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Molecular and Morphological Assessment of the Impact of Gas Flares on the Fungal Abundance and Diversity in soils of Niger Delta Area, Nigeria

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Abstract: *Fungal abundance and diversity in soils of three communities flow stations (ID, IR and OB) owned by an unnamed oil exploration firm operating in the Niger Delta area for about five decades were determined using nucleic acid analytical methods. Top soil (0-15 cm) samples were collected from mini pits using sterile bottles at 50, 100 and 150 meters from the flare barrier of each community flow station and soil samples from the control was taken at 2.5 kilometers during the dry and wet seasons. The fungal properties were determined first using the cultural method of serial dilution method which revealed the presence of the following fungi; Aspergillus sp, and penicillium chrysogenum at 50 m away from flare in all the three flow stations during the dry seasons and in the wet season Aspergillus sp, penicillium chrysogenum, Mucor sp., yeast cells, A. niger, and Rhizopus sp. At 100 m away from flare, Mucor sp, A. niger and rhizopus were isolated in all three-flow station in the dry season and in the wet season, Yeast cells, A. niger, Aspegillus sp. and Rhizopus were isolated. P. chrysogenum, Aspergillus sp. and yeast cells were isolated at 150 m away from flare in the dry season and in the same distance, yeast cells, A. niger aggregate, A. niger and P. chrysogenum were isolated. At the control site, during the dry season, A. niger, P. chrysogenum, Aspergillus sp., Rhizopus sp. and Botryodiplodia threobome were isolated in the dry and A. niger, P. chrysogenum and yeast in the wet season. Fungal DNA was extracted using Norgen fungal genomic DNA extraction kits for the molecular analysis to establish the true characteristics and species identity of the isolates earlier identified with cultural methods. This revealed the presence of the following fungi upon sequence blast on NCBI database; Aspergillus fumigatus (Aspergillus fumigatus brown 2 (abr2) gene, complete cds strain), Aspergillus fumigatus (Aspergillus fumigates strain IHM 15988 pigment biosynthesis protein), Fusarium phyllophilum , Fusarium graminearum, Aspergillus oryzae (a strain of Aspergillus niger) Aspergillus oryzae (niger). Findings from this work have shown that molecular technique gave the exact identity of fungal species isolated from study area. All six (6) molecularly identified fungi were isolated from the three-study flow station and in both seasons. The Aspergillus group was dominant as a result of their ability to adapt to the flare environment and all isolated fungi are good crude oil degraders.*

Key Words: fungal, diversity abundance, molecular, gas flare.

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INTRODUCTION

Nigeria is an oil and gas producing nation and their production is majorly carried out in the coastal area of the Niger Delta region. Flaring is a continuous trend in the Nigerian oil and gas industry and has impacted negatively on the environmental components and its associated biota (Enetimi and Sylvester, 2017). The produced gas is underutilized, and about 10 to 40% is flared into the environment (Obi *et al.*, 2021). Enormous amount of heat is also emitted in the process which causes varying degrees of environmental pollution. The magnitude of these effects on soil microbiological, meteorological, soil chemical and physical parameters have been adequately documented (Elenwo and Dimkpa, 2022; Atuma and Ojeh, 2013). There are noticeable effects resulting from oil exploration and gas flares on host Communities farmlands that may in turn affect the microbial quality of the farmlands which may result in low fertility of the soil (Elisha et *al.*, 2008). Microorganisms show variation in their activities and in their relationship with other faunas and floras due to their differences in structure and functions in the biological and agricultural ecosystems (Bogat and Walczak, 2022).

Soil fungi are part of the normal micro flora and are major actors in the nutrient cycling in the ecosystem. Their diversity is a good indicator of soil health especially in agriculture (Choi, 2003). Fungi are abundant and active in normal aerated soils and all the heterotrophic fungal mycelia in the soil spread over surfaces and helps bind mineral particles into aggregates (Singer and Munns, 1996). They are essential participants in the decomposition of many types of organic matter which are added to the soils (Prescott *et al.*, 1999). Fungi are eukaryotes and possess cell walls like higher plants; however, they do not contain photosynthetic pigment and are chemo-organo heterotrophs. Like many other heterotrophic organisms, fungi obtain their cell carbon predominantly from organic matter which serves as both carbon source and energy source and are partially assimilated into the cell material and partially oxidized to provide energy.

According to Lindley (1992), the widespread ability of yeast and filamentous fungi to transform hydrocarbons suggests that they may be involved in the recycling of naturally occurring hydrocarbons in the environment as well as in the bio deterioration of liquid fuels. The versatility of fungi in degrading hydrocarbon is due to the broad substrate specificity of their enzymes (Cerniglia and Sutherland 2010). Most filamentous fungi and yeast grow abundantly in soils contaminated by petroleum residues and research has shown that over 200 classes of fungi, yeast can break down hydrocarbons such as methane (Ekundayo and Obuekwe, 2001). Previous studies have shown that organic content, texture and pH, of some soil samples may have various impacts on soil microbial diversity. Soil samples collected at different depths in a core and from same location can also be completely different. These factors and more affect the microbial load and consequently the yields of DNA and RNA that can be obtained (Tseng *et al.*, 2021). Researchers like Broz et al. (2007) reported the examination of fungal abundance and diversity in soils using very sensitive molecular techniques.

In recent times there is increase in microbial identification in the natural environment using cultural method. But currently the use of the Nucleic acid analyses method to identify a whole microbial community will resolve and provide answers to questions that have been unanswered using standard cultural methods. The 16S and 18S regions of the ribosomal genes' sequences are the universally accepted phylogenetic markers for the study of microbial systematic and ecological surveys from the natural environments. Ribosomal molecules have highly conserved sequence domains interspersed with hyper variable regions and these variables domain differentiate one microbe from another and therefore, can be used as molecular markers to discriminate among taxa (Ferraz-Helene *et al.*, 2022). The molecular technique enables us to analyze the soil microbial community with more accuracy than cultural methods. This is because microbiological advances are closely linked with molecular biological technique (Nakats, 2006). In the last decade, platforms for accurate amplification, probe development and various quantitative polymerase chain reaction (PCR) technologies have gone through research revolution on fungal detection and identification.

The soil fungal community varies in their association at the various levels of the biological organization. The chemical composition of the soil affects the types of microorganisms and size of the microbial population in that soil environment and crude oil exploration in the Niger Delta which cannot be talked about without mentioning gas flaring remain the most notorious activity of the oil companies. It is one major activity in the Niger Delta capable of changing the chemical composition of the soil and as such affect microbial diversity in the community. Therefore, this research reports the impact of gas flare on the fungal diversity using cultural and molecular methods of identification for non-cultured fungi.

MATERIALS AND METHODS

Study Locations

Three flow stations of an oil exploration company in the Niger Delta Area of Nigeria were sampled for fungal abundance and diversity. The three flow stations where soil samples were collected were in Rivers, Delta and Bayelsa States all in the Niger Delta Area of Nigeria. The flow stations are designated as ID, IR and OB flow stations respectively for legal reasons. ID flow station lies within the swamp forest of the Niger Delta zone of Nigeria. Two communities lie within the facility and the area is characterized by streams and flood plain and is located 60km North West of Port Harcourt in Rivers State, IR flow station is located in Delta State of Nigeria. The area lies within the swamp forest of Niger Delta. IR area is a typical rainforest subjected to seasonal floods and inundations from River Niger and OB flow station is situated in Bayelsa State of Nigeria. The field lies in the fresh water swamp forest characterized by thick forests belt and low-lying lands which are subjected to seasonal flooding. It is criss-crossed by creeks and creek-lets, which receives some tidal water from the Atlantic Ocean through the southern axis. Patches of mangrove occupy the flanks of the creeks ecosystem. Three communities lie within the project area. There are two main climatic seasons of the year in the entire region and these are the dry and wet seasons.

Soil Sample Collection

Sampling was done twice, in dry season (late November, 2015) and raining season (early August) of the following year (2016). Soil samples were taken from 0-15 cm depth at 50, 100 and 150 m intervals from the flare barrier while the control was taken from 2km radius of each of the study communities. Therefore, soil samples were randomly taken from each distance, homogenized and stored in sterile bottles resulting in four (4) samples from each flow station, giving a total of 12 soil samples for each season and a total of 24 for both dry and wet seasons.

MICROBIAL ANALYSIS

Ten-fold serial dilution method was used and the diluted soil samples were cultured on Sabouraud and Potato Dextrose Agar for the isolation of fungi and yeast respectively. The morphologies of the isolated fungi were examined macroscopically and recorded accordingly as they were sub cultured. Wet preparations were made from the sub cultured fungi on clean grease free slides, covered with cover slips, and examined under the microscope and identified based on their cultural and microscopic characteristics using the methods described by Barnett and Hunter, (1998) and Nyongesa *et al.*, (2015).

Determination of Fungal Load

The fungi load in each sample was determined visibly by counting the colony forming unit after 24 hours. The microbial load/ml was determined by the formula of Cheesbrough (2002) as:

$$\text{Count/ml} = \frac{\text{Number of counted colonies on plate x volume plated x dilution factor}}{\text{Number of counted colonies on plate x } 1/10/10^3 \text{ cfu/ml}}$$

Molecular Identification of Fungal Isolates

Molecular analysis for fungal isolates was done to identify some of the fungi which the cultural method and biochemical tests carried out could not identify. The fungi to be identified using molecular methods were isolated and sub-cultured using the cultural technique.

Fungal DNA was extracted using Norgen fungal genomic DNA extraction kits. PCR amplification of the internal transcribed spacer region (ITS1 region) of the 18S rDNA of the fungal genome was carried out using the General multipurpose primers (Gmf1: TGTACACACCGCCGTC and Gmf2: CTGCGTTCTTCATCGAT).

The products gotten from the PCR amplification were purified using the Invitrogen PCR amplification kit. Gene enrichment was done using the Raindance Technology (RDT 1000) Raindance Sequence Enricher. Agarose Gel Electrophoresis of PCR Products was done by using 2% of Agarose Gel Electrophoresis containing 05µg/ml Ethidium Bromide (EtBr). The DNA sequencing was done using the Next Generation Sequencer (NGS) (solid 5500xl plat). The nucleotide sequences obtained from the various fungi isolates were analyzed by searching and comparing with other sequences from the National Center for Biotechnology Information (NCBI) website. A blast search was conducted on all the nucleotide sequences and the identities of the isolates were revealed (Stach *et al.,* 2001).

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RESULT

Fungal enumeration and Identification in Soils of ID flow stations at various Distances from Flare in the Dry and Wet seasons.

Fungal enumeration and identification in soils of the ID flow station with distances away from flare in the dry and wet seasons are summarized on table 1.The table showed that at 50m from flare the diversity of fungi isolated in the dry season, revealed the presence of yeast cells which had the highest value of occurrence of (72%), *Aspergillus sp* had (20%) and *Penicillium chrysogenum* was (8%). In the wet season, Yeast cells had the highest at (92.2%), *Aspergillus niger* (3.9%), *Aspergillus niger* (aggregate) (1.9 %), *Mucor sp* (1.9%) and *Penicillium chrysogenum* (0.1%), were revealed. During the dry season at 100 meters away from the flare, the isolated fungi were *Mucor sp* (50%), *Aspergillus sp* (49.1%) and *Rhizopus sp* (0.9%) while in the wet season, the fungi isolates were Yeast cells (96.1%). Aspergillus sp (1.9%), A. niger (0.9%), and Rhizopus sp (0.9%). 150 meters away from the flare in the dry season revealed the presence of the following fungi; Aspergillus sp (50%), Yeast cells (42%), and P. chrysogenum (8%), and in the wet season ,Yeast cells (92.4%), *A. niger* (aggregate) (3.8%), *A.niger* (2.5%) and *P. chrysogenum* (1.3%), were revealed. The control in the dry season had *Aspergillus sp* (92.5%), *P. chrysogenum* (7.5%) while wet season had *A.niger*, (92.5%) and *P. chrysogenum* (7.5%) isolated. ID flow station had fungal count ranging from 3 x 106 at 50 m from the flare to 79 $x10^6$ at 150 m away from the flare in the dry season and 51 $x10^6$ at 50 m from flare to 104 x 10^6 at 100 m from flare in the wet season.

Table 1: Fungal enumeration and Identification in Soils of ID Flow Station at various Distances from Flare in the Dry and Wet Season

Distance from Flare	Dry season			Wet season		
	CFU/g	PO (%)	Fungi	CFU/g	PO (%)	Fungi
50 m	$3x10^6$	72.0	Yeast cells	$51x10^6$	92.2	Yeast cells
		20.0	*Aspergillus sp.*		1.9	*A. niger aggregate*
		8.0	*P.chrysogenum*		3.9	*A. niger*
					0.1	*P. chrysogenum*
					1.9	*Mucor sp.*
100 m	10x 10^6	50.0	*Mucor sp*	104 $x10^6$	96.1	Yeast cells
		49.1	*Aspergillus sp.*		0.9	*A. niger*
		42.0	*Rhizopus sp*		1.9	*Aspergillus sp.*
					0.9	*Rhizopus sp*

150 m	$79x10^6$	42.0	*Yeast cells*	$79 x10^6$	92.4	Yeast cells
		8.0	*P.chrysogenum*		3.8	*A. niger aggregate*
		50.0	*Aspergillus sp.*		2.5	*A. niger*
					1.3	*P.chrysogenum*
Control	$71x10^6$	92.5	*A.niger*	$79 x10^6$	92.5	*A. niger*
		7.5	*P chrysogenum*		7.5	*P. chrysogenum*

Key: AT = Ambient Temperature, ST = Soil Temperature, Rad = Radiation,
CFU = Colony forming unit, PO = Percentage occurrence

Fungal enumeration and Identification in Soils of IR flow stations at various Distances from Flare in the Dry and Wet seasons.

Fungal enumeration and identification in soils of the IR flow station with distances away from the flare in the dry and wet seasons are presented on table 2. At 50 meters [distance] away from the flare in the dry season, the isolated fungi were *Aspergillus niger* aggregate (92%) and *P. chrysogenum* (8%). In the wet season at the same distance, Yeast cells (75.5%), *A. niger* (12.5%) and *A. niger* aggregate (12.0%) were isolated. At 100 meters distance away from the flare, in the dry season, only *Mucor spp* (100%) was isolated and in the wet season, *A. niger*(100%) was isolated. 150 meters away from the flare in the dry season had *Aspergillus spp* (100%) while in the wet season, yeast cells (82.40%), and *A. niger* (17.60%) were isolated. At the control, in the dry season, *Aspergillus spp* (100%) was isolated and in the wet season, yeast cells (100%) was isolated. The total fungal count ranged from $1 x 10^6$ at 100 m from flare to $300 x 10^6$ at 50 m from flare in the dry season and $33 x 10^6$ at 100 m from flare to $10^6 x 10^6$ at the control in the wet season. The *Aspergillus* group and the yeast cells in this study flow station were the dominant fungal species but was completely absent at 100 meters away from the flare, which had *Mucor spp* 100% at this distance in the wet season.

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Table 2 Fungal enumeration and identification of soils of IR station at various Distances from flare in the dry and wet seasons

Distance from Flare	Dry season			Wet season		
	Cfu/g	PO (%)	Fungi	Cfu/g	PO (%)	Fungi
50 m	300x 10^6	92 8	*A. niger aggregate* *P.chrysogenum*	49x10^6	12.5 12.0 75.5	*A. niger* *A. niger aggregate* *Yeast Cells*
100 m	1x 10^6	100	*Mucor sp*	33x10^6	100	*A. niger*
150 m	17x10^6	100	*Aspergillus spp.*	51 x10^6	17.60 82.40	*A. niger* *Yeast cells*
Control	5x10^6	100	*Aspergillus spp*	106 x10^6	100	*Yeast cells*

Key: AT = Ambient Temperature, ST = Soil Temperature, Rad = Radiation, Cfu = Colony forming unit, PO = Percentage occurrence

Fungal enumeration and Identification in Soils of OB flow stations at various Distances from Flare in the Dry and Wet seasons.

Fungal enumeration and identification in soils of OB flow station with distances away from flare in the dry and wet seasons are shown on table 3. *Aspergillus spp* (100%) was isolated at 50 m; in the dry season while Yeast cells (100%) was isolated at same distance in the wet season. At 100 meters from flare, *Rhizopus spp* (50%) and *Aspergillus spp* (50%) were isolated in the dry season and in the wet season; *Rhizopus spp* (100%) was isolated. At 150 meters away from the flare, yeast cells (100%) was isolated in the dry and wet seasons At the control, *Rhizopus spp* (50%), *Botryodiplodia threobome* (25%) and *Aspergillus spp* (25%) were isolated in the dry season, whereas at the same distance in the wet season, *Aspergillus niger* aggregate (50%) and Yeast cells (50%) were the isolated fungi. The least Cfu ranged from 2x106 at the control in the dry season to 300x106 at 150 m both seasons and at the control in the wet season.

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Table 3: Fungal enumeration and Identification in Soils of OB flow Station at various distances from Flare in the Dry and Wet Seasons

Distance from Flare	Dry season			Wet season		
	Cfu/g	PO (%)	Fungi	Cfu/g	PO (%)	Fungi
50 m	$54x10^6$	100	*Aspergillus spp.*	$54x10^6$	100	*Yeast Cells*
	$5x10^6$	50	*Rhizopus spp*	$80x10^6$	100	*Rhizopus spp*
100 m		50	*Aspergillus spp.*			
150 m	$300x10^6$	100	*Yeast cells*	$300x10^6$	100	Yeast cells
Control	$2x10^6$	50	*Rhizopus spp*	$300x10^6$	50	*A. niger*
		25	*Botryodiplodia threobome*		50	Yeast cells
		25	*Aspergillus spp.*			

Key: AT = Ambient Temperature, ST = Soil Temperature , Rad = Radiation, Cfu = Colony forming unit, PO = Percentage occurrence

Table 4; Molecularly Identified Fungi, at the various Stations, Distances and Seasons

The molecularly identified fungi isolated from the three (3) study flow stations and their seasons of sampling are presented on the table 4 below , *Aspergillus fumigates* and *Fusarium phyllophilum* strain NRRL were present at ID flow station, 50 and 150 m away from flare and IR 50 meter from flare during the rainy season and IR during the dry season. *Aspergillus fumigates*, *Aspergillus fumigates* strain IHM 15988 pigment biosynthesis protein 2 and *Fusarium phyllophilum* strain NRRL were isolated in ID 50 m, 100, and 150 m away from the flare and in IR flow station 100 and 150 meters from the flare during the rainy seasons while these organisms were not found in the dry season in any location. *Aspergillus fumigates* and *Aspergillus fumigates* strain IHM 15988 pigment biosynthesis protein 2 was found in 50,100 &150 meters from the flare in the dry and rainy seasons in the soils of the three-study flow station but IR in 50 meters from flare as well as the control in the dry season. *Aspergillus fumigates, Aspergillus oryzae (*a strain of *niger*), and *Aspergillus oryzae* (*niger*) were only in OB at 50 and 100 meters in the dry season. The combination of *Aspergillus fumigates*, *Aspergillus oryzae (niger)* and *Fusarium graminearum* chromosome 2, complete genome were found in ID 50 &100 in the rainy season and IR 50m in the dry season. The combination of *Aspergillus fumigates, Aspergillus fumigates* strain IHM 15988 pigment biosynthesis protein (2) *Fusarium phyllophilum* strain NRRL, *Fusarium graminearum chromosome 2,* complete genome and *Aspergillus oryzae (niger)* were found ID 50, IR 50, 150 and control in the rainy season and ID 50, 150 and OB 150 in the dry season.

Table 4: Molecularly Identified Fungi, Location and Seasons of Sampling

Season	Flow Station	Distance from Flare	Fungal Isolate
Wet	ID	50	A,B,C,D,F
		100	A,B,C,D,F
		150	ABC
		Control	-
	IR	50	A,B,C,D,F
		100	A,B,C
		150	A,B,C,D,F
		Control	A,B,C,D,F
	OB	50	A,B,D
		100	A,F
		150	A,B,D
		Control	A,B,D,F
Dry	ID	50	A,B,C,D,F
		100	A,B,E
		150	A,B,C,D,F
		control	-
	IR	50	A,B,C
		100	A,F,B,D
		150	A,B
		Control	A,B
	OB	50	A,E,F
		100	A,E,F
		150	A,B,C,D,E,F
		Control	-

Molecularly Identified fungi

Key: A > *Aspergillus fumigates* B > *Aspergillus fumigates* strain IHM 15988 pigment biosynthesis protein C > *Fusarium phyllophilum* strain NRRL D > *Fusarium graminearum* chromosome 2, complete genome E > *Aspergillus oryzae* (a strain of *niger*) F > *Aspergillus oryzae*

DISCUSSION

The *Aspergillus* group and yeast cells were found to be most dominant when compared to the other fungal isolates in all the stations among the diversity of fungi isolated and identified from soils of three flow stations of an oil exploration company operating in the Niger Delta area of Nigeria in the dry and wet seasons and at different distances away from the flare stack using the cultural methods. (Ukoima *et al.*,2016) in a similar study using the same method observed that *Rhizopus stolonifer* was the most prevalent fungus followed by *Aspergillus niger*. Researchers like (Nwaugo *et al.*, 2005; Ezeigbo *et al.*, 2013), observed increase in population of the soil microbes with increased distances from the flare. This does not corroborate with this study since this study didn't follow any trend in the population count which maybe resulting from flare design type in each of these flow stations. Abdukareem (2005) mentioned that the distribution of pollutants could be attributed to flare stack type and high temperatures also reduces microbial growth which eventually affect microbial diversity distribution. Isolation and identification were done culturally using biochemical processes in this study. Six fungal isolates were not clearly identified for example, *Aspergillus* aggregate and *Aspergillus spp* had similar morphological features as that of *Aspergillus niger* on PDA but beneath the plate it had different colors hence the term aggregate. According to Parenicova *et al.*, (2001) a group of different species of *Aspergillus* that are morphologically identical and different in strain can be called *Aspergillus* aggregate. Goffeau, (2005) also mentioned that *Aspergillus flavus* and *Aspergillus niger* are close relatives of *Aspergillus oryzae* because they contain syntenic genes from a singular ancestor. However, Nucleic acid analytical methods revealed identities for *Aspergillus spp*, to be *Aspergillus fumigates, Aspergillus fumigates* strain IHM 15988 pigment biosynthesis protein (2) *Aspergillus oryzae* (same score as *niger*), *Aspergillus oryzae (niger)* .Others identified molecularly were *Fusarium phyllophilum* strain NRRL and *Fusarium graminearum chromosome 2*, complete genome these were isolated but not identified hence the need for molecular method which was used to give a clear identity of these organisms. According to (*Balser et al.*,2010), often, microbial identity and function are misunderstood and only about 17% of fungi are isolated using cultural techniques. In a similar study to molecularly identify fungi capable of degrading hydrocarbon by (El Hanafy *et al.*, 2015) revealed the presence of fifteen (15) fungal isolates which included different strains of *Penicillium* and *Aspergillus spp*, but two strains that showed to be the most competent in decomposing crude oil are *Aspergillus niger* and *Penicillium commune*. ID and OB flow stations are marine environments and according to Arora (2003), fungi also degrade hydrocarbons in streams and lakes. *Aspegillus , penicillum* and yeast amongst others are some of the fungi used for bioremediation because they are good degraders of hydrocarbon.

CONCLUSION

Findings from this work have shown that molecular technique gave the exact identity of fungal species isolated from study area. All six (6) molecularly identified fungi were isolated from the three-study flow station and in both seasons. The study showed that fungal population increased

as distances increased in all three flow stations. Fungal load was higher in the wet season and favored fungal diversity. Fungal diversity isolated did not follow any particular trend in distribution and may not be influenced by season but may have been influenced by distance from flare. Fungal isolated may be dominant as a result of the ability to adapt the flare environment and are all good crude oil degraders.

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