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Screening, isolation and identification of microorganisms from petrochemical contaminated environment

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Abstract. Soil is comprised of minerals, soil organic matter, water and air. The composition and proportion of these components greatly influence soil physical properties like structure and porosity. Soil bacteria and fungi play pivotal roles in various biogeochemical cycles and are responsible for the cycling of organic compounds. The view on the microbiological safety of drinking water is changing. The demand for the total absence of any pathogenic organism is no longer significant in light of the new pathogens, some of which are capable of growing in drinking water systems. This is mainly due to many pollutants that are present at much higher concentrations in groundwater than they are in most contaminated surface supplies. In order to determine the microbes, soil and water samples were collected from petrochemical industry, Eleme, Port-Harcourt, Rivers State, Nigeria, for microbiological analysis. This was carried out by the isolation, assessment and characterization of the isolated organisms. The highest bacterial counts was determined in soil sample 1 (SS1) and water sample 4 (WS4) with microbial loads of 1.48 x 10^6 cfu/mL and 9.40 x 10^5 cfu/mL and the lowest count was found in soil sample 2 (SS2) and water sample 2 (WS2) with microbial load 2.90 x 10^5 cfu/mL and 3.67 x 10^4 cfu/mL. The highest fungal counts was determined in soil sample 2 (SS2) and water sample1 (WS1) with microbial loads of 1.76 x 10^6 cfu/mL and 2.17 x 10^6 cfu/mL and the least colonies was in soil sample 1 (SS1) and water sample 2 (WS2) with microbial counts of 1.75 x 10^5 cfu/mL and 4.30 x 10^4 cfu/mL. The results present that the presence of these microbes can be linked to the prehistory of the effects or contamination of surface and underground water in this region and could leads to water-borne diseases.

Keywords: Organic matter; Biogeochemical cycles; Pathogenic organisms; Pollutants, Microbes.

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Introduction

Soil is a natural body consisting of layers (horizons) of mineral constituents, each with different proportions and has some distinctive features which differentiate them from their parent materials due to the interaction between the lithosphere, hydrosphere and the biosphere It comprises of particles of broken rock that have been altered by chemical and environmental processes that include

weathering and erosion (Sabata and Nayar, 1995). Some of these are morphological, physical, chemical, and mineralogical characteristics (Bhatt et al., 1999). The soil contains about 10% of organic matter, this have a tremendous effect on soil chemical and physical properties. It consists of organic matter such as carbon, oxygen, hydrogen, nitrogen and smaller quantities of sulphur and other elements. The impact of soil on agricultural productivity and sustainability can be determined by analyzing the physical, chemical and biological parameters of the soil (Mohan et al., 2007).

In recent times, due to technological advancements, human activities and increasing in population density, the view on the safety of drinking water is not predictable. Due to the health effects of these waters, the demand for the total absence of any pathogenic organisms is no longer significant in light of the new pathogens, some of which are capable of growing in drinking water systems. According to the new European Union Council Directive 98/83/EC, water for human consumption must be free from any microorganisms and parasites and from any harmful substances or pollutants which concentrations either either low or high can lead or constitute a potential danger to human health (European Union Council, 1998). To combat this, this agency, that is, Environmental Protection Agency used a microbial risk assessment approach. It has been defined that an annual risk of 1.034 (one infection per 10,000 consumers per year) should be acceptable for diseases acquired through potable or drinking water. The value (4×10^{-3}) has close proximity to the annual risk of infection from waterborne disease outbreaks in the United States (USEPA, 2006).

Microbiological risk assessment is a major tool for decision making in the regulatory area. The problem is, however, that the key data to perform this assessment are mostly missing. Few epidemiological studies associating the incidence of disease to the pathogen densities have been reported. Several outcomes, from asymptomatic infection to death, are possible through exposure to microbes. The issue of dose-response relationships is alarming. The relationship is applicable to few pathogens when the dosage concentration is low most especially for viruses and protozoan (Kolade, 1982). The calculated tolerable concentrations are also low and monitoring of these pathogens in drinking water becomes impracticable (Mark et al., 2002).

Pathogenic bacteria can be transmitted through water; these are known as water-borne bacteria. The most important pathogenic bacteria transmitted by the water route is *Salmonella typhi*, this causes typhoid fever and *Vibrio cholerae*, the organism causing cholera (Arvnabh et al., 2001). Soil bacteria and fungi play pivotal roles in various biogeochemical cycles (BGC) (Ali, 1991; Roy and Kumar, 2002; Hemant et al., 2012) and are responsible for the cycling of organic compounds. Soil microorganisms also 10 influence above-ground ecosystems by contributing to plant nutrition (Tebutt, 1983), plant health (Kumar, 1997), soil structure and soil fertility. Our knowledge of soil microbial diversity is limited in part by our inability to study soil microorganisms (Bassey, 2008). They also carried out research and discovered that in 1 g of the soil sample, there are 4000 different bacterial “genomic units” based on DNA-DNA re-association. It has also been estimated that about 5000 bacterial species have been described (Rahda et al., 2007). Approximately 1% of the soil bacterial population can be cultured by standard laboratory practices.

In the attempt to analyse the microbes present in soil and water, soil and water samples were collected from a petrochemical contaminated environment. The research project was conducted with following objectives, isolation of different bacteria found in the soil and water samples collected, assessment of microbial loads in the soil and water samples collected and characterization of the isolated organisms.

Materials and methods

Collection of samples

Different soil and water samples were collected from petrochemical industry, Eleme, Port-Harcourt, Rivers state, Nigeria for microbiological analysis. These samples were transferred into a clean sample bottles and stored at 4 °C.

Preparation of potato dextrose agar (PDA) and nutrient agar (NA)

About 39 g of potato dextrose agar (PDA) was accurately weighed and dissolved in 1000 mL of deionized water. About 28 g of nutrient agar was accurately weighed and dissolved in 1000 mL of distilled water. To prevent bacterial and fungal contaminations, 2 mL of tetracycline and nystatin was added. The media were prepared, mixed thoroughly and sterilized by autoclaving at 121 °C for 15 min.

Isolation of fungi and bacteria from the collected soil and leaf samples

The microorganisms, fungi and bacteria were isolated by serial dilution plate method. About 1 g of the sample was accurately weighed and suspended in 9 mL of double distilled water to make microbial suspensions (10^{-1} to 10^{-3}). Dilutions of 10^{-2} and 10^{-3} were used to isolate both fungi and bacteria. 1 mL of microbial suspension of each concentration were added to sterile Petri dishes (triplicate of each dilution) containing 15 mL of sterile PDA and NA for bacteria and fungi isolation and incubated for four days at 4 °C (bacterial) and 37 °C for 24 h (fungal). The growth of the organisms were counted using colony counter and the microbial loads were calculated in CFU/mL although no growth was observed on fungal plates after 4 days of incubation. Therefore, only bacterial colonies were sub-cultured by streaking on new solidified nutrient agar plates to obtain pure cultures. After pure isolates have been obtained, they were stored in slant bottles for characterizations.

Characterization of the bacterial isolates

Colonial characteristics of the bacterial isolates were determined using parameters such as size, elevation, pigment, surface, opacity, edge and shape. Cellular characteristics of the isolates were determined through the following experiments:

Gram's staining

Smear of each bacterial isolate was prepared on a clean slide. In preparing the smear a drop of sterile distilled water was placed in the middle of the slide. A sterilized inoculating needle was used to pick from the bacterial colony and rubbed on the slide containing a drop of sterile distilled water. The bacterial cells were spread into a thin smear, air dried and heat fixed (Fawole and Oso, 2001). The heat fix smear was stained with crystal violet for 1 to 2 min after which the stain was poured off. The smear was rinsed off with Gram's iodine and the iodine was allowed to react for 1 minute with the smear. The slide was then washed with 95% alcohol until the violet was seen to stop running from the slide. The slide was rinsed with gentle running tap water and counterstained with safranin for 1 to 2 min. The slide was rinsed with water, blotted dry and examined under microscope with oil immersion. Gram positive cells appeared purple while gram negative cells appeared pink.

Motility test

The hanging-drop method was used to determine the motility of the bacterial isolates according to the method of Olutiola et al. (1991) and was examined immediately under the X40 objective lens.

Spore staining

Heat-fixed smear of each isolate was prepared in a slide. Malachite green solution was added to the smear and steamed for 10 minutes. The stain was not allowed to dry out. The stain was then

washed off with cold water. The smear was counterstained with safranin solution for 15 s. It was wash with water, blotted dry and examined under the microscope with the oil-immersion objective (Olutiola el al., 1991). Spores appeared green and bacterial cells appeared red.

Capsule staining

Air-dried smear of each isolate was prepared and fixed on a slide. Crystal violet was applied on the slide for 2 min and the slide was then steamed for 40 min. The crystal violet was rinsed off with copper sulphate solution. Each slide was blotted carefully, dried in the air and examined under the microscope using oil immersion lens. Bacterial cells appeared deep violet while the capsules appeared pale violet.

Catalase test

The catalase test was carried out according to the method of Fawole and Oso (2001) using a thick emulsion of each test organism and several drops of 3% hydrogen peroxide were added on each of the slides.

Oxidase test

A filter paper was soaked in 1% sodium oxalate solution. A portion of each bacterial colony was picked and rubbed on the filter paper. A blue colour change within 10 s indicated the production of the enzyme oxidase.

Methyl red test

Accurately 10 mL of glucose phosphate broth was prepared into different test tubes. The test tubes were then inoculated with different bacterial isolates. The test tubes were incubated for 3 days at 37 ^{0}C. After 3 days, 5 drops of methyl red indicator was added to 5 mL of each cultured broth. Acid production was indicated by a yellow colouration.

Indole test

1% tryptone broth was prepared in different test tubes. The test tubes were inoculated with each bacterial isolate. The tubes were then incubated for 48 h at 35 °C. After incubation, 2 mL of chloroform was added to each broth culture and was shook gently. 2 mL of Kovac's reagent was added to the broth culture and shook gently. The tubes were allowed to stand for 20 min in order to permit the reagent to rise to the top. A red colour at the reagent layer indicated indole production.

Starch hydrolysis

The starch hydrolysis was carried out according to the method of Fawole and Oso (2001).

Citrate utilization

Citrate agar plates were inoculated with the bacterial isolates using streaking method. The plates were incubated at 37 °C for 24 h. Colour change from green to blue on the plates indicated citrate utilization by the test organisms.

Sugar fermentation

The sugar tested for fermentation included fructose, maltose, lactose, sucrose and glucose. Nutrient broth containing 0.5% of each of the sugar was prepared. Two drops of 0.01% phenol red indicator was added to each of the broth media. 10 mL of each of the broth media was dispensed into test tubes containing inverted Durham tubes. The media setup was sterilized by steaming for 30 min on three successive days.

Each indicator-sugar-broth was inoculated with a loopful of each of the bacterial isolates. One test tube of each medium was left uninoculated as a control. The test tubes were incubated at 35 °C for 4 days. Growth occurred in the inoculated tubes and absent in uninoculated tubes. The change in colour from red to yellow indicated acid production and the presence of air space in the Durham tubes.

Oxygen relationship

McCartney bottles containing sterile nutrient agar were used. The bottles were inoculated while at semi-solid state with each of the bacterial isolate using stabling technique. The agar in the McCartney bottles were allowed to solidify and incubated at 37 °C for 48 h. Anaerobes grew at the bottom of the bottles, aerobes grew on the surface and facultative

anaerobes grew from the bottom through the bottles to the top (Fawole and Oso, 2001). The fungal isolates were identified with the help of Bunett et al. (1998) and Mark et al. (2002).

Results and discussion

The isolation of microorganisms of the collected different soil and water samples from petrochemical industry, Eleme, Port-Harcourt, Rivers State, Nigeria was carried out using nutrient agar and potato dextrose agar as media. Many organisms were isolated from both soil and water samples in Table 1. The highest bacterial counts was determined in soil sample 1 (SS1) and water sample 4 (WS4) with microbial loads of 1.48 x 10^6 cfu/mL and 9.40 x 10^5 cfu/mL, respectively, followed by soil sample 3 (SS3) and water sample 1 (WS1) with microbial count of 6.70 x 10^5 cfu/mL and 3.10 x 10^5 cfu/mL, respectively, then soil sample 4 (WS4) and water sample 5 (WS5) with microbial loads of 3.90 x 10^5 cfu/mL and 2.80 x 10^5 cfu/mL, respectively. The lowest fungal count was found in soil sample 2 (SS2) and water sample 2 (WS2) with microbial load 2.90 x 10^5 cfu/mL and 3.67 x 10^4 cfu/mL and the least bacterial colonies was in soil sample 5 (WS5).

Also, from Table 1, a quite a number of organisms were isolated from both soil and water samples for fungi. The highest fungal counts was found in soil sample 2 (SS2) and water sample 1 (WS1) with microbial loads of 1.76 x 10^6 cfu/mL and 2.17 x 10^6 cfu/mL, respectively, followed by soil sample 4 (SS4) and water sample5 (WS5) with microbial count of 4.50 x 10^5 cfu/mL and 5.60 x 10^5 cfu/mL, respectively. The least bacterial colonies was in soil sample 1 (SS1) and water sample 2 (WS2) with microbial counts of 1.75 x 10^5 cfu/mL and 4.30 x 10^4 cfu/mL, respectively.

There are a quite numbers of microorganism, bacteria and fungi isolated from the samples (soil and water) collected from the petrochemical industry, Eleme, Port-Harcourt, Rivers State, Nigeria. These organisms were shown in Table 2.

Table 1. Results of microbial counts of the bacterial and *E. coli* form colonies.

Samples	DF	NA (cfu/mL)	PDA (cfu/mL)
SS1	10^{-2}	2.28×10^5	2.14×10^4
	10^{-3}	1.48×10^6	1.75×10^5
SS2	10^{-2}	4.10×10^3	1.70×10^4
	10^{-3}	2.90×10^5	1.76×10^6
SS3	10^{-2}	1.54×10^5	4.0×10^4
	10^{-3}	6.70×10^5	3.3×10^5
SS4	10^{-2}	9.80×10^3	9.30×10^3
	10^{-3}	3.90×10^5	4.50×10^5
SS5	10^{-2}	3.23×10^4	2.70×10^4
	10^{-3}	2.49×10^5	2.0×10^5
WS1	10^{-2}	2.63×10^5	3.50×10^5
	10^{-3}	3.10×10^5	2.17×10^6
WS2	10^{-2}	1.09×10^4	3.1×10^4
	10^{-3}	3.67×10^4	4.3×10^4
WS3	10^{-2}	1.10×10^4	4.69×10^4
	10^{-3}	2.84×10^4	1.78×10^5
WS4	10^{-2}	1.72×10^5	6.2×10^4
	10^{-3}	9.40×10^5	4.0×10^5
WS5	10^{-2}	4.00×10^3	1.80×10^3
	10^{-3}	2.80×10^5	5.60×10^5

Table 2. Bacteria and fungi probable organisms isolated from respective soil and water sample.

Sample	Bacteria	Fungi
SS1	*Pseudomonas aeruginosa*, *Bacillus cereus*, *Acetobacter* spp	*Aspergillus niger*, *Penicillium chrysogenum*
SS2	*Lactobacillus* spp, *Pseudomonas putida*, *Nictrobacter* spp, *Staphylococcus aureus*	*Aspergillus niger*, *Aspergillus flavus*
SS3	*Staphylococcus aureus*, *Acetobacter* spp, *Nictrobacter* spp	*Aspergillus vasisolor*, *Aspergillus flavus*
SS4	*Bacillus cereus*, *Bacillus substilis*, *Pseudomonas aeruginosa*	*Aspergillus niger*, *Penicillium chrysogenum*
SS5	*Nitrobacter* spp, *Staphylococcus aureus*	*Aspergillus niger*, *Aspergillus flavus*
WS1	*Clostridium* spp, *Staphylococcus aureus*, *Pseudomonas aeruginosa*	*Aspergillus vasisolor*, *Aspergillus niger*
WS2	*E. coli*, *Staphylococcus aureus*, *Lactobacilli* spp	*Aspergillus niger*, *Aspergillus flavus*
WS3	*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Lactobacilli* spp	*Aspergillus niger*, *Aspergillus flavus*
WS4	*Pseudomonas putida*, *Clostridium* spp, *Staphylococcus aureus*	*Aspergillus flavus*, *Aspergillus vasisdor*
WS5	*Pseudomonas putida*, *E. coli*, *Pseudomonas aeruginosa*	*Aspergillus niger*, *Penicillium chrysogenum*

The results obtained showed that there will be highest microbial activities of bacteria in the soil and water samples collected form soil sample 1 (SS1) and water sample 4 (WS4) because they had the highest microbial counts while the least microbial activities will be in the soil and water samples collected from soil sample 5 (SS5) and water sample 3 (WS3) because they had the least microbial loads. Also, this means that there will the highest microbial activities of fungi in the soil and water samples collected from soil sample 2 and water sample 1 because they had the highest microbial counts while the least microbial activities will be in the soil and water samples collected from soil sample 1 and water sample 2 because they had the least microbial loads. *Escherichia coli* are the most widely adopted indicator of faecal pollution and they can also be isolated and identified simply, with their numbers usually being given in the form of faecal coliforms/100 mL of wastewater (Parihar et al., 2003). Outbreaks of these diseases can occur as a result of, drinking water from wells polluted by a combination of different wastewater microorganism species, eating contaminated fish, or indulging in recreational activities in polluted water bodies containing water borne pathogen. *E. coli* cause urinary tract infection and diarrheal (Hemant et al., 2012).

A large variety of bacteria, fungi, viral and protozoan pathogens are capable of initiating waterborne infections. Some are primarily the enteric bacterial pathogens including classic agents such as *Vibrio cholerae*, *Salmonella* spp., *Shigella* spp., and newly recognized pathogens from faecal sources like *Campylobacter jejuni* and enterohemorrhagic *E. coli*. The survival potential of these bacteria increases in biofilms and due to their stages as VBNC (viable but non-culturable) cells. Several new bacterial pathogens such as *Legionella* spp., *Aeromonas* spp, *Pseudomonas aeruginosa* and *Mycobacterium avium* have a natural reservoir in the aquatic environment and soil. These organisms are introduced from the surface water into the drinking water system usually in low numbers. They may survive and grow within the distribution system biofilm (Wilson et al., 1983). The presence of bacteria is of great importance in the water industry with regards to water-borne diseases. Some of such diseases are

dysentery, typhoid fever, paratyphoid fever, cholera, infantile paralysis, poliomyelitis, infectious hepatitis, guinea worm, amoebic dysentery, amongst others. Transmission of the causative micro-pathogenic organism is through direct or indirect contamination of water source by human excreta most especially the microorganisms present in the soil and water (Edema et al., 2001). Since it is extremely difficult to isolate and identify different forms of pathogens, the microorganisms which are of significance to water quality are those of enteric pathogenic origin.

Water-borne diseases are diseases contracted through the ingestion of contaminated water. Table 3 present some of such diseases and their causative agents (Mills, 2000).

Table 3. Some water-borne diseases and their causative agents.

Disease	Causative organism
Bacterial dysentery	*Shigella dysentera*
Typhoid fever	*Salmonella typhii*
Para typhoid fever	*Salmonella paratyphii*
Cholera	*Vibrio cholera*
Amoebic dysentery	*Entamoeba histolytic*
Infantile paralysis (Poliomyelitis)	Poliomyelitis virus
Infectious hepatitis	Hepatitis virus
Guinea worm	*Dracunculus mendenensis*

Conclusion

Due to the long time the indigenous bacteria have had to degrade the organic matter originally present, subsurface environments contain little organic matter. Furthermore, when percolating through the porous media, water containing organic matter encounters attached bacteria which remove most of this organic matter. The microbial studies can be utilized for the prevention of any pathogenic diseases caused by the microbes found in soil and water. Regular check up of the aquatic life can help in maintaining ecological balance.

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Conflict of interest

The authors declare that they have no competing interests.

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