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Original Research

Impact of Chronic Crude Oil Pollution on Nitrification in Edaphic Systems

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Abstract

Nitrification plays a key role in regulating soil fertility, concentration of soil inorganic nitrogen and production of the highly potent greenhouse gas, nitrous oxide, from soil. This study investigated the effect of long-term crude oil pollution on the activity, abundance and diversity of autotrophic nitrifying bacteria in impacted soils from Bomu and Abonema in the Nigerian Niger Delta compared to unpolluted systems. Nitrifier activity was investigated by determining the soil oxidation potentials while the most probable number microtechnique using Winogradsky media was used to ascertain nitrifying bacteria abundance and soil DNA analysis alongside plate cultures defined nitrifying bacterial diversity. Nitrifier activity was found to be higher in the polluted soils than in the unpolluted soils while quantification of the nitrifying bacteria revealed that polluted samples had higher nitrifier counts. Statistically significant differences existed amongst nitrifier counts and activity ($p \leq 0.05$) between polluted and unpolluted systems with positive correlation between nitrifying bacteria counts and oxidation potential. The results highlighted the dominance of cultivable *Nitrosomonas* and *Nitrobacter* in both polluted and unpolluted soils while DNA analysis revealed that *Nitrospira* was the dominant nitrifier genus in unpolluted soils and *Nitrobacter* in polluted soils. *Nitrospira* was not detected the polluted ecosystems. No ammonia oxidisers were detected via metagenomics analysis. The ammonia oxidisers proved to be more sensitive to chronic crude oil pollution than the nitrite-oxidising bacteria. Long term crude oil exposure produced a 3 – 5 times increase in the activity of nitrifying bacteria in polluted soils with approximately twice the abundance when compared to the unpolluted systems.

Keywords: *Ammonia-oxidising bacteria; Ammonia oxidation potential; Crude Oil; Nitrification; Nitrite-oxidising bacteria; Nitrite oxidation potential*

INTRODUCTION

Nitrogen is a key element for life in any ecosystem. Although, 78% of the atmosphere is made up of nitrogen gas, it remains the limiting nutrient in most edaphic systems. It is considered the nutrient that exerts the strongest influence on plant growth and crop yield (Robertson and Groffman, 2007; John *et al.,* 2011). It circulates through the ecosystem via the nitrogen cycle which begins with the conversion of atmospheric nitrogen into readily utilisable forms via the activities of the diazotrophs followed by nitrification and then, denitrification (Hai, 2009). Nitrification, alongside denitrification, is pivotal to the regulation of the inorganic nitrogen concentration in soil, leaching of nitrate into the aquifer and the production of greenhouse gases of concern. Imbalances between nitrification and denitrification have been linked to the production of nitrous oxide (N_2O), a major greenhouse gas that significantly influences the degree of depletion of ozone in the stratosphere as well as nitrogen dioxide (NO_2) and nitric oxide (NO) which are not necessarily greenhouse gases but contribute substantially to the formation of tropospheric ozone, considered a greenhouse gas. Although, there are multiple sources of N_2O, its only known sink in the biosphere is microbial activity – the conversion of N_2O to dinitrogen gas, N_2 (Barnard *et al*., 2005; Halin *et al.,* 2017). The significance

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of the nitrification process is highlighted by the fact that observation of nitrification activity is typically recommended in routine monitoring for soil quality (Bloem and Breure, 2003).

Nitrification is the second stage of the autotrophic nitrogen cycle and is simplified into two main stages – ammonification, driven by ammonia-oxidising bacteria (AOB), and nitrite oxidation, driven by the nitrite-oxidising bacteria (NOB). Ammonification is the rate limiting step in nitrification (Kucharski *et al.,* 2010). It is a two-step process that involves the transformation of reduced forms of nitrogen – ammonia (NH_3) or ammonium (NH_4^+) – to nitrites (NO_2^-). The nitrites yielded in ammonification are converted to nitrates during nitrite oxidation (Robertson and Groffman, 2007). Participating AOB microbial genera found in biomes and engineered systems include *Nitrosomonas, Nitrosospira* and *Nitrosococcus* (Treusch *et al.,* 2005; Robertson and Groffman, 2007; Tourna *et al.,* 2008; Urakawa *et al*., 2019). Four groups of bacteria oxidise nitrites; the most studied genus amongst the nitrite-oxidising bacteria (NOB) is *Nitrobacter. Nitrospira, Nitrococcus* and *Nitrospina* are other three nitrite oxidiser genera (Schleper and Nicol, 2010; Prosser and Nicol, 2012). *Nitrobacter* is hitherto the only recorded nitrite-oxidising genus cultured from soil (Wertz *et al.,* 2012; Attard *et al*., 2011). The AOB and NOB occur across the Proteobacteria and Nitrospirota phyla.

The Nigerian Niger Delta is amongst the ten most important wetland and marine biomes worldwide but has, sadly, been grouped globally as one of the top five ecosystems most extensively ravaged by petroleum pollution. Studies have shown that around 1.8 million metric tonnes of petroleum have been spilled into the Niger Delta environment over the past five decades (Kadafa, 2012; UNEP, 2011). The impact of consistent unremediated spillage has left the Niger Delta ecosystem critically vulnerable. This level of pollution has resulted in biodiversity loss which in turn severely affects ecosystem services (Kadafa, 2012; John and Okpokwasili, 2012). Microorganisms tend to be the most under-appreciated part of this loss. The microorganisms involved in nitrogen cycling, particularly nitrification, have been described as one of the most sensitive groups to environmental disturbance (Hai *et al*., 2009; Bissett *et al*., 2013; Urakawa *et al*., 2019). Despite this, Scott *et al*. (2014) opine that the effect of oil contamination on nitrogen cycling processes remains poorly understood. A comprehensive grasp of the biogeochemical implications of spillage has, therefore, become imperative. This study, therefore, looked to investigate the effect of long term exposure to crude oil on the activity, abundance and diversity of aerobic nitrifying bacteria in impacted soil in the Nigerian Niger Delta.

MATERIALS AND METHODS

Sample Collection

Composite sampling method was used. Samples were collected, per location, at the surface up to 20cm in depth using a sterile handmade auger at three points within a 3 m^2 area. Upon collection, the samples were put in sterile black polyethylene bags which were subsequently transferred to the research laboratory. Unpolluted samples were obtained from sites in the same area with zero documented incidents of oil contamination. A total of 15 samples per site were used to form 3 composite samples each.

Soil was sampled from oil-polluted wetland sites in Bomu area of Ogoniland (Gokana LGA, Rivers State, Nigeria) with coordinates, 4.6340°N, 7.3559°E and Abonema (Akuku-Toru LGA, Rivers state, Nigeria) at 4°43’25.42″N, 6°46’44.73″E. Both areas not only have several decades of oil spillage but of oil bunkering activities as well which informed the choice of these areas for this study.

Soil Physicochemical Properties

A pH meter (Wintab digital pH meter, Germany) was employed in ascertaining the pH levels in the collected samples in a 1:5 soil-water suspension while the hydrometer method of Bouyoucos (1962) defined soil texture. The Dichromate Wet Oxidation method was utilised in determining soil organic carbon content (Walkey and Black, 1934). One mole ammonium acetate at pH 7 was used to extract exchangeable sodium and potassium from samples then their concentrations were ascertained by the Flame Photometric technique outlined by AOAC (1975). Electrical conductivity was determined as described by Jackson (1962) while the ammonium nitrogen content was measured by the nesslerisation method (Keeney and Nelson, 1982). For the determination of moisture content, approximately 2 g of sample was dried in a hot air oven (DHG-9023A, Hinotek, China) at 105 °C until a constant weight was attained. Drying was done until a constant weight was achieved. The total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAH) contents were also determined using gas chromatograph fitted with a flame ionisation detector (Agilent 6890N, USA) in a capillary column.

Isolation of Nitrifying Bacteria

Isolation was done using specialised media. About 10 g of soil was suspended in 100 ml of sterile normal saline and shaken vigorously. The suspension was then serially diluted and aliquots from selected dilutions were inoculated unto sterile modified Winogradsky Phase I and II media for ammonia oxidation and nitrite oxidation respectively via the

spread plate technique (Odokuma and Okpokwasili, 1996; APHA, 2005). Incubation was at 28 °C for 7 – 10 days in the dark. Discrete colonies were aseptically sub-cultured unto agar plates before being transferred to slants.

Enumeration of Nitrifying Bacteria (Ammonia-Oxidising Bacteria and Nitrite-Oxidising Bacteria)

Nitrifying bacteria were enumerated via the Most Probable Number (MPN) technique using 96-well microtitre plates according to methods stipulated by Deni and Penninckx (1999) and Rowe *et al.* (1977). Each sample was analysed in triplicate sets of 3. A constant mass was achieved by air drying the samples at 30 ℃ then all the nitrite and nitrate in the soil was removed by suspension of 5g of the dried sample in 45 ml of 1 mM sterile phosphate buffer at neutral pH. The suspension was shaken at 100 rpm for 2 h. Samples from the suspension were then diluted in microtitre plates containing Winogradsky phases I and II broth as required. The control microtitre wells contained Winogradsky broth alone. A known amount of 0.05 ml of the sterile the Winogradsky broth was placed into each of the 8 by 12 wells of a sterile microplate using a micropipette, then 0.05 ml aliquots of the soil suspension were pipetted into each of the first eight wells. Serial dilutions were then performed by using sterile micro-pipettes to deliver 0.05 ml from one well to the next across the plate resulting in 12-fold serial dilutions with eight replicates of each dilution.

After incubation aerobically at 28 °C for 28 days, an indicator containing 0.1 g of diphenylamine in 50 ml concentrated sulphuric acid was used to detect nitrite or nitrate production as indicated by a dark blue colouration. The most probable number (MPN) was determined using the table of MPN values (Cochran, 1950). The table determined the P values; based on the MPN table, P_1 being the number of positive wells in the least-concentrated dilution in which either all the wells are positive or the greatest number are positive, and P_2 and P_3 being the number of positive wells in the next two higher dilutions, respectively. The total count was expressed as MPN per gram of dried soil (Mekki *et al*., 2006).

Determination of Ammonia Oxidation Potential (AOP)

Ammonia Oxidation Potential expressed as the production of nitrite over time (nitrite accumulation) is indicative of the activity of ammonia-oxidising bacteria (AOB). AOP was measured using the short-term nitrification assay described by Hart *et al. (*1994). Approximately, 15 g soil was shaken in 90 ml of 0.5 mM phosphate buffer containing 0.2 ml of 0.25 M ammonium sulphate, and 1 ml of 1.0 M potassium chlorate at about 200 rpm for about 8 – 10 hours. The chlorate inhibited the biological conversion of nitrite to nitrate (Belser and Mays, 1982). The rate of nitrite accumulation is considered representative of the ammonia oxidation potential. The nitrite produced was measured by filtering the sample through 0.45µm filters then analysing the filtrate using the spectrophotometric method described by Narayana and Sunil (2009). Analysis was done approximately every one (1) hour for a 6 – 8 h period.

Soil samples were dried at 105 °C to determine dry weight, and ammonia oxidation rates normalised to soil dry weight (dw). Ammonia oxidation potential in microgram nitrogen (inorganic) per gram of dry weight per hour (***µg***-N/g-dw/h) was calculated as the slope of the linear regression of accumulated nitrite mass against time adjusted against soil dry weight.

Determination of Nitrite Oxidation Potential (NOP)

Nitrite oxidation potential (NOP) defines the consumption of nitrite with time and it is indicative of the activity of the nitrite-oxidising bacteria (NOB). NOP was calculated by shaking 15g samples for 8 – 10 hours at 200 rpm in 90 ml of 0.5 mM phosphate buffer with sodium nitrite and 150µg nitrapyrin to inhibit ammonification. The mixture was filtered and the nitrite content of 5ml filtrate samples collected hourly was assayed using the spectrophotometric method of Narayana and Sunil (2009). The NOP in microgram nitrogen (inorganic) per gram of dry soil weight per hour (***µg***-N/g-dw/h) was determined as the slope of the graph of nitrite content recorded against time standardised with the soil dry weight (dw).

Characterisation of Nitrifying Bacteria

Characterisation of cultivable nitrifying bacteria

The identities of the bacteria isolated using selective culture media were confirmed based on their morphological, microscopic, and biochemical properties as proposed by Cheesbrough (2006) and Holt *et al.* (1994).

Culture independent technique – Quantification via metagenomics analysis

Basic quantification of the nitrifying bacterial groups relative to other soil bacteria was done by analysing entire soil DNA. The FastDNA SPIN kit was employed in soil DNA extraction. The purity and integrity of the DNA obtained were analysed with the NanoDrop 2000 spectrophotometer and via quantification and visualisation on 1 % agarose gel using a UV transilluminator respectively. The PCR primers 341F and 805R were used for amplification under the following conditions: initial denaturation at 94˚C for 5mins, 36 cycles of denaturation at 94 ˚C for 30 s, annealing at 56 ˚C for 30 s, elongation at 72 ˚C for 45 s, then final elongation at 72˚C for 7 minutes with hold temperature at 10 ˚C. The DNA amplicons obtained were resolved in 1 % agarose gel then sequenced using the BigDye Terminator 117 v3.1 cycle sequence kit

(Applied Biosystems Inc., USA). AMPure XP beads were used for DNA purification.

Data Analyses

SPSS® 21.0 and Microsoft Excel® 2016 were used for analyses. Following basic statistical distribution analysis, Pearson's correlation analysis was used to define the relationships between bacterial counts and oxidation potentials for polluted and unpolluted systems across the study sites. The data was further analysed using one-way and two-way analysis of variance (ANOVA) to ascertain whether counts, AOP and NOP levels differed significantly from polluted to unpolluted systems and if significant difference existed between the two systems. Statistical significance of data sets was determined at $p \leq 0.05$.

RESULTS

Physicochemical Properties of test soils

The results showed that the soil from Bomu was loamy sand while that from Abonema was sandy. The physicochemical characteristics of the soil samples collected are outlined in Table 1. There were marked differences in the physicochemical characteristics of the polluted and the unpolluted soil samples. Unpolluted soils had a nearer neutral pH of 6.9 – 7.68 while the polluted soil had a pH of 6.25 – 6.60 making it only slightly more acidic. As expected, the polluted soil has about 150 – 300 times more total petroleum hydrocarbon and polycyclic aromatic hydrocarbon concentrations than the unpolluted sample. Bomu soil had higher hydrocarbon content than Abonema but both areas had levels much higher than the acceptable levels of 5000 mg/kg TPH recommended by the Department of Petroleum Resources (DPR, 2002).

Table 1: Physicochemical Characteristics of Soil Samples

Parameters	BOMU		ABONEMA	
	Mean values for Polluted Soil	Mean values for Unpolluted Soil	Mean values for Polluted Soil	Mean values for Unpolluted Soil
pH	6.250 ± 1.552	7.680 ± 1.233	6.600 ± 2.01	6.920 ± 0.722
Moisture (%)	5.280 ± 1.027	3.690 ± 0.20	5.980 ± 1.488	2.300 ± 0.151
Electrical Conductivity (μs/cm)	158.000 ± 10.02	45.000 ± 0.023	1240.000 ± 63.05	120.000 ± 21.01
Total Organic Carbon (TOC) (%)	8.590 ± 2.566	5.060 ± 1.83	8.360 ± 0.988	3.150 ± 1.093
Ammonium Nitrogen (mg/kg)	0.743 ± 0.082	1.273 ± 0.098	0.772 ± 0.11	1.845 ± 0.78
Exchangeable Na^+ (mg/kg)	0.625 ± 0.05	0.495 ± 0.062	0.541 ± 0.17	0.411 ± 0.135
Exchangeable K^+ (mg/kg)	0.499 ± 0.07	0.836 ± 0.05	0.388 ± 0.071	0.746 ± 0.09
Total petroleum hydrocarbons (mg/kg)	18526.117 ± 974.225	124.702 ± 16.173	16832.710 ± 519.73	55.077 ± 34.015
Polycyclic aromatic hydrocarbons (mg/kg)	9368.378 ± 113.892	60.956 ± 8.911	4394.173 ± 206.142	17.420 ± 8.22

Nitrifying Bacteria Activity – Oxidation Potentials

The variation in nitrifying bacteria activity in the samples is highlighted in Figures 1 and 2 which show the nitrite accumulation over time in polluted and unpolluted soils for Bomu and Abonema. From these values, the ammonia oxidation potential (AOP) for the two types of sample were calculated. The nitrite utilisation with time for Bomu and Abonema, from which NOP values were obtained, are illustrated in Figures 3 and 4 for polluted and unpolluted systems respectively. Comparisons of mean values for NOP and AOP in polluted and unpolluted systems are depicted in Figure 5. Evaluation across sites show that polluted soils displayed higher nitrifier activity than unpolluted soils.

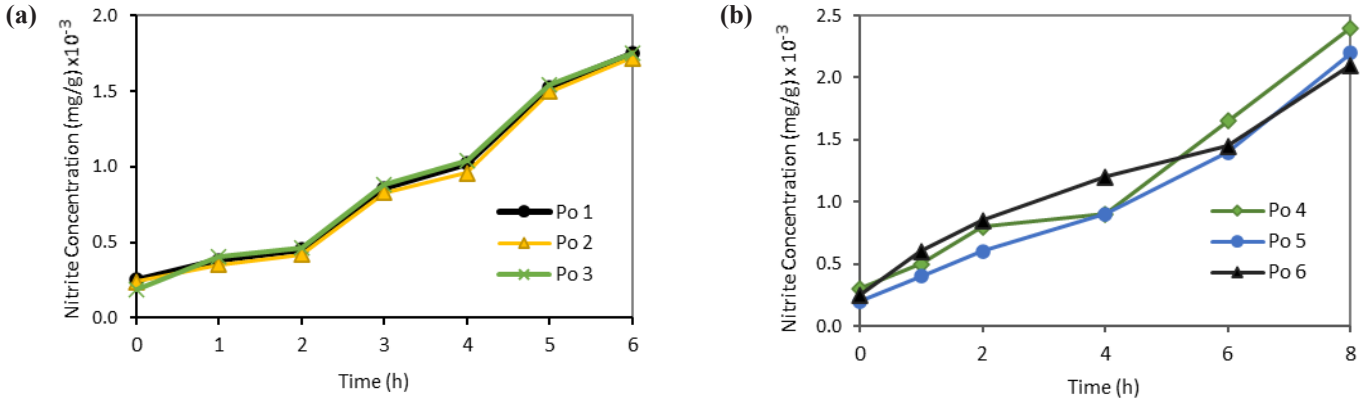

Fig. 1. Nitrite accumulation over time (AOP measure) in polluted soil samples from Bomu (a) and Abonema (b)

*Values are means of triplicates; Po represents sample set codes

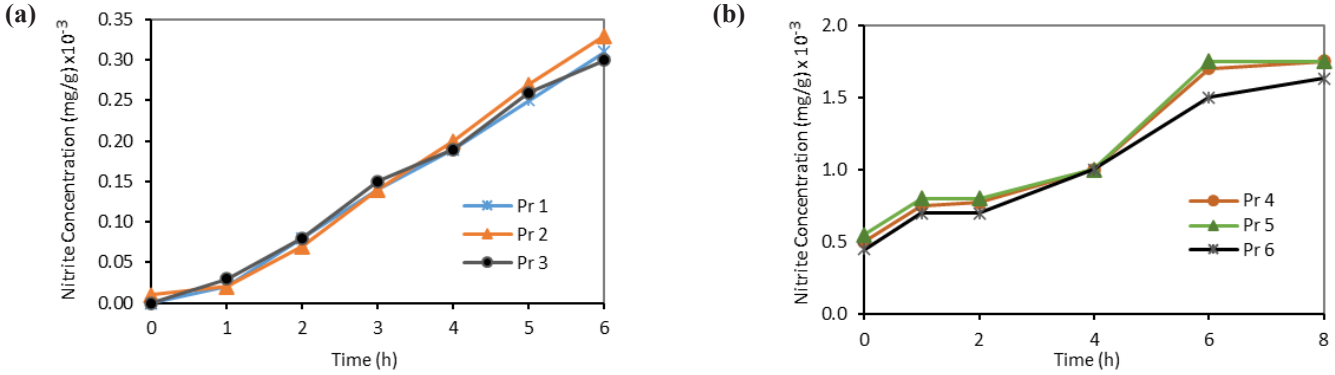

Fig. 2. Nitrite accumulation over time (AOP measure) in unpolluted soil samples from Bomu (a) and Abonema (b)

*Values are means of triplicates; Pr represents sample set codes

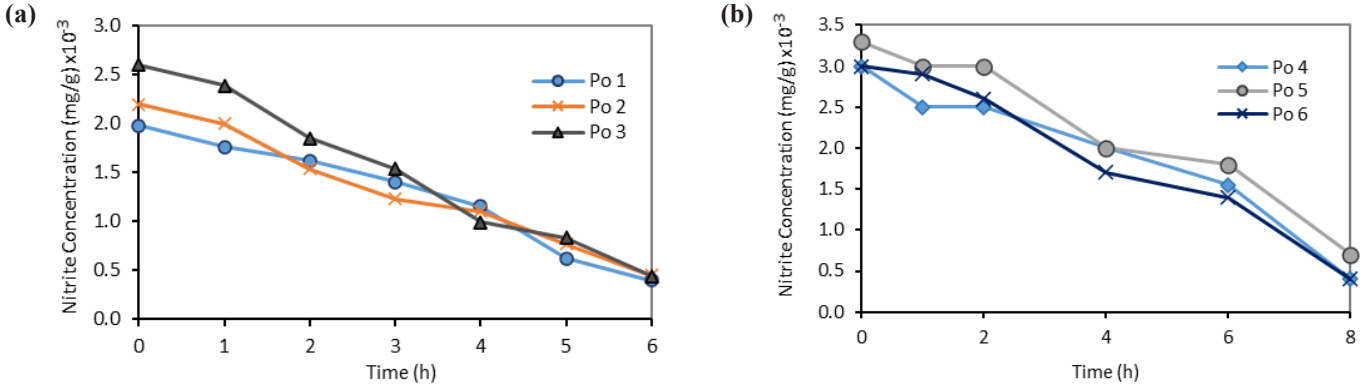

Fig. 3. Nitrite utilisation over time (NOP measure) in polluted soil samples from Bomu (a) and Abonema (b)

*Values are means of triplicates; Po represents sample set codes

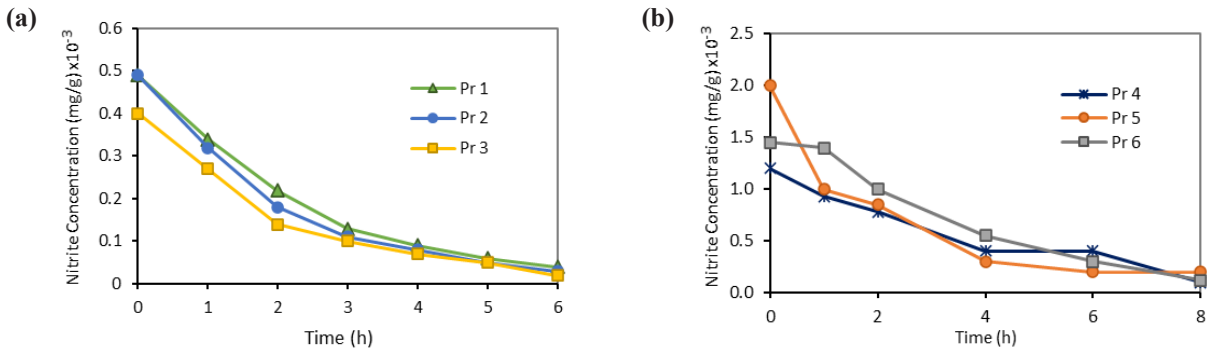

Fig. 4. Nitrite utilisation over time (NOP measure) in unpolluted soil samples from Bomu (a) and Abonema (b)

*Values are means of triplicates; Pr represents sample set codes

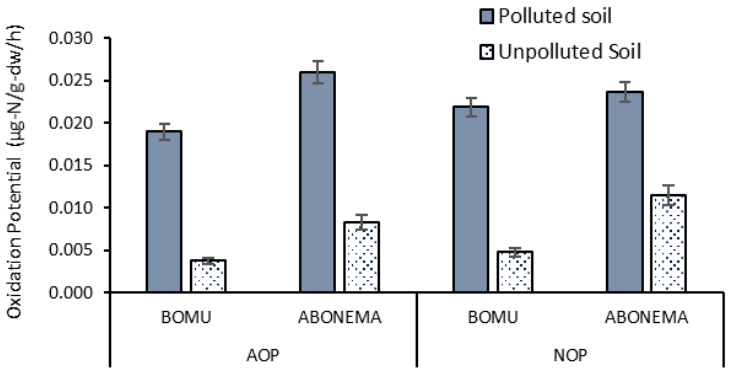

Fig. 5. Comparison of mean AOP and NOP in polluted and unpolluted soils from Bomu and Abonema.

*Values are means of means of triplicates

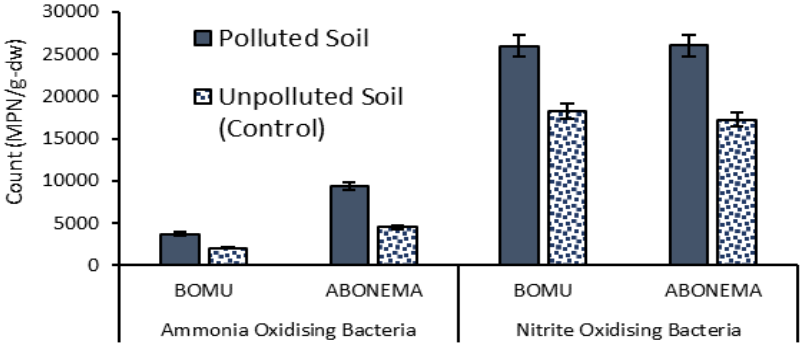

Fig. 6. Comparison of mean AOB and NOB counts in the soil samples from both sites.

*Values are means of means of triplicates

The mean AOP obtained for polluted and unpolluted soils were 0.01895 µg N g^{-1} ds h^{-1} and 0.00376µg N g^{-1} ds h^{-1} respectively for Bomu (18.95 ng N g^{-1} ds h^{-1} and 3.76 ng N g^{-1} ds h^{-1}) and for Abonema, 0.02592 µg N g^{-1} ds h^{-1} (25.92 ng N g^{-1}ds h^{-1}) and 0.0083µg N g^{-1} ds h^{-1} (8.3 ng N g^{-1} ds h^{-1}) respectively. The mean NOP values recorded for polluted and unpolluted soils in Bomu were 0.002187 µg N g^{-1} ds h^{-1} and 0.00472µg N g^{-1} ds h^{-1} (21.87 ng N g^{-1} ds h^{-1} and 4.72 ng N g^{-1}ds h^{-1}) respectively while Abonema presented values of 0.0236 µg N g^{-1} ds h^{-1} (23.6 ng N g^{-1} ds h^{-1}) and 0.0115 µg N g^{-1} ds h^{-1} (11.5 ng N g^{-1} ds h^{-1}) respectively for polluted and unpolluted systems.

Generally the samples showed greater NOP than AOP. Both the NOP and AOP were roughly five times higher in the polluted soil than in the unpolluted for the Bomu site while they were only about two to three times greater in polluted and unpolluted samples from Abonema.

Ammonia and Nitrite Oxidiser Abundance and Diversity

The nitrite oxidisers occurred in much higher numbers than the ammonia oxidisers. Polluted soil samples showed greater counts across both groups than unpolluted samples. A comparison of the mean AOB and NOB counts in unpolluted and polluted soil samples from both sites is provided in Figure 6. In the samples of polluted soil from Bomu, the nitrite oxidisers were 7 times more than the ammonia oxidisers while in the unpolluted samples, they were about 9 times more. NOB counts in polluted soil was about 1.5 times more than in the unpolluted while the AOB in the polluted soil had counts that were twice the numbers recorded in unpolluted soil samples. The nitrite oxidisers were almost three (3) times more than the ammonia oxidisers in polluted samples from Abonema while in the unpolluted samples, they were about 4 times more. Furthermore, amongst the Nitrite-oxidising bacteria (NOB), counts were one and a half (1.5) times more in polluted sample than in unpolluted while the Ammonia oxidisers recorded counts approximately two (2) times higher in polluted samples than in unpolluted. AOB counts for unpolluted soil averaged at 2.02 x 10^3 MPN/g-dw for Bomu and 4.472 x 10^3 MPN/g-dw for Abonema differing slightly from polluted soil which averaged at 3.693 x 10^3 MPN/g-dw and 9.306 x 10^3 MPN/g-dw for Bomu and Abonema respectively. For the NOB, the mean count for polluted soil was 2.595 x 10^4 MPN/g-dw for Bomu and 2.599 x 10^4 MPN/g-dw for Abonema while the mean count for unpolluted soil was 1.823 x 10^4 MPN/g-dw for Bomu and 1.725 x 10^4 MPN/g-dw for Abonema.

The cultivable isolates obtained were the same for both Bomu and Abonema. *Nitrosomonas* dominated in both unpolluted and polluted soil amongst the cultivable ammonia oxidisers while *Nitrobacter* dominated among the cultivable nitrite oxidisers. Metagenomic soil DNA analysis provided a total of 23180 – 57364 sequence reads across the samples. A higher abundance of bacteria was seen in the polluted soils but the nitrifying bacteria–related groups showed greater abundance and diversity in the unpolluted soils. Groups related to the NOB were more readily detected (Figure 7). *Nitrospira* and *Nitrobacter* were the only nitrifying bacteria genera obtained. *Nitrospira* dominated in the unpolluted samples from both Bomu and Abonema while *Nitrobacter* was detected in samples from Bomu – both polluted and unpolluted – and the unpolluted samples from Abonema. It was the dominant genus in the polluted soil ecosystems. Only orders and families related to NOB were detected. *Candidatus Nitrososphaera*, a member of the ammonia-oxidising archeae (AOA) was found only in unpolluted samples from Abonema at comparatively low abundance levels (0.185 %). The absence of detectable AOB-related groups indicated that the occurrence of the taxa out of the total reads obtained was insubstantial.

Relationship between Bacterial Counts and Oxidation Potentials of Test Soils

Bomu study site

The relationship between bacterial counts and oxidation potentials in the polluted and unpolluted systems was investigated using Pearson's Product Moment Correlation co-efficient. The analyses revealed a strong positive correlation between nitrite oxidiser counts and nitrite oxidation potential (NOP) in both polluted and unpolluted soils as well as between ammonia oxidisers and ammonium oxidation potential (AOP) in the polluted soil alone. These all had r values of 0.5 – 1.0 with shared variance values of 72.25%, 45.02% and 94.9% respectively. The relationship between ammonia oxidisers and ammonium oxidation potential (AOP) in the unpolluted environment showed a weak positive correlation with an r value ranging from 0.1 – 0.29 and a shared variance of 2.6 %. Generally, the higher the ammonia-oxidising and nitrite-oxidising bacteria counts, the higher the oxidation potential. Results from single factor ANOVA showed that there was no significant difference at 95% confidence interval in both AOB and NOB counts between the polluted and unpolluted soils, however, there were significant differences between the two in the case of the Ammonia Oxidation Potential and Nitrite Oxidation Potential.

Abonema study site

The investigation using Pearson's Product Moment Correlation co-efficient revealed a positive correlation between AOB counts and ammonia oxidation potential (AOP) in both the polluted and unpolluted samples from Abonema. An r value of 0.873 was recorded for the polluted system and an r value of 0.317 for the unpolluted system. The polluted samples showed a stronger correlation with a shared variance of 76.2% while the unpolluted soils had a shared variance of 10.05%. The relationship was slightly different with the nitrite oxidisers. Results obtained indicated that in unpolluted soil, there was a strong positive correlation between nitrite

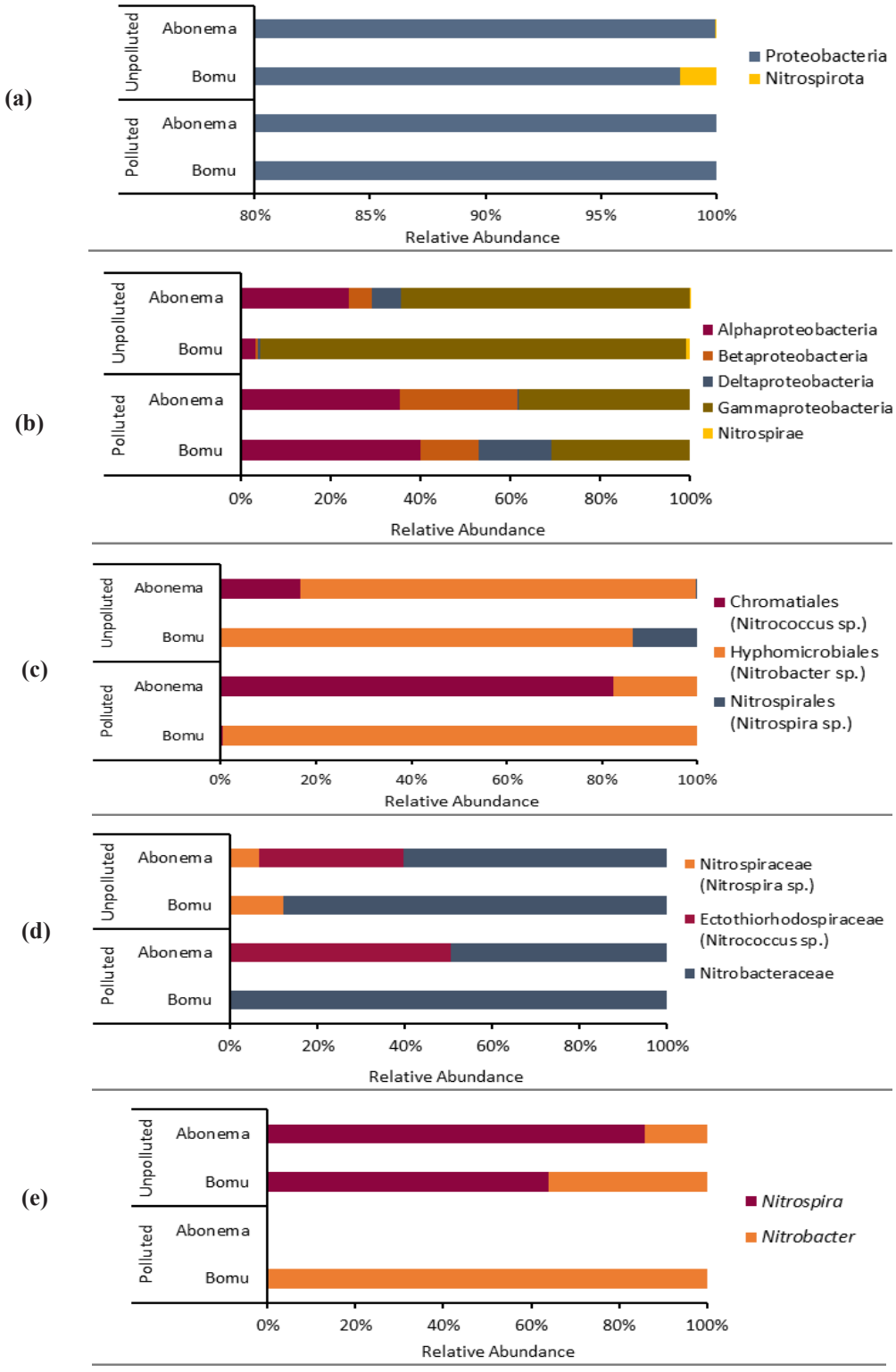

Fig. 7. Relative abundance of detected nitrifying bacteria related groups (based on amplicon sequences) in Abonema and Bomu study sites at the (a) phylum, (b) class, (c) order, (d) family and (e) genus taxonomic ranks. The order Hyphomicrobiales is also known as Rhizobiales. The family, Nitrobacteraceae is also known as Bradyrhizobiaceae. *Nitrospira* and *Nitrobacter* were the only nitrifying bacterial genera detected

oxidiser counts (NOB) and nitrite oxidation potential (NOP) with a shared variance of 61.62% (r value = 0.785) however, for the polluted soil, the correlation between nitrite oxidiser counts (NOB) and nitrite oxidation potential (NOP) was only marginally positive with an r value of 0.547 and a shared variance of 29.92%.

Results from single factor ANOVA at 95% confidence interval revealed that there was significant difference between the polluted and unpolluted systems for all the parameters considered – AOB, AOP, NOB and NOP.

Comparison between Nitrification Statistics of Bomu and Abonema Study Sites

Investigation using two-way analysis of variance at 95% confidence interval suggested that for all the parameters being considered, on the average, the interaction between polluted and unpolluted systems for Abonema was not significantly different from the interaction between polluted and unpolluted systems for Bomu. When considering the individual relationships for each parameter between the two sites, there was a significant difference at the 95% confidence interval. This means that AOB measures in polluted soil in Bomu was different from AOB measures in polluted soil in Abonema and so forth.

DISCUSSION

The unpolluted soil had 1.69% less moisture than the polluted soil in Bomu and 3.68% less in Abonema. This is unusual as the presence of hydrocarbons have been shown to increase soil temperature – to an extent mostly as a result of increased microbial activity - as well as soil hydrophobicity thereby reducing its water retention capacity (Aislabie *et al.,* 2004) resulting in lower moisture content which in turn affects oxygen availability (Schjønning *et al.,* 2003). Total organic carbon levels were higher in the polluted soils while the ammonium nitrogen were higher in the unpolluted soils. Enhanced microbial activity due to the presence of the pollutant tends to bring about increased nitrogen utilisation in the polluted soils and sediment thus the lower ammonium nitrogen (Roy *et al*., 2010; Tan *et al*., 2014). Wang *et al.* (2010) and John *et al.* (2016) found that soil ammonium and pH were significantly lower in polluted soil compared to unpolluted. Abdulrasheed *et al*. (2018) have also pointed out that soil pH significantly impacts on nitrite oxidisers in tropical soils and Oje *et al.* (2015) maintain that following crude oil exposure, soil ammonium concentration increased from day 1 – 168 after which there was a decrease.

The current study established that long term exposure to crude oil modified the nitrification process. The findings indicated that the initial disruption reported by other researchers seemed to give way to increased proliferation and hyperactivity on the part of the nitrifying bacteria. The results further highlighted the high sensitivity of the nitrifying bacteria to long term crude oil exposure with the ammonia oxidisers being more sensitive than the nitrite oxidisers as underscored in several other similar studies (Amadi *et al*., 1996; Horel *et al*., 2014; Dos Santos *et al*., 2012, Wertz *et al*., 2012; Kurola *et al.,* 2005). Ammonia oxidisers are sensitive to three major after effects of a crude oil disturbance: Toxicity, oxygen limitation resulting from the rapid proliferation of hydrocarbonoclastic bacteria and competition for basic nutrients, particularly ammonium (Bissett *et al*., 2013). The hydrocarbons constituents of crude oil are thought to compete against ammonia for the active site of ammonia monooxygenase (AMO) enzyme (Deni and Penninckx, 1999). This is particularly true of the aromatics that specifically deplete cellular reductants essential for ammonia oxidation while alkynes permanently inhibit ammonia monooxygenase (Bissett *et al*., 2013). Studies carried out by Kucharski *et al.* (2010) showed that diesel oil and petrol greatly inhibited the nitrification process with diesel oil having a stronger inhibitory effect than petrol. As contact time increases though, this inhibitory effect on nitrifier activity and abundance, as with the current study, gave way to hyperactivity and enhanced proliferation.

The values obtained for AOB and NOB counts in both Bomu and Abonema in the present study were in tandem with values reported in crude oil contaminated land farming soil by Kurola *et al.* (2005) who obtained AOB counts of 4 – 9 x 10^5 cells g^{-1} dry soil which is much higher when compared with this study but similar in multiples to Qiu *et al.* (2010) who found AOB counts in eutrophic lake sediment to be within values of 1.14 – 1.24 x 10^5 cells g^{-1} dry weight of sediment. Similarly, in crude oil contaminated soil, John and Okpokwasili (2012) obtained counts for *Nitrosomonas* sp. (AOB) of 7.8 x 10^5 cfu/g and for *Nitrobacter* sp. (NOB) 5.6 x 10^5 cfu/g while Philips *et al.* (2000) observed values of 1.1 x 10^4 cells g^{-1} in unpolluted sediment and Hesselsøe *et al.* (2001) recorded AOB counts ranging from 1.57 – 1.81 x 10^3/g in sandy loam farm soil. Féray *et al.* (1999), likewise, determined that average NOB value using MPN-PCR method were in the magnitude of 10^3 cells g^{-1} dry weight of sediment in freshwater sediment; lower than observed in the present study. Increased nutrients in form of the hydrocarbons could account for the increased numbers of nitrifying bacteria in the polluted soils when compared to unpolluted; bacteria have adapted to the long-term chronic disturbance and may now be able to utilise the crude oil as a carbon source due to synthesis of relevant degradative enzymes.

The AOP levels obtained for unpolluted soils in the present study compared favourably with the values obtained by Hayashi *et al.* (2016) where the AOP in somewhat unpolluted tundra mineral soil was found to range from 2.3 to 14.1 ng N g^{-1} ds h^{-1} (0.0023 – 0.0141 µg N g^{-1} ds h^{-1}). Alves *et al.* (2013), in 15 °C artic soil got nitrification potential rates of 10 – 200 ng N g^{-1} ds h^{-1} (0.01 – 0.2 µg N g^{-1} ds h^{-1}) following addition of 1.7 – 2.5 mM ammonium while the observed rates on moss tundra on dry mound was about 20 ng N/g ds h^{-1} (0.02 µg N g^{-1} ds h^{-1}). It could be said that the AOP in these studies

compared to the current study are roughly similar taking into cognizance the temperature and substrate conditions. Kurola *et al.* (2005) obtained an average AOP of 0.05 – 0.28 µg NO_2^--N g^{-1} dry soil/h (50 – 280 ng NO_2^--N g^{-1} dry soil/h) in oil contaminated land farming soil. These values are much higher than obtained in the current study which may be due to the period of exposure; the soils used in the case of Kurola *et al.* (2005), unlike in the current study, had been subjected not only to oil spillage but had also been farmed for over ten years during which there was regular amendment with urea, superphosphate and other similar nutrient sources. Studies for NOP are not as readily available. Laanbroek and Woldendorp (1995) found that NOP values in extensively used pasture soil averaged at 295 x 10^6 – 382 x 10^6 L/g-dry wt soil/h. The higher oxidation potential observed in polluted soils is explained by increased organic content which results in increased bacterial numbers. Hayashi *et al*. (2016) confirm that organic soils have greater rates of nitrification than mineral soils. The increased activity (AOP and NOP) obtained in the current study could be due to the over-expression of the relevant genes following environmental stress or the expression of a stress-induced gene. Several studies have recorded this phenomenon in plants (Khan *et al*., 2015; Kumari *et al*., 2016; Bhauso, 2014; Borkotoky *et al*., 2013) and certain bacteria and fungi (Poole, 2012; Gasch and Werner-Washburne, 2002, Viveiros *et al*., 2007).

AOB counts obtained from Bomu were 82.8% higher in the polluted soils compared to unpolluted while NOB counts were only 42.3% higher in the polluted samples. With the Abonema soils, an increase of over 100% was observed in AOB counts while NOB counts were higher in polluted soils by only 50.7 %. The stronger response of the AOB to crude oil pollution is also reflected in their activity – AOP values in polluted soils from Bomu were on average almost 5 times those in the unpolluted soil. NOP values showed an increase as well, albeit lower, being 4.5 times more on average in the polluted soil. Polluted soils samples from Abonema had an AOP mean value three times that recorded in unpolluted samples whereas NOP values were only two (2) times the mean values recorded in the unpolluted samples. These findings are akin to those found in similar studies: Wertz *et al.* (2012) reported that over time, stressors will generally increase the abundance of ammonia-oxidising bacteria and greatly increase AOP. These same stressors have limited impact on the size of nitrite oxidisers. This would indicate less sensitivity on the part of the NOB compared to AOB. Orji *et al.* (2011) observed that the rate of ammonium consumption (representative of ammonia oxidiser activity) in chronically contaminated soils were greater than in uncontaminated soils. It can be observed that while the AOP values for unpolluted samples from both sites in the present study are similar, the values for polluted samples are three times higher in Bomu than in Abonema even though the oxidiser counts are higher in Abonema than in Bomu samples. Environmental factors may be responsible for this. The concentration of the hydrocarbons present in the soil could be pertinent as well.

Unlike the present study, most studies involve acute short term exposure to hydrocarbons which have been shown to result in a reduction in nitrifier abundance and activity, however, in these cases as well the AOB are still shown to be the more sensitive group. In investigations by Fuller and Scow (1997) hydrocarbons were found to inhibit ammonia oxidation and as a result, reduced the energy supply to nitrite oxidisers. Their findings show that NOP was not as sensitive to hydrocarbon contamination as AOP – after 28days of incubation upon exposure to 30 – 60µg TCE per ml, soil NOP activity diminished by 43% of those recorded in the control experiment. While AOP reduced by 50% in the presence of a considerably lower concentration of 0.81µg of TCE per ml. The work of Deni and Penninckx (1999) and Odokuma and Okpokwasili (1996) confirmed these findings. However, in the work of John and Okpokwasili (2012), the ammonia oxidiser, *Nitrosomonas* showed better tolerance for the components of petroleum than the nitrite oxidiser, *Nitrobacter*. Dos Santos *et al.* (2012) also recorded lower ammonia oxidation potential (AOP) in polluted sites when compared with unpolluted sites. These findings did not tally with those in the current study which recorded higher oxidation potentials and higher nitrifier abundance in polluted soils. This disparity may highlight the difference between acute and chronic petroleum hydrocarbon exposure. The differences found in nitrifying bacteria responses to chronic and acute crude oil pollution are clearly indicative of their ability to adapt to the disturbance.

The investigation of the relationship between nitrifying bacteria counts and oxidation potentials (activity of nitrifiers) in polluted and unpolluted systems revealed that even though there were significant differences between polluted and unpolluted systems for oxidation potential (both AOP and NOP), there were no significant differences in the nitrifier counts between the two Nitrifying potentials are considered a stronger, more accurate quantitative measure of nitrifier activity than biomass. This is validated by the conclusions drawn by Hayashi *et al*. (2016) that nitrifying bacterial counts are not a true representation of oxidation potentials. Alves *et al.* (2013) however, opined that the opposite was the case. Marton *et al.* (2015) on the other hand, observed that nitrifying bacterial counts corresponded significantly with oxidation potential especially in polluted soil. This is similar to the findings of Im *et al.* (2014) that AOB gene copies correlated with the performance of the nitrification process in a nitrition reactor. Based on the results obtained from Abonema, it can be concluded that the counts play a stronger role in determining oxidation potential in the polluted soil compared to the unpolluted. The results of the shared variance from statistical analysis in this current study demonstrates that even though there is some correlation, the nitrifying bacteria abundance is not the sole determinant of the oxidation potential.

This study found *Nitrosomonas* and *Nitrobacter* to be the dominant cultivable genera amongst the ammonia oxidisers and nitrite oxidisers respectively in both polluted and unpolluted soils. This result may not be considered a true measure of nitrifier diversity in these systems as most groups

of the nitrifying bacteria are considered both fastidious and unculturable and would be more adequately characterised using molecular based techniques. The findings, however, buttress those of Bernhard *et al*. (2016) that after an oil spill, the ammonia oxidiser, *Nitrosomonas* was the dominant species as a result of increased nitrogen and carbon availability while *Nitrosospira* dominated under low nitrogen and carbon conditions. The two genera, *Nitrosomonas* and *Nitrosospira* are the main ammonia oxidisers found in soil ecosystems while Wertz *et al.* (2012) confirmed *Nitrobacter* as the only genus of NOB cultured from soil systems. Metagenomics analysis determined the relative abundance of members of the nitrifying bacterial groups within the soil microbial community in the present study. Only groups relating to the nitrite oxidisers were readily detected. This may be because AOB tend to occur in quite low numbers in the environment as shown by the MPN values obtained in the present study which may bring about poor detection. The use of ammonification gene primers would be useful here to aid detection by amplification. Certain researchers have confirmed that, similar to the present study, *Nitrospira* spp., though uncultivable, will often be detected in the analysis of soil DNA alongside *Nitrobacter* (Wertz *et al.,* 2012; Attard *et al*., 2011). Other NOB genera like *Nitrospina* are more commonly associated with marine ecosystems (Prosser, 2007).

The lower statistical impact of bacterial counts on oxidation potential observed in unpolluted soil can be explained by the presence of a wider diversity of microorganisms than would be found in petroleum polluted soils. In soil, more organisms are able to convert ammonia to nitrites than nitrites to nitrates and thus have greater impact on the AOB/AOP relationship than on the NOB/NOP relationship. Banerjee and Silciliano (2012) and Hayashi *et al.* (2016) reported that edaphic factors played a stronger role in determining AOP than species abundance. Ling-Zhi *et al*. (2014) as well corroborate that AOB abundance and oxidation potential were significantly correlated with total organic nitrogen (TON), ammonium nitrogen and nitrate content of the soil as well as the pH. This is similar to the conclusions of Hayashi *et al.* (2016) that AOP correlated more strongly with soil moisture content and ammonium nitrogen than with AOB counts. Petersen *et al*. (2012) further observe that the functional gene abundance was more important than cell numbers in the prediction of potential nitrogen cycling rates. The results from both sites indicate an increased capacity on the part of the nitrifiers as a means of adapting to the new environmental conditions. These findings underlined those of Deni and Penninckx (1999) who stated that these bacteria adapted to hydrocarbon pollution by increasing their affinity for ammonia which would, in turn, increase their activity and the average amount of nitrites and nitrates produced in the system. Studies on NOB and NOP are not particularly extensive and so only limited statistics were available for comparison.

The study generally highlighted the sensitivity of the nitrifying bacteria to long term crude oil exposure with the ammonia oxidisers being more sensitive than the nitrite oxidisers Chronic exposure to crude oil instigated the increased proliferation of nitrifying bacteria with enhanced activity such that approximately twice the number of nitrifying bacteria had three to five times the level of activity observed in the unpolluted systems. This could prove problematic if the nitrates are produced faster than the less sensitive denitrifiers can handle. The production of excess nitrates in any soil ecosystem due to imbalances between nitrification and denitrification has far-reaching consequences for climate change, human health and water security.

CONCLUSION

There have been very limited studies on the response of nitrifying bacteria to crude oil exposure in tropical soils particularly as regards the nitrite-oxidising bacteria. This study has provided valuable information as to the effect of crude oil spills on nitrification in the long term and has defined the relationship between nitrifying bacteria abundance and activity. Long term exposure to crude oil in soil modified the nitrification process impacting on both the diversity and abundance of relevant bacterial genera as well as their activity. The prevention of spills into the environment and adequate clean-up where there has been spillage is crucial to the continued provision of niche ecosystem services like nitrification.

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The authors declare that no known conflicts of interest exist.

AUTHORS' CONTRIBUTIONS

Authors GCO, CBC and AUO conceived and designed the study; Authors GCO and CBC provided supervision while Author AUO carried out the laboratory and data analysis, conducted the literature searches and wrote the first draft of the manuscript. All the authors reviewed the results and approved the final draft of the manuscript.

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