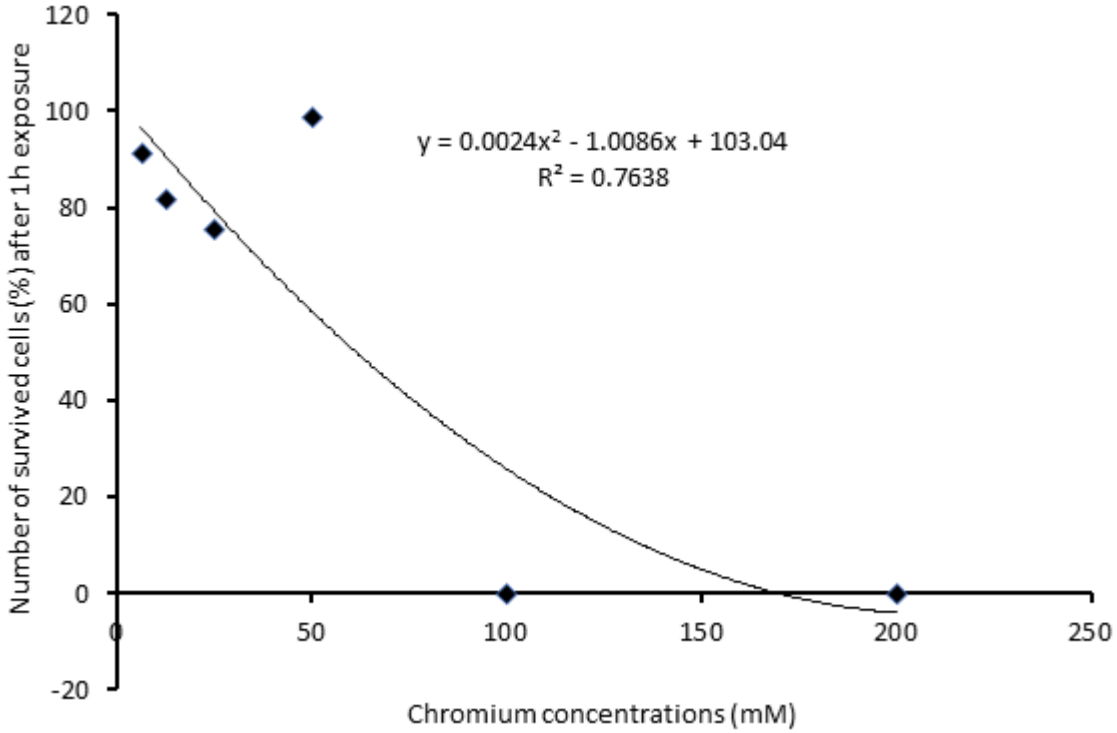

Fig 4:- Quadratic Regression Line Fit Plot of Percent Survivability of *Pseudomonas fluorescens* OWLB1 after a 1-h Exposure to Various Concentrations of Chromium

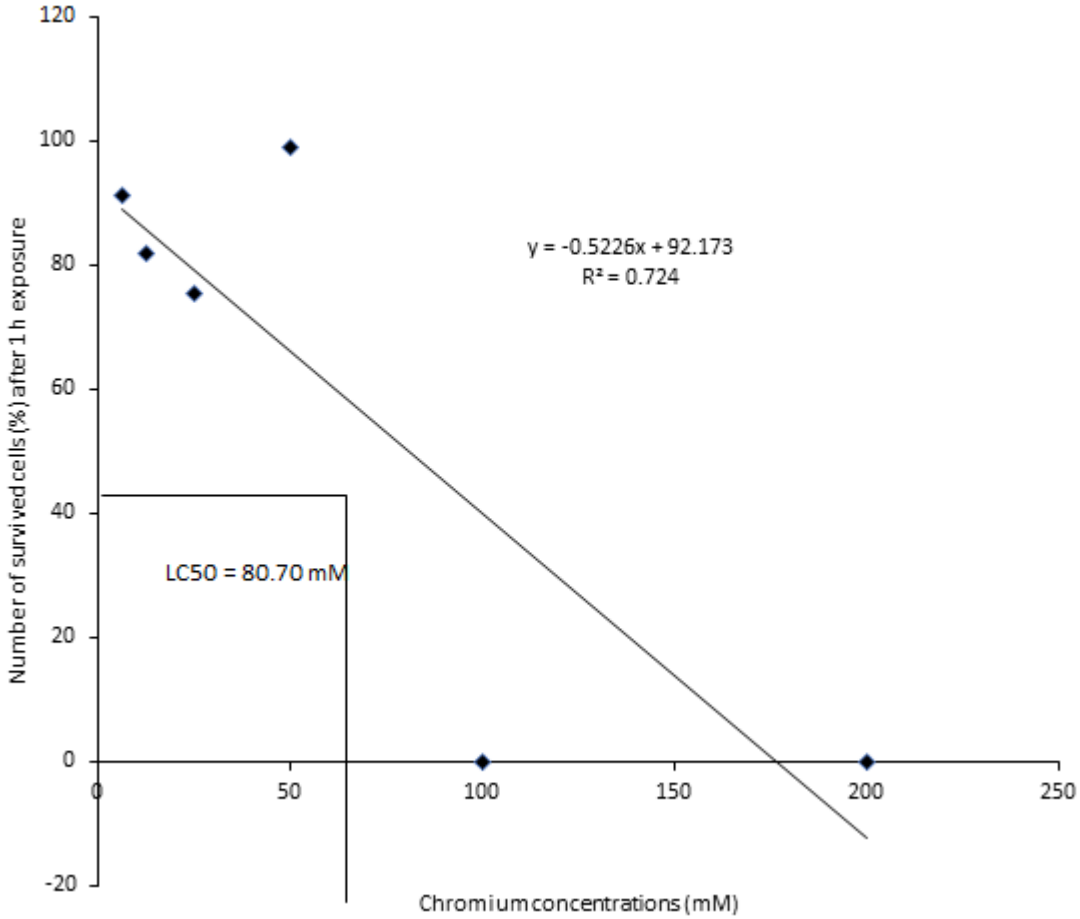

Fig 5:- Linear Regression Line Fit Plot Showing Lethal Concentration 50 (LC_{50}) of Chromium to *Pseudomonas fluorescens* OWLB1 After 1 h Exposure

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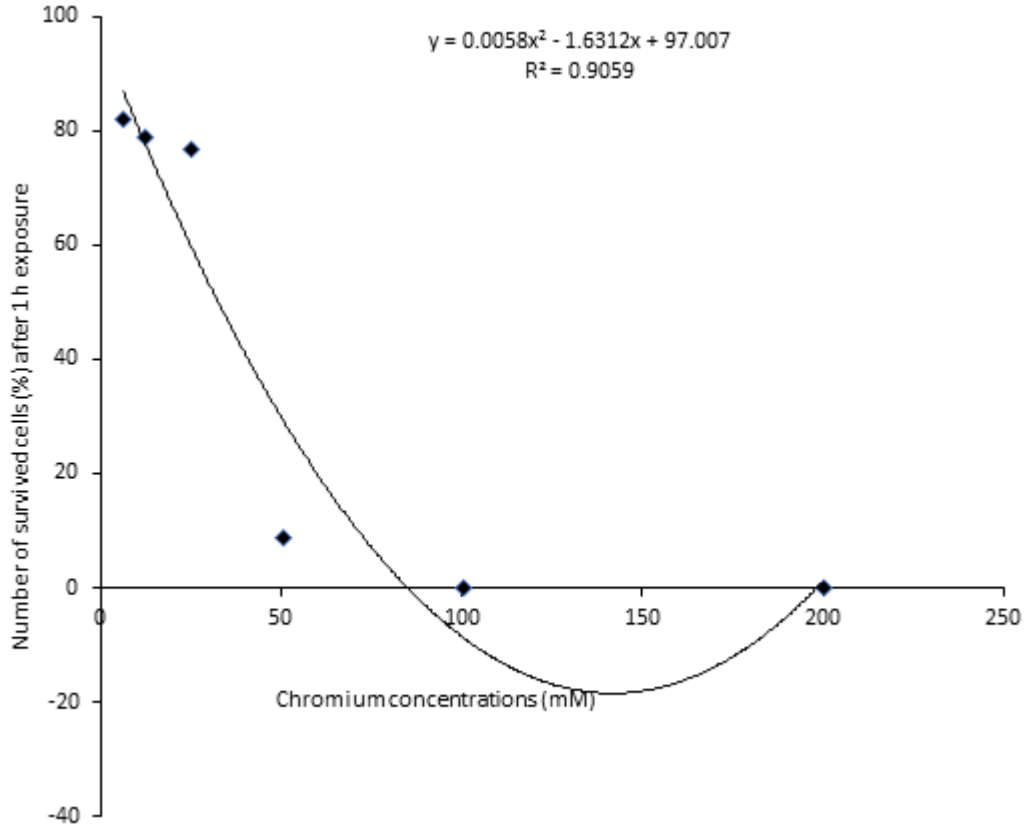

Fig 6:- Quadratic Regression Line Fit Plot of Percent Survivability of *Vibrio fluvialis* OWPB63 after a 1-h Exposure to Various Concentrations of Chromium

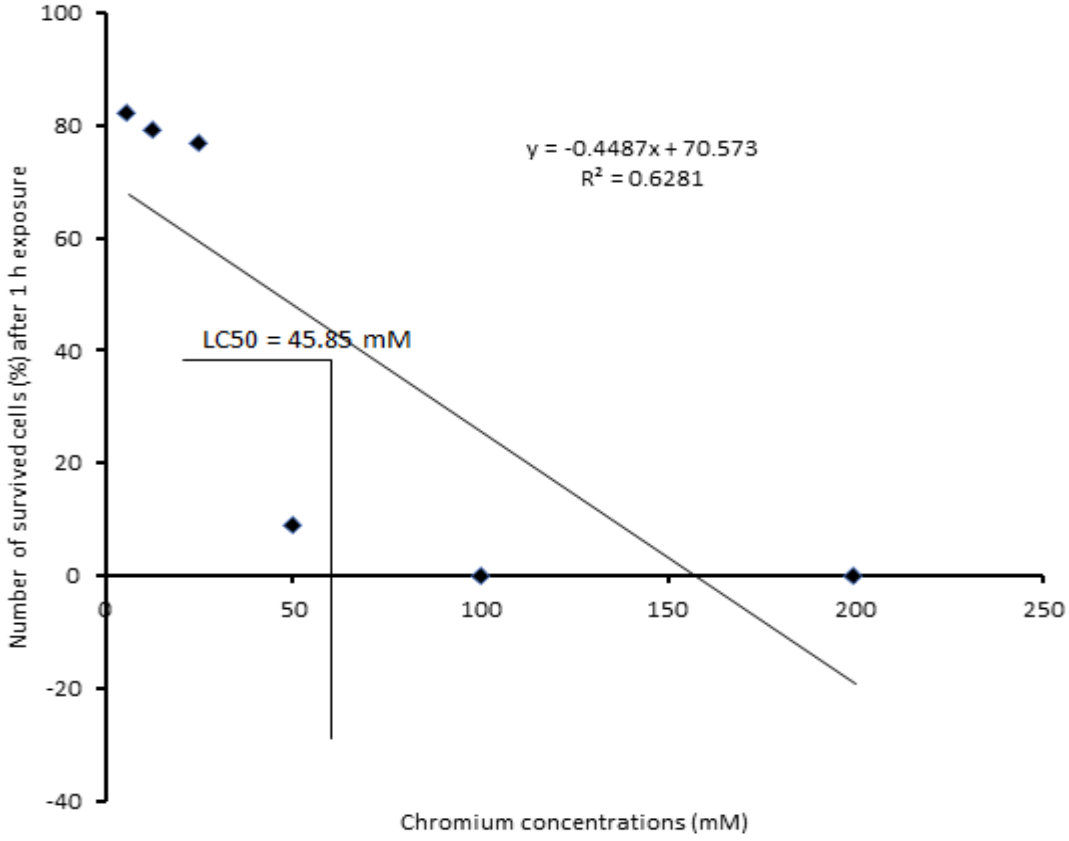

Fig 7:- Linear regression line fit plot showing lethal concentration 50 (LC_{50}) of chromium to *Vibrio fluvialis* OWPB63 after 1 h exposure

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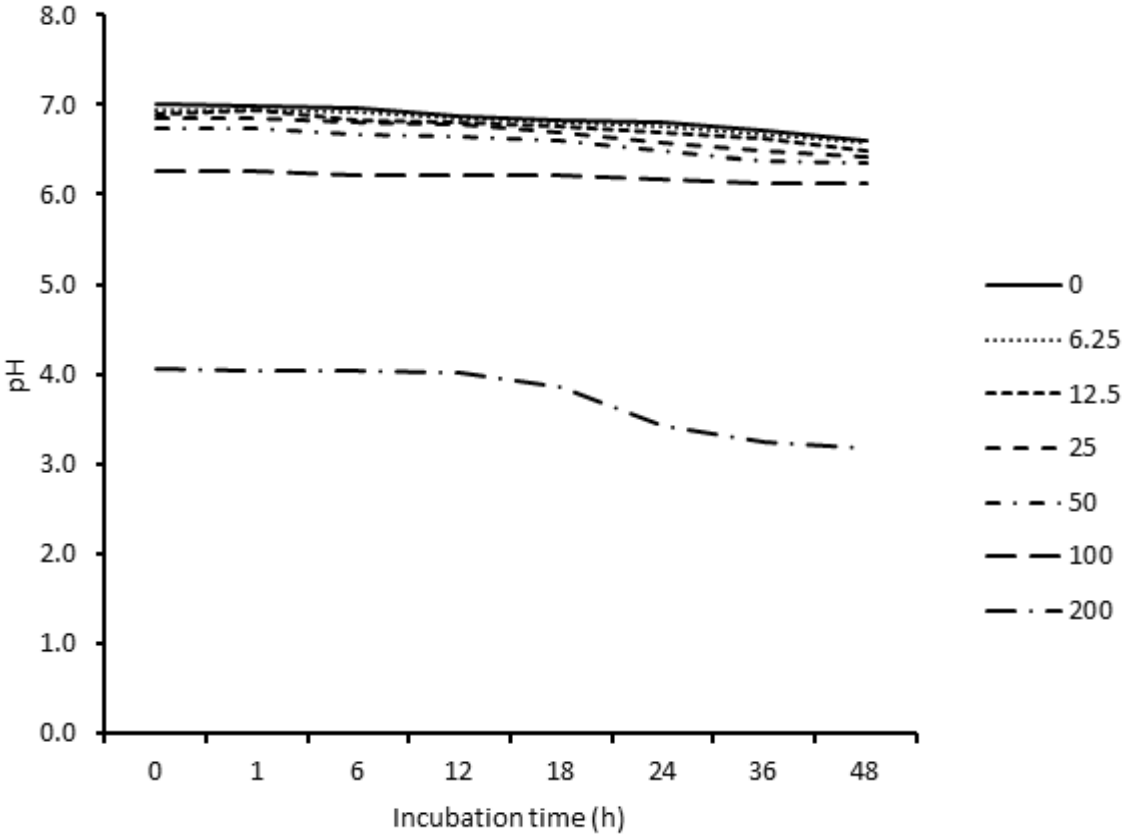

Fig 8:- Effect of Chromium on pH Dynamics During Growth of *Pseudomonas fluorescens* OWLB1 in Olive Oil Minimal Medium. Legend Indicates Chromium Concentrations [mM].

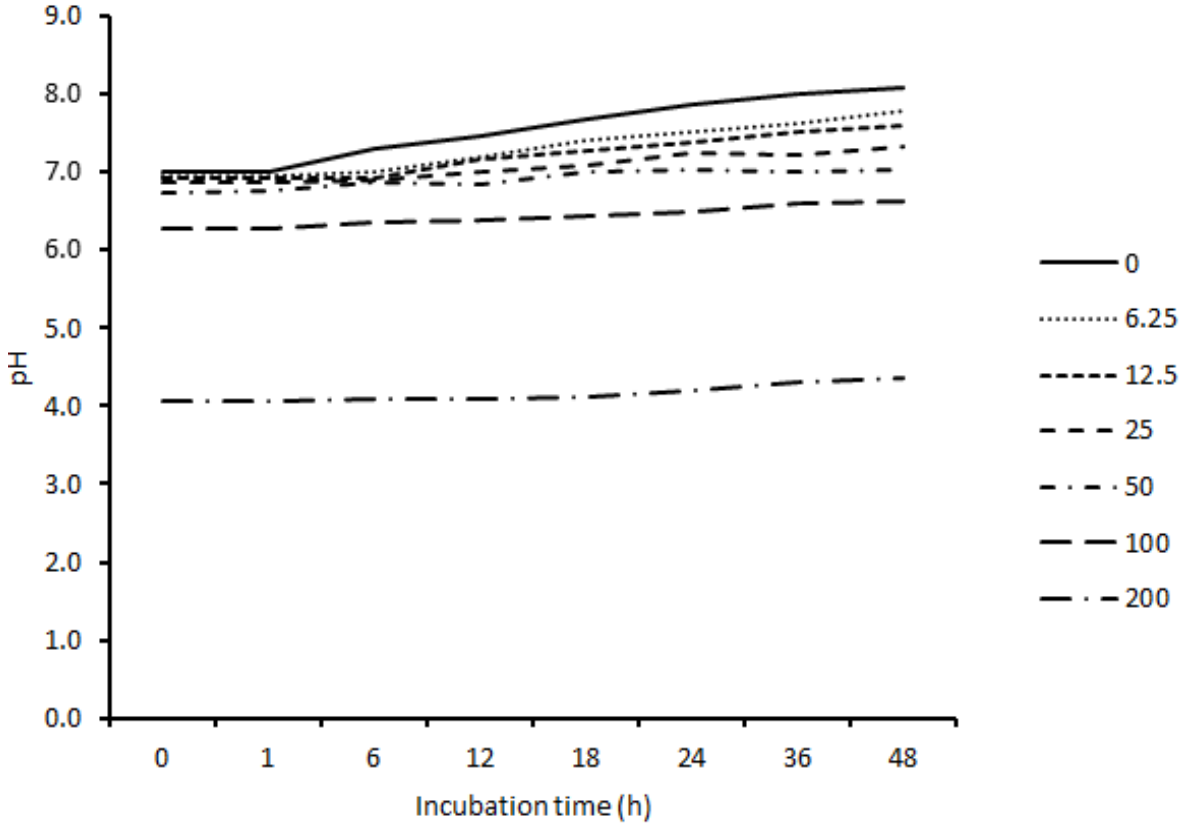

Fig 9:- Effect of Chromium on pH Dynamics during Growth of *Vibrio fluvialis* OWPB63 in Casein Minimal Medium. Legend Indicates Chromium Concentrations [mM]

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V. DISCUSSION

The results of the influence of chromium on the growth parameters of the test bacteria showed that chromium concentrations above 12.5 % did not permit growth of the test bacteria, However, exposure time played a significant ($p < 0.05$) role in the determination of its toxic effect. Acclimation period of *Pseudomonas fluorescens* OWLB1 in olive oil minimal medium was extended from 1 h in the control medium to 6 h and 12 h in media containing chromium concentrations of 6.25 and 12.5 mM. Chromium concentrations above 25 mM did not permit growth of the bacterium. Quite significantly, chromium concentration of 50 mM increased the number of survived cells higher than that of the control. This observation suggests that this concentration of trivalent chromium might be necessary for triggering the formation of metalloproteins necessary for metal immobilization thus reducing toxicity and permitting growth [12]. It is also possible that by reason of the nutritional significance of chromium in this oxidation state, their presence increases the activity of relevant enzymes active in protein synthesis. Stationary phase of growth was reached between 18 and 24 h in both control and the test media that permitted growth of the bacterium. Analysis of variance revealed that both time and toxicant concentrations significantly ($p < 0.05$) influenced the responses of *Pseudomonas fluorescens* to chromium exposure. The results of regression analysis of data of influences of chromium *on Pseudomonas fluorescens* present an R^2 value of 0.7638 in the quadratic model. This value differs from the one obtained by the linear regression plot of 0.724 which gives the lethal concentration 50 (LC50) of chromium as 80.70 mM. These results suggest that the linear regression model alone was able to explain 72.4 % of the growth responses of the bacterium in relation with chromium concentrations and that 27.4 % of the responses may be due to other causes presumably exposure time and pH reductions.

Reports on the enhancing influence of pH on metal toxicity to bacteria have been well documented [12,32]. Both reports working on cadmium suggested that low pH increases cadmium toxicity in particular, or heavy metals in general, because at low pH, the concentration of dissociated or free metal ions increases, thus increasing the rate of uptake by test bacteria. By concentration-dependent killing, the toxic metal interacts optimally with biological targets and uncouples relevant physiological functions or induces a general oxidative stress in aerobic bacteria, such as the one considered in this study.

The lower coefficient of determination, R^2 value of 0.6281 obtained by linear regression of chromium concentrations against growth of *Vibrio fluvialis* OWPB63 and a much higher one (0.9059) obtained by quadratic line fit plot suggest that chromium was more toxic to this bacterium than to *Pseudomonas fluorescens*. This is further confirmed by the lower LC50 value of 45.85 mM. The results also suggest that 90.59 % of the growth inhibition responses of *Vibrio fluvialis* were due to chromium intoxication and that the responses were only marginally linear.

While the pH reductions in the control and lower concentrations of chromium in *Pseudomonas fluorescens* test media were near neutrality at the end of the study period, that in the 200 mM test medium dropped to 3.18. Analysis of variance of data revealed that both time and metal concentration made significant ($p = 0.000$) contributions to the pH responses but toxicant concentration mediated by far the greater part of the toxic effect. This result confirms the earlier suggestion of pH-enhanced chromium toxicity but indicate that the pH enhancement is concentration-dependent since low chromium concentrations did not significantly influence growth of the bacterium. The high LC50 value confirms this.

The initial exposure of *Vibrio fluvialis* cells to chromium concentrations less than 200 mM resulted in reductions in initial medium pH. Incubation of bacteria for 48 h resulted in the increase of the pH to slightly alkaline values with chromium concentration 6.25 mM producing a pH of 7.77. This pH increase above neutrality is presumably a result of production of alkaline protease by the bacterium. Production of alkaline protease by species of *Vibrio* especially *Vibrio mimicus* has earlier been reported [33]. In their report, optimal protease was attained at pH 9.0. In our earlier study, *Vibrio fluvialis* strain OWPB63 produces alkaline protease at pH 8.09 [4]. Quite significantly medium pH with chromium concentration of 200 mM which was initially (0 h) 4.00 only increased to 4.35 at the end of the study. Analysis of variance revealed that exposure time and toxicant concentrations were significantly ($p = 0.000$) responsible for pH dynamics in the test media with chromium concentration.

VI. CONCLUSION

The results of this study suggest that chromium exert toxicity on the selected hydrocarbonoclastic bacteria that was time and concentration dependent. *P. fluorescens* survival rate was higher and better than that of *V. fluvialis* with concentrations of 50mM and less. As the concentrations increased, a gradual decrease in microbial activities as seen in the reduced growth rates, extended acclimatization period and short exponential phases were observed. The LC50 for *V. fluvialis* was 45.85mM while that of *P. fluorescens* was 80.70mM. Interestingly, pH values between 4.0 and neutral appeared to favour growth of both isolates at higher concentrations of chromium. Chromium although is a non-essential heavy metal, our findings suggest that higher concentration > 50mM is associated with microbial toxicity.

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