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EFFECT OF CONCENTRATION OF GROWTH MEDIA ON CADMIUM TOXICITY TO ISOLATED RIVER WATER BACTERIA (*Bacillus* species).

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Abstract

The effect of concentration of growth media on cadmium toxicity to isolated river water oligotrophic *Bacillus* species was done *in vitro* using dehydrogenase assay. This was done in sterile nutrient broth of different strengths ranging from normal strength NB (representing eutrophic condition), and nutritionally deficient nutrient broth strength of NB/40, NB/100, NB/200, NB/500, NB/1000, and NB/10000 (representing oligotrophic conditions). The different concentration ranges of the metal (cadmium) were utilized ranging from 0.002mM to 0.2mM. The results obtained were based on visual observations of color intensities from a spectrophotometric reading after 24 hours incubation for different concentrations. Cadmium exposure showed that media dilution strength of NB/40 was consistent in toxicity trend with increasing cadmium concentrations and was found to represent a true oligotroph best suited for the dehydrogenase assay of metal toxicity to *Bacillus* species.

Key word: Oligotroph, Eutroph, *Bacillus* species, Dehydrogenase assay and Spectrophotometer.

Introduction

The river environment is complex and constantly changing, often contains low nutrient concentrations (oligotrophic environment) and exposes microbes to many over-lapping gradients of nutrients and other environmental factors including chemicals such as metals, phenols, herbicides and pesticides. The growth of microorganisms depends on nutrient availability, microbial response to prevailing environmental conditions and nature as well as concentration of available toxicants. Microorganisms have been found to be sensitive to different types of pollutants, antibiotics, xenobiotics, poly aromatic hydrocarbons (PAHs) and heavy metals [13]; [16]. These investigations have shown that the numbers and the diversity of bacterial communities can decrease as a response to toxic pollutant and the remaining bacterial communities can have genetic and physiological characteristics differing from reference communities. The toxicity of a metal depends on the metal

itself, its total concentration and bioavailability to the particular organism, as well as the organism itself [14].

A major challenge for ecotoxicologists is the demonstration of ecological effects and causes of contamination in field situations. This is due to the incapability of establishing direct causal relationships between the presence of specific toxic compounds and changes observed in the field using ecological or ecotox method. This hampers extensive application of many ecotoxicological risk assessments. Measurement of microbial dehydrogenase enzymes as a measure of total biological activities might be a useful approach for risk assessment because in microbial respiration, the dehydrogenase participates in the transport of electrons from substrate to final electron acceptor in an electron transport system [10]. In addition, microbial dehydrogenases are intracellular, rapidly degraded following cell death and are common throughout microbial species [10]. Dehydrogenase enzymes are also specific to target substrates and are more rapid and less costly [5]. Dehydrogenase assay therefore is a promising tool for assessing the toxicity effects of metals on microbial community under different nutrient conditions.

Materials and methods

Isolation and screening for test organisms:

The microorganism used for the assay was isolated from Otammiri river (5^0 $28^1$$40^{11}$N, $7^0$$2^1$$34^{11}$E), a highly impacted aquatic environment which flows through Owerri Municipality in Imo State, Nigeria. The water sample was collected as described by [12] and transferred to the laboratory within one (1) hour of collection.

Isolation was carried out using spread plate technique as described by [15]. The medium used was nutritionally deficient agar medium (NA/100), prepared by diluting nutrient broth (Lab M) than the usual manufacturer's guide for the growth of fastidious organisms. Oligotrophs are able to grow in nutritionally deficient media and even in distilled water, their rate of growth is slower and several days may be required for them to reach confluence. This was solidified with 5g of agar No.1 (Lab M) and poured in sterile petri-dishes. Ten micro-litres (0.01ml) of the homogenized river water sample were spread-inoculated into the sterile nutritionally deficient plate (NA/100), and incubated at room temperature of (28 ± 2^0C) for 2days. The bacterial colonies were purified by sub-culturing several times onto freshly prepared nutritionally deficient nutrient agar plates (NA/100). Purified isolates were further screened for oligotrophy.

The purified bacterial river water isolates were screened for oligotrophy as described by [15]. Screening was done by sub-culturing the purified isolates onto agar media of decreasing strengths-4%w/v agar and then 2%w/v agar in distilled water. Thereafter, the isolates that survived were transferred from a 100-fold (NB/100) nutrient broth medium of strength to a deficient nutrient broth NB/10,000. After each transfer, cultured plates were incubated at room temperature (28 ± 2^0C) for two (2) days on a laboratory bench. The bacterial isolates with true oligotrophic strains were confirmed routinely in quarter strength nutritionally deficient liquid medium (NB/10,000 dilutions) and were maintained in the same medium throughout the experiments. True oligotrophic strains were identified using the method as described in [2]. These isolates were also maintained in nutritionally deficient agar (NA/100) slants stored at 4^0C and served as the stock for toxicity test.

Preparation and standardization of oligotrophic inoculums

The confirmed river water oligotrophic inoculums were prepared and standardized as follows: A loopful of cells from the stock culture was inoculated into 25ml sterile deficient nutrient broth (NB/40) contained in 100ml Erlenmeyer flask. The flask was incubated at room temperature (28 ± 2^0C) with intermittent shaking on a rotary incubator at 150rpm for 22hrs which corresponded to the mid exponential phase of the culture. Thereafter, cells were harvested by centrifugation (4000rpm, 10minutes). The harvested cells were washed twice in 10ml sterile distilled water by centrifugation (4000rpm for 10mins) and re-suspended in 10ml sterile distilled water. Re-suspended cells were standardized spectrophotometrically by diluting with sterile water to obtain an OD of 0.1 at 540nm.

Preparation of different strengths of growth media.

Sterile nutrient broth of different strengths ranging from Normal strength NB, (representing eutrophic condition), and nutritionally deficient nutrient broth strengths of NB/40, NB/100, NB/200, NB/500, NB/1000 and NB/10,000 (representing oligotrophic conditions) were utilized for the assay. Normal strength growth medium was prepared according to manufacturer's instructions by dissolving 13g of nutrient broth powder (Lab M) into 1000ml of distilled water. 0.5ml of stock concentrations of nutritionally deficient nutrient broth strengths of NB/10, NB/25, NB/50, NB/125, NB/250 and NB/2500 were prepared and used to obtain nutritionally deficient Nutrient broth strengths of NB/40,

NB/100, NB/200, NB/500, NB/1000, and NB/10000 respectively, in 2ml final volume made up with distilled water contained in 10ml test tubes. (See appendix 1 for protocol of nutrient dilutions).

Preparation of metal (cadmium)

Stock concentration (10mM) of the metal (cadmium) was prepared by dissolving exactly 0.257g of cadmium salt ($CdSO_4.H_2O$) in distilled water contained in 100ml volumetric flask and were made up to the 100ml mark with sterile distilled water. Other concentrations were obtained by further diluting the stock concentration (10mM) in sterile distilled water to obtain 0.002mM, 0.02mM and 0.2mM using the formula; $C_1V_1=C_2V_2$. From the stock solution, 0.002ml, 0.02ml and 0.2ml were respectively transferred into 9.998ml, 9.98ml and 9.8ml of distilled water contained in 20ml test tube. These gave metal concentrations of 0.002mM, 0.02mM and 0.2mM respectively.

Toxicity Assay

The toxicity of the metals to the organism was assayed using dehydrogenase activity as the end point as described by [9]. The toxicity assay was done in a 2ml reaction volume contained in 10ml screw-capped test tubes. The reaction consisted of standardized inocula (0.1 OD), graded concentrations of cadmium salt [0.2mM, 0.02mM, 0.002mM, 0.0mM (control)], nutrient broth of varying concentrations (NB, NB/40, NB/100, NB/200, NB/500, NB/1000, and NB/10000) and distilled water in 2ml final volumes contained in 10ml test tubes. The protocol for the experiment is shown in appendix 1. The tubes were incubated at room temperature of (28 ± 2^0C) for 24hr on a laboratory bench. Thereafter, 0.1ml of 0.2% w/v INT in deionized distilled water was added to each of the tubes and homogenized. The reaction mixtures were further incubated statically in the dark at room temperature (28 ± 2^0C) for another 24hr after which the reaction was stopped by addition of 1% (v/v) Triton $X\text{-}_{100}$ and the mixtures were allowed to stand for 5mins. The amount of triphenyl formazan produced (TPF) was determined based on visual observations of color intensity after extraction with 4ml of amyl alcohol after 24hr. The color intensities were graded as (+++), (++), (+), (-) respectively representing heavy growth, medium growth, light growth, and no growth observations using INT as indicators. The intensity of the red color formazan formed is directly proportional to the enzymatic activity present in the tube.

Result

The results of the biochemical characteristics and the effects of media concentration on *Bacillus* species are shown in Table 1 and 2 respectively. The results were however obtained based on visual observation of color intensity and the spectrophotometric analysis after 24hr incubation. The results of the morphological and biochemical characteristics of the oligotrophic isolate are shown on Table 1. The organism was a gram positive, motile, oval, and non capsulated bacteria with catalase and methyl red positive that has the ability to ferment glucose and xylose. The organism was tentatively identified as *Bacillus* species according to [2].

The effects of media concentration on *Bacillus* species are shown in Table 2. In the absence of the metal (control), the organism grew in all the media dilutions and at the normal concentration of the nutrient broth; there was growth observation for all cadmium concentrations. At media dilution of NB/500 and above, no growth of organism was observed for different concentrations of cadmium exposure. At 40-fold media dilution strength (NB/40), there was consistency in toxicity trend with increasing cadmium concentrations and was chosen to be best suitable for the dehydrogenase assay following the consistent pattern of toxicity of increasing cadmium concentrations to *Bacillus species*. The color intensities; (+++), (++), (+), (-) are respectively representing heavy growth, medium growth, light growth, and no growth observed when INT was used as indicator. With the result obtained in this study using *Bacillus* species (oligotrophic strains), it is a prove that toxic metals may be detected using dehydrogenase assay as *Bacillus* species (oligotrophic strains) are susceptibility to heavy metals.

Table 1: Morphological and biochemical characteristics of oligotrophic isolate.

TESTS	RESULTS
Cell Morphology and Microscopic Characteristics	
Cell Morphology: Creamy, raised, slimy colonies on Nutrient agar	
Motility	+
Gram Stain	+
Spore (Oval and Central)	+
Flagellum	+
Capsule	–
Biochemical and Carbohydrate Fermentation Characteristics	
Catalase	+
Oxidase	–
Coagulase	–
Indole	–
Methyl Red	+
Voges Proskaeur	–
Citrate	–

Hydrogen Sulphide Utilization	Nd
Nitrate Reduction	–
Urease	–
Triple Sugar Iron	Nd
Glucose Utilization	+
Sucrose	–
Arabinose	–
Lactose	–
Mannose	–
Xylose	+

Key: (+) Positive test, (-) Negative test, (Nd) Not done.

Table 2: Effects of concentration of growth media on response of *Bacillus* species to cadmium exposure

Media dilutions	Contr	0.002mM	0.02mM	0.2mM
Normal concentration of nutrient broth (NB)	+++	+++	++	++
NB/40	+++	++	+	-
NB/100	+++	++	-	-
NB/200	++	+	-	-
NB/500	++	-	-	-
NB/1000	+	-	-	-
NB/10000	+	-	-	-

The color intensities; (+++), (++), (+), (-) are respectively representing heavy growth, medium growth, light growth, and no growth.

Discussion

This study demonstrated the toxic effects of heavy metals to *Bacillus* species of Otammiri river water isolates under nutrient deficient (induced oligotrophic) conditions. Bacterial isolates of river water, sediments and soil have been reported by [11]; [10]; and [14] for the study of metal toxicities.

The effects of the media strengths (NB, NB/40, NB/100, NB/200, NB/500, NB/1000 and NB/10000) were monitored by both visual observations and spectrophotometrically; and were compared by the effects on the toxicity of cadmium (Cd^{2+}) concentrations of the ranges of 0.002-0.2mM. Reaction mixture (distilled water + microbial inocula + nutrient broths of normal strength) samples without cadmium components and 10000-fold (NB/10000) of nutrient broth without cadmium ions served as control. In a similar research carried out by [11], *Bacillus* species was proved suitable for study of metal toxicity at exponential phase under nutrient deficient conditions. [14] also studied metal toxicities using oligotrophic bacterial strains of bacteria namely *Sphingomonas paucimobilis* KPS01, *Burkholderia cepacia* KPC01 and KPC02 isolated from soils for biological monitoring of heavy metals.

The results of the studies revealed an increase in the toxicity pattern along the horizontal rows, and this was observed for all the treatments which must have resulted due to the addition of appropriate metal concentrations. Also, the increase in the toxicity trend across the vertical columns obtained corresponded to an increase in the metal toxicities as the media concentrations reduces up to 10000-fold as reported by [14]. The *Bacillus* species used in this study was found to be more susceptible to heavy metal under oligotrophic than feasting condition. This greater susceptibility may be due to their need to efficiently take up a wide range of nutrient in order to be able to survive in nutritionally deficient environment. This observation is supported by the work of [14] which indicated that the susceptibility of oligotrophs to heavy metals was dependent on the amount of nutrients available. Metals added to ordinary media may interact with media components and cause significant effects on assay systems. However, the nutritionally deficient medium NB/40 used in this study as induced oligotrophic condition could prevent such effects because of the low amount of interfering components in the medium. *Bacillus* species however was found to be highly susceptible to heavy metals and showed a high potential for the assessment of metal toxicity. Previous authors have

reported on this phenomenon by different organisms [7]; [8]; [1]. They reported that low pH (media compositions) affects the network or chemistry of cell wall as well as physico-chemistry and toxicity of heavy metals.

Conclusion

The results of this study have shown potential toxicity of the heavy metals studied using *Bacillus* species under a deficient 40-fold nutrient broth medium (NB/40). The dose response relationships of metal ion (Cd^{2+}) and dehydrogenase activity of *Bacillus* species studied was different in various treatment options of media concentrations with varying concentrations of the media strengths. The observed increase in toxicities of metal (cadmium) at high concentrations when applied singly is consistent with many reported cases of microbial response to single metal exposure [6]; [4]; [10].

Thus, the microbial biomass is a sensitive parameter and has shown positive responses as an indicator of changes in organic matter composition as observed by [3]. There is need therefore, to consider the test organism, medium for bio-assays to further investigate the toxicity of pollutants; and it is wise to underline that the biological activities of particular heavy metal can be compared only between identical systems; otherwise false conclusions may be derived.

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Appendix

Appendix 1: Protocol for media dilutions

Stock dilution (ml)	**NB/2.5**	**NB/10**	**NB/25**	**NB/50**	**NB/125**	**NB/250**	**NB/2500**
Vol. of nutrient broth (ml)	**80**	**20**	**8**	**4**	**1.6**	**0.8**	**0.08**
Vol. of distilled water (ml)	120	180	192	196	198.4	199.2	199.92
Final vol. (ml)	200	200	200	200	200	200	200
Dilution in culture tube (ml)	NB/10	NB/40	NB/100	NB/200	NB/500	NB/1000	NB/10,000