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Impact assessment of cement industry activities on soil nitrogen-fixing bacteria

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Abstract

Nitrogen fixing bacteria play a vital role in transforming atmospheric nitrogen into inorganic form easily available for plant use. However, environmental pollution due to effluent from industrial activities could accumulate in soil, significantly altering the soil chemical and microbiological characteristics, such as nitrogen fixing bacteria. The effect of cement dust on nitrogen-fixing bacteria is presented in this study. Homogenized soil samples 0-20 cm in depth from the cement factory and other communities 200m-2km away from the factory were evaluated for soil physicochemical properties and nitrogen-fixing bacterial bioload using standard procedures. Soil pH, total nitrate, and total phosphate decreased as distance increased away from the factory. Elevated conductivity values of 605.94-621.80(µs/cm) was recorded for soil samples from the factory, indicating the presence of higher dissolved solutes. Total culturable *Azotobacter* count increased as distance increased away from the factory lcation, with SR1 (Akinbo) recording the highest of 10^4-10^5 cfu/g, while *Azospirillium* and *Clostridium* count significantly reduced. Pollution due to cement production activities may not have had a significant negative effect on the bioload of nitrogen-fixing bacteria. A higher amount of nitrate in soil samples around the cement plant showed that nitrogen-fixing activities occurred at a lower rate compared to the locations farther away from the cement plant.

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Introduction

Nitrogen accounts for the highest amount of atmospheric gas; however, nitrogen gas is absent in the soil's parent material (Sundaraj, 2004). The availability of nitrogen in the soil is mostly dependent on the degradation of organic matter, the application of synthetic fertilizers, and the activities of nitrogen-fixing bacteria through the nitrogenase enzymes (Mlitan *et al.,* 2013). Nitrogen is a common soil nutrient element required in large quantities by plants. Many plant species in diverse habitats usually contain 1-3% nitrogen on a dry weight basis. The growth of higher plants in many ecosystems is limited by the nitrogen supply (Ijah and Antai, 2003). Nitrogen-fixing bacteria have the capacity to transform atmospheric nitrogen into inorganic compounds that can be used by plants (Bilen, 2010). They carry out this activity by encoding an enzyme, nitrogenase, that converts nitrogen gas to ammonia (NH_3) (Majolagbe *et al.,* 2013; Frederick *et al.,* 2014). The activities of nitrogen-fixing bacteria are very important because they ensure the reduction in the use of nitrogen fertilizers in plant agriculture. The spread of dust and gases such as sulphur dioxide, and nitrogen dioxide have been the leading consequence of cement production in the environment (Amani *et al*., 2018; Sandar *et al.,* 2019). These dust particles are spread over large areas through wind and rain with subsequent accumulation in soil and plants (Aneja, 2003; Bilen, 2010; Orji *et al.,* 2016). Significant changes in pH and accumulation of emitted metals in the soil as a result of dust from cement production and other industrial activities may affect the composition and physiological processes in microorganisms and enzymatic activity (Biyik *et al*., 2005; Stanley *et al*., 2014). Nitrogen-fixing bacteria (NFB) are part of the bacterial communities in the soil with a very important role in oxidizing ammonia to nitrate, contributing significantly to soil health and fertility. This process of biological nitrogen fixation (BNF) is critical in the sustainable crop production system, as it accounts for 65% of nitrogen utilized in agriculture (Matiru and Dakora, 2004). Important biochemical reactions of BNF occur mainly through the symbiotic association of N_2-fixing microorganisms with legumes that convert atmospheric elemental nitrogen (N_2) into ammonia (NH_3) (Matiru and Dakora, 2004). Rhizobia (species *of Rhizobium, Mesorhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium, and Sinorhizobium*) form intimate symbiotic relationships with legumes by responding chemotactically to flavonoid molecules released as signals by the legume host. The bacteria that change nitrogen gas from the atmosphere into soluble nitrogen usable by plants are Nitrogen Fixing Bacteria (NFB). They convert nitrogen gases from the atmosphere into nitrogen compounds that are utilized by plants in the soil. More than 90% of all nitrogen fixation is attributed to the NFB; hence they play an important role in the nitrogen cycle and general soil fertility. The effect of cement dust pollution negatively impacts soil fertility as toxic heavy metals are released into the soil, bringing about changes in the physicochemical properties of soil and inhibiting the activities of the soil fertility enzymes and microorganisms responsible for the nitrogen cycle (Ibanga *et al.,* 2008; Frederik *et al.,* 2013). This study reports changes in the nitrogen-fixing bacteria bioload in soil due to pollution by cement production.

Materials and methods

Sampling area and sample collection

The study was carried out with samples from communities around the Lafarge Cement production plant, Ewekoro Local Government Area, Southwestern Nigeria. The cement factory is 5 km north of Ewekoro (Hussein *et al*., 2017) with a coordinate of latitude 6°55′59.99″N and longitude 3°12′60.00″N. The soil samples were collected from Akinbo, SR1 (200 m from the cement factory) and Egbado SR2 (2 km from the cement factory) and locations around the cement-producing community of Ewekoro SR3, using augers at 0-10 and 10-20 cm depths respectively. Soil samples from Egbado SR2 were considered as the control sample since the location is the farthest away from the cement plant. The soil samples were randomly collected in triplicates at each sampling site and mixed together representing a composite sample for each sampling site.

Determination of total heterotrophic bacterial count

The total heterotrophic bacterial count present in the soil samples was determined using the standard dilution plate count technique as described by Amani *et al.* (2018). Ten grams of soil samples were introduced into a conical flask and 100 mL of sterile saline was aseptically added.

This was vigorously agitated for 20 minutes. The aliquot (0.1 mL) of the various dilutions was spread on the surface of solidified sterile nutrient agar in Petri dishes. The Petri dishes were incubated for 24-48 hours at 28°C in a bacteriological incubator.

Determination of total nitrogen-fixing bacteria

Jensen's Medium was used for the enumeration and isolation of NFB. Other solid agar media used were *Azospirillum* (Hurst *et al*., 2000) and the *Azotobacter* medium.

The *Azospirilium* medium comprised of malic acid (5 g/L), dipotassium hydrogen phosphate (0.5 g/L), ferrous sulphate (0.5 g/L), manganese sulphate (0.1 g/L), magnesium sulphate (0.1 g/L), sodium chloride (0.1 g/L), bromothymol blue (0.002 g/L), calcium hydroxide (4 g/L). *Azotobacter* medium, previously reported by Onyenze *et al.* (2013), was prepared using the following formulation; dipotassium phosphate (1 g/L), ferrous sulphate (5 g/L), soil extract (5 g/L), mannitol (2 g/L), sodium chloride (0.2 g/L), and agar (15 g/L). *Clostridium* medium was compounded using the following formulation; potassium dihydrogen phosphate (1.5 g/L), disodium hydrogen phosphate (4 g/L), ammonium chloride (0.5 g/L), magnesium chloride (0.18 g/L), calcium chloride dehydrate (2.4 g/L), zinc sulphate (2.8 g/L), resazurin (indicator) (2.0 µg/L), biotin (5.02 µg/L), thiamine (20 µg/L), folic acid (20 µg/L), sucrose (20 g/L), cysteine (2 µg/L). Individual nitrogen-fixing bacteria were isolated by the spread plate method on a solid medium. Discrete colonies formed were sub-cultured on nutrient agar, and the pure cultures obtained were subjected to Gram stain reaction and different biochemical tests for identification (Chikere *et al.,* 2009; Nwachukwu *et al*., 2001).

Determination of soil pH and soil conductivity

The pH of the samples was determined using a digital pH meter (ADWA 3015, United Kingdom). The previously standardized electrode of the pH meter was dipped into the diluted soil sample, swirled gently, and allowed to stand until a stable/constant reading was obtained. At each point, three values were obtained and the mean of the values was used. Five (5) grams of the soil sample were diluted in 10 mL distilled water and swirled gently for proper distribution. The electrode of a conductivity meter was dipped into the diluted soil, and reading was noted immediately after a constant value appeared. The redox potential electrode was dipped into the same sample solution to determine redox potential. Three (3) values were obtained at each point and the mean of the triplicates was used as real values.

Determination of total nitrogen content

The total nitrogen in the soil was determined by Macrokjeldahl digestion and distillation method. Twenty-five (25) grams of air-dried soil samples were weighed in duplicates on a filter paper and placed in a dry 500 ml Kjedahl flask. A mixture of copper sulphate, selenium, and sodium sulphate was added as a mixture of catalysts, and thereafter 30 ml of concentrated tetraoxosulphate (vi) acid was added. This was swirled while being gently heated until a grey colour developed. The hot mixture was cooled, 100 mL of tap water was added, and the entire mixture was transferred to a conical flask. Sodium hydroxide (10 mL) was then added, liberated ammonia was collected in a boric acid solution, and distillation was carried out. The reaction mixture containing catalysts and tetraoxosulphate (vi) acid was titrated against 0.05N hydrochloric acid and the point where the grey colour turned blue was considered the endpoint.

Determination of total phosphorus content

The method used was the colorimetric method as described in United Nations Environmental Programme (UNEP, 2004). Fifty milliliters (50 mL) sample solution was pipetted into a clean conical flask. This aliquot was autoclaved with $K_2S_2O_8$ and

H_2SO_4 for 30 minutes at 121^{0}C. Five milliliters (5 mL) of ammonium molydbate was added to the autoclaved mixture to form heteropolymolybdophosphoric acid and was reduced with stanneous chlorides in an aqueous sulphuric acid medium at 30^{0}C to form a molybdenum blue complex. The resulting blue colour was measured spectrometrically at 660 nm and compared to identically prepared standard (water). The detection limit of this method is 0.005 mg/l/kg (UNEP, 2004).

Determination of percentage total organic carbon (% TOC)

Total organic carbon is an alternative analytical method for measuring petroleum hydrocarbons using the wet oxidation technique, as previously reported by Nelson and Sommers (1975). A 0.1 molar concentration of the sample slurry was pipetted into a clean pyrex conical flask. Five milliliter (5 mL) potassium dichromate ($K_2Cr_2O_7$) solution and 7.5 mL concentrated sulphuric acid was added. The mixture was heated on an electro-thermal heater for 15 minutes to reflux. The sample was cooled to room temperature and diluted to 100 ml with distilled water. 25 ml of the sample solution was titrated with standard ferrous ammonium sulphate using ferion as an indicator. A blank containing oxidant and sulphuric acid was titrated as in the sample and the titre values were recorded.

Results and discussion

The physico-chemical indices of the soil under investigation showed that the pH of SR1 and the control, SR2, were slightly acidic, while soil samples from SR3 were slightly alkaline between 7.5-7.8 (Table 1).

Table 1. Physico-chemical properties of soil samples.

Soil Sample	Depth (cm)	pH	Conduc-tivity (µs/cm)	Redox potential	Total Nitrate	Total phosphate	Total Organic Carbon (%)
SR1 Akinbo	0 - 10	6.50	315.67	35.7	26.19	19.86	10.47
	10 - 20	6.70	320.12	11.6	27.80	19.20	10.46
SR2 Egbado (Control)	0 -10	6.50	418.26	12.95	24.60	10.00	8.21
	10 -20	6.28	425.17	20.60	18.50	10.08	8.07
SR3 Ewekoro	0 - 10	7.50	621.80	83.59	42.96	26.27	10.00

This result agrees with the observations of Lamare and Singh (2020) and Soladoye *et al.* (2020). In their report, the authors observed that the pH values of soil around cement-producing communities were generally alkaline, tending toward neutral as the distance from the factory increased. A study by Jain and Jain (2006) also reported a moderate decrease in soil pH as the distance from the cement plants increased. Soil pH can affect enzyme activity by influencing the concentration of inhibitors or activators in the soil solution and the effective concentration of the substrate. It controls the growth and metabolic activities of non-symbiotic microorganisms in the soil (Merlo and Susana, 2014).

The conductivity level of the soil samples studies revealed that the samples from SR3 had the highest conductivity of 605.94 µS/cm-621.60 µS/cm (Table 1). Amos *et al.,* 2015 made similar observations of elevated conductivities in soil samples within the Ashaka Cement factory area. The elevated conductivity values strongly indicate the presence of higher dissolved solute (excess salts), which may hinder plant growth as a result of loss of equilibrium in soil and water balance (Lamare and Singh 2020).

The total nitrate concentrations in SR1 (Akinbo) and SR2 (control) were between 18-50 mg/kg – 27.80 mg/kg of soil, and this is far below the nitrate concentration in SR3, 42.96 mg/kg. Ajon and Chagbe (2018) cited a similar trend of low nitrogen with increasing distance from the cement plant in their studies on the soil chemical property of Dangote Cement, Gboko.

Table 2. Bacterial counts of various groups of microorganisms isolated from the soil samples.

Soil	Depth (cm)	Heterotrophic Bacterial Count (Cfu/g)	Culturable *Azospirilium* Count (Cfu/g)	Culturable *Azotobacter* Count (Cfu/g)	Culturable *Clostridium* Count (Cfu/g)	Culturable NFB Count (Cfu/g)
SR1 Akinbo	0 - 10	1.20×10^9	5.9×10^6	6.6×10^4	$5.1x\ 10^3$	5.3×10^6
	10-20	2.45×10^7	8.2×10^6	5.6×10^5	1.08×10^4	7.5×10^7
SR2 Egbado	0 –10	2.17×10^7	6.7×10^4	$5.1. \times 10^4$	8.6×10^3	5.7×10^5
	10-20	1.82×10^5	4.6×10^4	1.4×10^4	6.5×10^3	2.6×10^5
SR3 Factory	0 –10	1.49×10^9	6.5×10^5	9.3×10^3	2.40×10^4	3.0×10^5
	10-20	1.69×10^7	6.8×10^5	1.28×10^2	9.6×10^5	5.5×10^6

Legend: NFBC: Nitrogen Fixing Bacteria.

The low nitrate concentration in SR1 (Akinbo) and SR2 (Control) could be due to agricultural activities and high organic matter decomposition (Periaswamy *et al.*, 1983) leading to the rapid absorption of nitrate by plants. Plants absorb nitrate in the assimilation process which is later converted into nitrogen-containing molecules for their growth.

Phosphate concentration was lowest for the Control, SR2 with 10.00 mg/kg, while SR3 had the highest phosphate concentration of 26.27 mg/kg (Table 1). Khamparia *et al.* (2012) reported a decrease in soil phosphate level with increasing distance away from the cement plant. Phosphate is the naturally occurring form of phosphoric acid; it contains phosphorus that stores and transfers energy in the plant for growth. It is required for the metabolic activities of non-symbiotic nitrogen fixers and also acts as a catalyst for nitrogen fixation (Lawal and Yusuf, 2021). Its availability in soil depends on the pH and the form present in the soil.

A higher concentration of phosphate in SR3 may not result in the availability of phosphate due to the formation of chelates when calcium, a component of cement dust, comes in contact with phosphorus in the soil. This may as well lead to the formation of calcium phosphate, making phosphate unavailable for plant use (Lamare and Singh, 2020).

Soil contains a large variety of organic materials such as simple sugars, carbohydrates, organic acids, etc., and they are required by non-symbiotic nitrogen-fixing bacteria for respiration. Soil samples from SR1 had the highest soil organic carbon 10.46% -10.47%, the values significantly decreased in SR2 (Control) to 8.07%-8.21%, and while SR3 was 10.00%-10.26% (Table 1).

Microbiological studies

The total heterotrophic bacterial counts in the soil of the study areas are shown in Table 2. SR3 recorded the highest bioload of heterotrophic bacteria, 1.69×10^7-1.4×10^9 cfu/g, while SR2, the Control sample, had the least count of $1.8x10^5 \times 2.17x10^7$ cfu/g. SR1, about 200m away from the cement factory, had more heterotrophic bacterial count than SR2 (Control), 2km away from the factory. The heterotrophic bacterial population at the factory, SR3, may not be unlikely, considering the pH of 7.5 – 7.8 (Table 1). Bilen *et al.* 2019, reported a significant positive increase in the bacterial population with increasing distance from the cement factory at a pH of 7.0 – 7.6.

The total culturable *Azospirillum* counts (Table 2) showed that SR1 had the highest *Azospirillum* bioload of 10^6 cfu/g than samples from SR2 (control) and SR3.

However, no significant difference was observed between the bioload of *Azospirillium* in SR1 and SR3. Furthermore, the highest total culturable *Azotobacter* count of 10^4-10^5 cfu/g was observed in SR1 (Table 2), while SR3 had the least bioload of *Azotobacter* counts between 10^2-10^3 cfu/g. SR1 and SR3 had the highest total *Clostridium* bioload of between 10^4-10^5cfu/g bioload, while SR2 (control) had the least bioload of *Clostridium* (10^3).

Table 3. Biochemical identities of bacterial isolates from various soil locations.

Isolate code	Source of Isolation	Medium of Isolation	Gram Reaction and morphology	Biochemical characteristics of Isolates												
				Motility	Catalase	H_2S	Coagulase	Indole	Methyl Red	Voges Praukaeur	Glucose	Sucrose	Lactose	Mannitol	Galactose	***Probable Indentities***
IA 115	SR1	Azospirillum Medium	Gram negative Rods in singles	+	+	+	ND	-	+	_	AG	AG	A	A	AG	*Azospirillum* sp.
IA 118	SR1	Azospirillum Medium	Gram negative rods in singles and cluster	+	+	+	ND	-	+	-	AG	AG	A	A	AG	*Azospirillum* sp.
IA 119	SR1	Nitrogen fixing bacteria medium	Gram positive Rods	+	+	+	ND	_	_	_	AG	AG	_	A	_	*Bacillus* sp.
IA 146	SR1	Nitrogen fixing bacteria medium	Gram negative rods in singles and cluster	+	+	+	ND	+	_	+	AG	AG	A	A	AG	*Pseudomonas aeruginosa*
IA 149	SR1	Azotobacter medium	Gram negative Rods	_	+	_	ND	+	+	+	AG	AG	AG	AG	AG	*Azotobacter* species
IA 150	SR1	Azotobacter medium	Gram negative Rods	_	+	_	ND	+	+	+	AG	AG	AG	AG	AG	*Azotobacter* sp.
IA 154	SR1	Clostridium medium	Gram positive rods	+	_	+	+	_	+	_	AG	A	A	A	AG	*Clostridium* species
IE 218	SR2	Azospirillum Medium	Gram negative Rods	+	+	+	ND	-	+	_	AG	AG	A	A	AG	*Azospirillum* species
IE 219	SR2	Azospirillum Medium	Gram negative Rods	+	+	+	ND	-	+	_	AG	AG	A	A	AG	*Azospirillum* species
IE 226	SR2	Nitrogen fixing bacteria medium	Gram positive Rods	+	+	+	ND	_	_	_	AG	AG	_	A	_	*Bacillus* species
IE 228	SR2	Nitrogen fixing bacteria medium	Gram positive Rods	+	+	+	ND	_	_	_	AG	AG	_	A	_	*Bacillus* species
IE 229	SR2	Azotobacter medium	Gram negative Rods	+	+	+	ND	+	_	+	AG	AG	A	A	AG	*Pseudomonas aeruginosa*
IE 236	SR2	Clostridium medium	Gram positive thick Rods	+	_	+	+	_	+	_	AG	A	A	A	AG	*Clostridium* species
FAC 301	SR3	Azospirillum Medium	Gram negative rods	+	+	+	ND	-	+	_	AG	AG	A	A	AG	*Azospirillum* species
FAC 316	SR3	Azotobacter medium	Gram negative rods	+	+	+	ND	+	_	+	AG	AG	A	A	AG	*Azotobacter* species
FAC 320	SR3	Nitrogen fixing bacteria medium	Gram negative rods	+	+	+	ND	+	_	+	AG	AG	A	A	AG	*Pseudomonas* aeruginosa
FAC 340	SR3	Nitrogen fixing bacteria medium	Gram negative rods	+	+	+	ND	+	_	+	AG	AG	A	A	AG	*Pseudomonas aeruginosa*
FAC 360	SR3	Clostridium medium	Gram positive rods	+	_	+	+	_	+	_	AG	A	A	A	AG	*Clostridium* species

Note: SR1- Akinbo; SR2- Egbado (Control); SR3- Factory; ND- Not Done, AG- Production of Acid And Gas, and A- Acid Production Only.

Interestingly, in all the study areas, the soil level of 10-20 cm had a higher bioload of *Clostridium* than the soil level of 0-10 cm. *Clostridium* is a facultative anaerobe that grows at both low and high oxygen tension. However, better growth is achieved at low oxygen tension. The variations in the microbiological bioload counts could be attributed to the changes in the soil's physicochemical properties. Obaroh *et al.*

(2016) with public health interest in cement dust, reported that a total of twelve bacterial isolates were characterized which included*; Micrococcus* species, *Kleibsella oxytoca*, *Clostridium* species, *Proteus mirabilis*, *Enterobacter cloacae*, *Citrobacter* species, *Cryseobacterium meningosepticum*, *Pseudomonas* species, *Klebsiella ornithinolytica, Pantoea, Acinetobacter baumanii,* and *Serratia liquefaciens*. Seven species were Gram negative, there are: *Proteus mirabilis, Enterobacter cloacae, Citrobacter species, Pseudomonas species, Klebsiella ornithinolytica, Pantoea species, Serratia liquefacien*s (Obaroh *et al*., 2016). Similarly, several species of diverse hetrotrophic bacteria were detected in the various soil samples as depicted in Table 3.

The total culturable nitrogen-fixing bacteria (NFB) counts as studied in this present investigation showed that all the locations had adequate bioloads of nitrogen-fixing bacteria that are responsible for the reduction of atmospheric nitrogen in the soil (Onyenze *et al.,* 2013), thereby maintaining soil health. However, the soil samples from SR1 had the highest bioload of NFB counts (10^6-10^7cfu/g), while SR2 (control) and SR3 had NFB bioloads of 10^5cfu/g (Table 2).

Conclusion

From the results obtained in the studies, it could be concluded that cement production activities at Lafarge Cement plant, Ewekoro may not have had much negative impact on the nitrogen-fixing bacteria in the soil around the cement plant and the nearby communities considered at the time of this study. Adequate bioloads of nitrogen-fixing bacteria were observed and a higher amount of nitrate in soil samples around the cement plant showed that nitrogen-fixing activities occurred at a lower rate than in the locations farther away from the cement plant.

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