ISSN 2989-0705 (Online)|Vol 3|Issue 1|January 2025

Biodegradation of Polycyclic Aromatic Hydrocarbons in Fuel Station Soil: Impact of Immobilized Bacteria on Microbial Communities

D. Senevirathna[1], S.N.R.D.S. Jeewakarathne[1], K. Balasayanthan[1], M.S. Vanderwall[1], M.N. Fathima Shahani[1], S.M. Fernando[1], H.O.T.O. Perera[1] and R.B.N. Dharmasiri[1*]

[1]School of Science, Business Management School (BMS), Sri Lanka.

[*]nadeema.d@bms.ac.lk

Abstract

Polycyclic aromatic hydrocarbons (PAH) are made with carbon and hydrogen with two or more fused benzene rings. These substances are known to be environmental pollutants as they cause serious damage to the health of humans and other living organisms due to their carcinogenic properties. The incomplete combustion of fossil fuels and other organic substances can also produce PAHs, which are naturally occurring substances of crude oil and other petrochemical products. Forest fires and volcanic eruptions are examples of natural sources of PAH emissions. Anthracene and pyrene are two pollutant PAHs that are found in the environment. The aim of this study is to isolate and identify PAH degrading soil bacteria from polluted areas. For this analysis soil samples from three different geographical locations such as Peliyagoda, Jaffna and Gampaha were collected from fuel stations and isolated bacteria that are capable of degrading the above-mentioned PAHs. According to the primary screening and secondary screening *Microbacterium paraoxydans*, *Burkholderia multivorans*, *Staphylococcus haemolyticus* strains had above 80% degradation percentage for anthracene whereas DG2, and DJ1 had 70% degradation for pyrene. Furthermore, the Antibiotic susceptibility test demonstrates that bacteria were inhibited by certain antibiotics such as erythromycin, ampicillin, and gentamycin. Antagonistic tests proved that there is no inhibition of bacteria surrounding them which proved that it can be used in the bacterial consortium. Toxicity assay was done using mung seeds and brine shrimps proving that there is no toxicity to the environment by the introduction of these microbes. In conclusion, the bacteria identified in this study possess the potential to serve as biological agents for the effective treatment of anthracene and pyrene PAHs in contaminated soils, thus contributing positively to environmental remediation efforts.

Keywords: Polycyclic Aromatic Hydrocarbons, Anthracene, Pyrene, Degradation percentage, Toxicity assay

1. Introduction

Soil pollution has become a severe problem due to its formation from both natural and anthropogenic sources that include both industrial and agricultural activities.[1] This can further affect the soil layer strength on top of the soil reducing the fertility and the biological activity of the soil. Soil pollution is regarded as the contamination of soil with toxic substances, chemicals and certain substances that can affect the soil fertility and its quality.[2] Contaminated soil further affects the quality of groundwater as these pollutants can leach down with time, thus affecting both humans and the ecosystem.[3] The increased amount of environmental contamination is the main reason for the prevalence of Polycyclic aromatic hydrocarbons (PAHs) in soils. The power industry and large thermal companies are the most effective sources of pollution with PAHs in the environment.

The quality of agricultural production has a direct impact on human exposure, which elevates the likelihood that PAHs may accumulate in soil. To minimize the hazards of contaminated affected soils, a better understanding of the interconnected issues is crucial.[4] PAHs are toxic substances in nature that are broadly found in the aquatic systems and terrestrials. PAHs are composed of more than two fused benzene rings and are a result of

incomplete combustion of fossil fuels, coal and crude oil.[5] Over-exposure to these PAHs can lead to kidney damage, liver damage and cataracts.[6] Certain polycyclic aromatic hydrocarbons are suspected carcinogens, and prolonged exposure can lead to adverse health effects including cancers and reproductive disorders. The incomplete combustion of fossil fuels and other organic substances can also produce PAHs, which are naturally occurring substances of crude oil and other petrochemical products. Forest fires and volcanic eruptions are examples of sources of PAH emissions, but the number of manmade sources like incomplete combustion or the spilling of fossil fuels, have increased and is still a significant environmental burden. 16 PAHs were nominated as to be harmful pollutants by the US United States Environmental Protection Agency (USEPA) in 1997 based on their proven carcinogenicity and mutagenicity as well as their high of occurrence in the ecosystem.[7] PAHs are degraded by different types of bacteria by secreting enzymes such as peroxidases and oxygenases.[8] *Sphingomonas* and *Rhodococcus* are two types of bacteria capable of degrading PAHs and are responsible for the production of biosurfactants. This causes tension in water molecules to be minimized and causes the entrapment of PAHs freely available on the surface for degradation by bacteria.

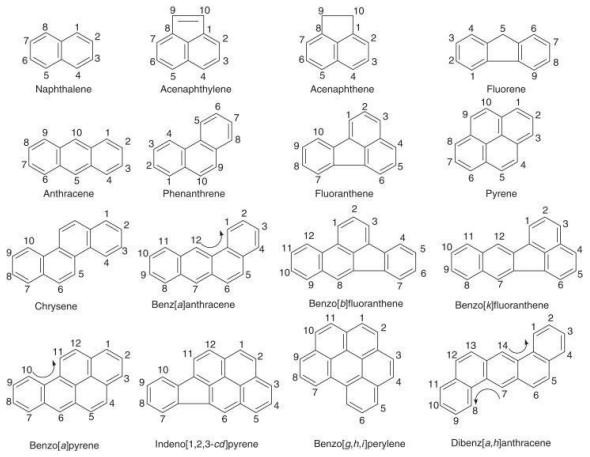

Figure 1. 16 types of priority pollutant PAHs present in environment.[9]

2. Methodology

2.1 Sample Collection. Samples were collected from three different sub-locations Gampaha (7°5'4"N 80°4'45"E), Jaffna (9°39'59.99" N 80°00'0.00" E) and Peliyagoda (6°57'21"N 79°53'10"E). These were collected into a Ziplock bag under hygiene conditions and placed in refrigerator.

2.2 HPLC environmental analysis. Each soil sample weighed around 4 g, which was then dissolved in 10 mL of hexane and shaken at 180 rpm for three minutes. After that, the supernatant was separated and allowed to evaporate. The residues were then thoroughly cleaned with 1 mL of acetonitrile. After passing through 0.22 µm nylon syringe filters, the samples were collected and placed in HPLC vials. Pyrene and anthracene concentrations of 1.25 mg were tested independently. After that, 5 mL of acetonitrile were added to the PAHs to prepare a stock concentration of 250 ppm. Pyrene and anthracene, two common PAHs, were produced at concentrations ranging from 250 ppm to 50 ppm [10].

An Agilent was used to analyze the filtered samples and assess the extract's pyrene and anthracene concentration. A 90:10 (acetonitrile: water) combination was used as the mobile phase for the PAHs at a flow rate of 3 mL per minute. The wavelength of the HPLC was 254 nm.

2.3 Soil Bacteria Isolation

2.3.1 Serial dilution. After weighing around 5 g of each sample, it was put into the conical flask and shaken for an hour at 180 rpm. After being cleaned, the soil samples were diluted to a 10^{-10} ratio.

2.3.2 Spread plate technique. An aliquot of 100 µL of each diluted sample was aseptically dispensed onto the center of a nutrient agar (NA) plate. The bacterial suspension was then evenly spread across the agar surface using a sterile glass spreader. After that, the plate was securely closed and parafilm-sealed. all the inoculated plates were incubated at room temperature for 24 hours.

2.3.3 Streak plate technique. Following incubation, bacterial colonies with distinct morphologies were chosen from the spread plates. The chosen colonies

then underwent streaking for isolation. Each of the chosen colonies underwent a 24-hour incubation period at 30°C using the same streaking approach.

2.4 Plate Assay (Primary Screening)

2.4.1 Bacterial Starvation in Bacto-Bushnell Hass (BBH) agar plates. Following the preparation of the solidified BBH agar plates, a single colony of each bacterial isolate was inoculated into 25 designated sections on separate BBH agar plates After inoculating each plate, The plates were then securely sealed with parafilm, incubated at room temperature for three days under starvation circumstances[11].

2.4.2 Starved bacteria being transferred into Anthracene and pyrene plates. First, an acetone solution containing 100 ppm of anthracene and pyrene was prepared. Subsequently, 100 µL of pyrene and anthracene solution were applied separately to solidified BBH agar plates. To evaporate the acetone, the plates were gently swabbed with a cotton applicator and left partially uncovered for a brief period. After the acetone had evaporated, the plates were inverted, and 25 equal-sized squares were marked on each plate. Each bacterial colony, which had been subjected to starvation conditions, was then carefully transferred into the corresponding square on the plate. Once all colonies were inoculated, the plates were sealed with parafilm and incubated at room temperature for 24 hours.

2.5 *Spectrophotometric analysis.* After sterilizing the test tubes, 2% methylene blue was added, and the PAH was added as a spike. An inoculation loop was used to add the corresponding bacterial colonies to the medium. The incubated samples were used for the spectrophotometric analysis. Before analysis, a blank sample was added, and negative results were recorded. Each experimental sample was examined in triplicate, and the average percentage of deterioration was calculated.

2.6 DNA extraction. Three of the finest bacteria for breaking down PAHs were subcultured, and the bacterial colonies were gathered into sterile Eppendorf tubes. After adding 1 mL of 0.9% NaCl to each tube, the tubes were centrifuged for three minutes at 13,000 rpm, and the resulting supernatant was discarded. This process was carried out three times, with the resulting supernatant being discarded. After that, 20 µL of Tris-EDTA (TE) buffer was added to each tube, and they were completely mixed by vortexing. Following freezing of the tubes on ice for 15 minutes, they were thawed in a warm water bath for another 15 minutes at 95 °C. The samples were then centrifuged for five minutes at 13,000 rpm and the acquired supernatant of each sample was put into a fresh Eppendorf tube, and each tube was well mixed with 20 µL of 100% ethanol. Then, the three Eppendorf tubes were securely sealed and stored at -25°C[11].

Table 1. PCR components, their volumes and concentrations for preparing master mix for bacterial DNA.

Component	Stock Concentration	Working Concentration	Volume required
Go taq green PCR master mix	2×	2×	12.5µL
Nuclease free water	-	-	8.5µL
Forward primer (F2 7)	100M	10M	1µL
DNA template	-	-	2µL
Reverse primer (R1 492)	100M	10M	1µL
PCR mix			25µL

Table 2. Thermal cycling conditions

Step	Temperature	Time
Initial Denaturation	94ºC	3 minutes
Denaturation	94ºC	30 seconds
Annealing	59ºC	1 minute
Extension	72ºC	1 minute
Final extension	72ºC	10 minutes

PCR amplification was carried out in a 25 µL reaction volume using a thermal cycler according to the above-mentioned PCR conditions (Table 2) which included 35 cycles of denaturation, annealing, and extension. For visualization of the PCR products, 1 % agarose gel was loaded with 2 µL of a 1KB DNA ladder and 2 µL of PCR products. The gel was set up to run for forty minutes at 55 volts. A gel documentation system was then used to visualize the PCR amplicons and to determine their sizes with reference to DNA ladder. Following the trimming of the sequencing data with the BioEdit program, the species were identified using the BLAST tool in NCBI.

2.7 Antibiotic susceptibility test. Following the preparation of NA plates, a standardized bacterial inoculum was evenly spread across the surface of each plate. The antibiotic discs were placed onto the inoculated NA plates and incubated overnight. The inhibitory zone diameter around each antibiotic disc was then measured. These readings were then compared to the standards established by the Clinical and Laboratory Standards Institute (CLSI).

3. Results and Discussion

3.1 *HPLC (*High-pressure liquid chromatography) *analysis.* The findings of the environmental analysis test using HPLC showed that the soil samples contained 50 ppm of pyrene and anthracene.

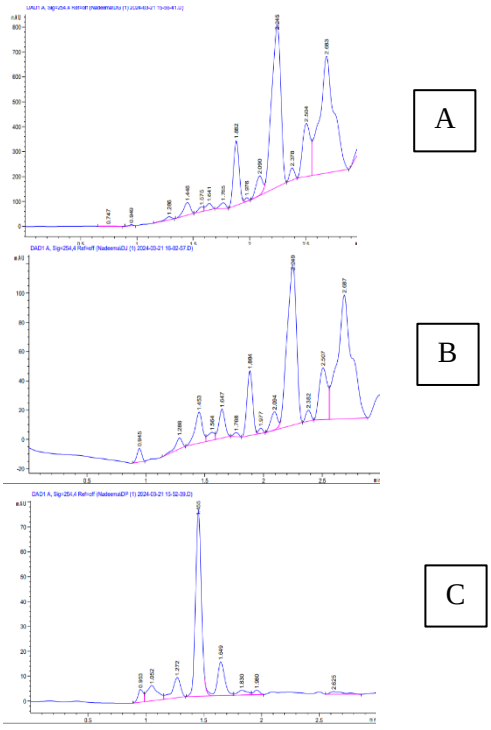

Figure 2. HPLC results for *Microbacterium paraoxydans* (A), *Burkholderia multivorans* (B), *Staphylococcus haemolyticus* (C) samples.

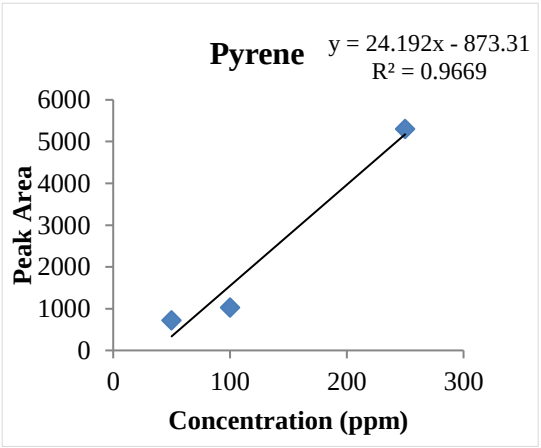

Figure 3. Standard curve of Pyrene

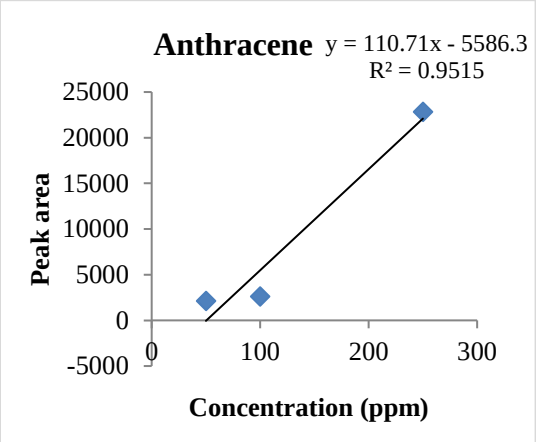

Figure 4. Standard curve of Anthracene

Table 3. Sample concentration

PAHs	DP /ppm	DG /ppm	DJ /ppm
Pyrene	49.902	75.242	45.263
Anthracene	56.749	62.199	55.752

The standard concentrations (50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm) were used to produce the standard curve using HPLC.

Following the incubation of spread plates, bacterial growth exhibited characteristic colony morphologies. Table 4 lists the several bacterial colonies that were discovered based on their morphology.

3.2 Population Density. The population density of each of the eight colonies is displayed in Table 5, with DJ 2 10^{-10} having the highest population density (6 $X10^{11}$). Single bacterial isolates of the morphologically distinct bacterial colony were obtained following the incubation of streak plates. This facilitated the identification of the bacterial colonies as distinct individual isolates that require more research.

Table 4. Different morphologies obtained from spread plate assay.

Colony	**Size**	**Colour**	**Texture**	**Elevation**	**Form**	**Margin**
DJ1 10^{-10}	small	white	smooth	convex	round	entire
DJ2 10^{-10}	small	white	smooth	convex	round	entire
DG2 10^{-10}	moderate	faded white	mucous	raised	irregular	lobate
DP1 10^{-10}	moderate	creamy white	smooth	raised	irregular	entire
DG3 10^{-5}	small	yellow	mucous	raised	irregular	entire
DG2 10^{-5}	small	faded white	mucous	umbonate	irregular	undulate
DJ1 10^{-10}	large	white creamy	smooth	convex	irregular	entire
DP2 10^{-10}	small	light orange	smooth	flat	round	entire
DJ3 10^{-5}	small	light yellow	smooth	raised	round	entire
DG1 10^{-10}	moderate	faded yellow	smooth	raised	irregular	lobate

Equation 01.

$$\text{Population Density}(\text{CFU/mL}) = \frac{\text{Number of isolated colonies}}{\text{Amount of diluted sample used in spread plate (mL) x Dilution factor}} \text{ x 1 mL}$$

Table 5. Calculation of population density

Colony samples	Number of Isolated Colonies in the sample	Dilution Factors	Population Density (CFU/mL)
DG1 10^{-10}	212	10^{-10}	2.12 $X10^{11}$
DP2 10^{-10}	2	10^{-10}	$2X10^{11}$
DJ2 10^{-10}	6	10^{-10}	6 $X10^{11}$

3.3 Primary screening technique (Plate assay)

Table 6. Plate assay results for DG1 10^{-10}, DP2 10^{-10}, DJ2 10^{-10}

Bacterial sample	Pyrene	Anthracene
DG1 10^{-10}	25/25	16/25
DP2 10^{-10}	2/25	25/25
DJ2 10^{-10}	5/25	19/25

As indicated in Table 6, all bacterial strains demonstrated positive screening results, allowing for additional screening to be carried out. Additionally, using phenanthrene and naphthalene as the only carbon sources, the replica plate findings demonstrated that over 80% of the isolated bacteria could thrive on BBH agar medium supplemented with 100 ppm anthracene and pyrene.

3.4 Spectrophotometric Analysis. Figure 5 displays the percentages of bacterial strains that degraded PAHs. This guarantees that The bacterial strains could break down PAH contaminants. These bacterial species caused a color change in the BBH broth by reducing the methylene blue indicator as a result of the oxidation of PAH. The UV-Vis spectrophotometer can detect this color shift as an absorbance value, and the overall color of the isolates change from blue to colorless suggesting their potential as hydrocarbon oxidisers.[8]

More than 80% of anthracene could be broken down by the bacterial strains DJ3 10^{-10}, DG2 10^{-5}, DG3 10^{-5} along with DG2$10^{-5}$ degraded more than 80% of pyrene.

Pyrene is broken down by bacterial strains at a lower rate than anthracene given pyrene contains four fused benzene structures, whereas anthracene has three. Consequently, the bacterial strains may readily degrade the structure of anthracene, unlike pyrene.[10]

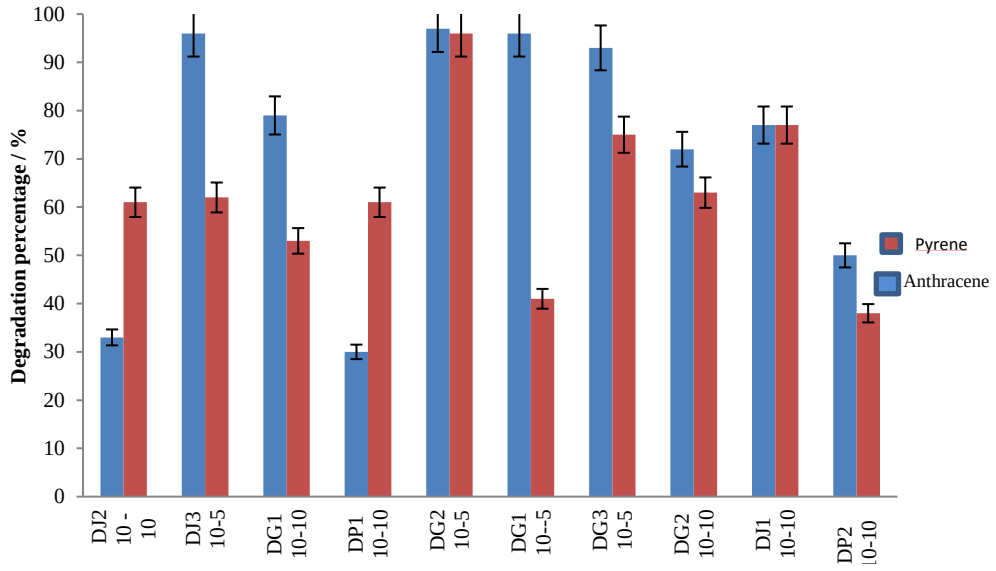

Figure 5. PAHs degradation percentage of bacteria from fuel station soil samples measured at a wavelength of 609 nm.

Table 7. Accession numbers of the PAHs

Sample name	**Bacterial strain**	**Accession number**
DJ2	*Microbacterium paraoxydans* strain	PQOO8540
DG1	*Burkholderia multivorans* strain	PQ008496
DP2	*Staphylococcus haemolyticus* strain	PQ002183

The antimicrobial susceptibility test shows that the tested bacterial strains have a high level of antibiotic resistance. Table 8 shows the larger diameters in ampicillin inhibitory zones in strains DP2, DJ2, and DG1 suggested effectiveness of the antibiotic to the bacterial strains.

Strains DP2 and DJ2 lacked Erythromycin inhibition zones, whereas strains DP2 and DJ2 further lacked Chloramphenicol and Gentamycin inhibition zones, suggesting further antibiotic resistance. Additionally, vancomycin exhibited greater zone of inhibitions in all three bacterial samples. Conversely, remaining bacterial colonies had respectable inhibition zones, suggesting that appropriate antimicrobial agents may affect their growth. The data revealed, most of the tested strains exhibited resistance to at least one antimicrobial agent highlighting the significance of selecting antibiotics with caution when treating illnesses caused by certain bacterial strains.

Table 8. Antibiotic susceptibility readings for DP2, DJ3, DJ2, DG3 samples.

ABST	DP2	DJ2	DG1
Tetracycline	2.50	3.00	2.10
Ampicillin	0.00	0.00	0.00
Vancomycin	1.90	2.40	2.40
Erythromycin	0.00	0.00	1.1
Chloramphenicol	0.00	0.00	3.00
Gentamycin	0.00	0.00	2

Table 9. Toxicity assay done for every hour

Zootoxicity					
Time	**PAH**	**DG1**	**DP2**	**DG3**	**DJ2**
11.30am	Pyrene Anthracene	10 10	9 10	9 10	10 10
12.30pm	Pyrene Anthracene	10 9	10 10	10 9	10 9
1.30pm	Pyrene Anthracene	10 9	9 10	9 9	9 9
2.30pm	Pyrene Anthracene	10 9	9 9	9 9	9 9
3.30pm	Pyrene Anthracene	10 9	9 9	9 8	9 9
4.30pm	Pyrene Anthracene	10 9	9 9	9 8	9 8

Table 10. Phytotoxicity results

Phytotoxicity			
Plant no.	**Height/cm**	**Negative/cm**	**Positive/cm**
1	11.5	28.2	8.2
2	16.6	23.9	9.3
3	19.8	25.7	9.5
4	17.6	23.0	9.0
5	19.4	27.8	9.6
6	18.2	20.3	7.1

Phytotoxicity was performed where the bacterial samples were added to the soil followed by the planting of mung seeds which were watered daily. The plant heights were measured to assess if these PAHs had an impact on the plant growth (Table 10). Growth measurements indicated that the PAHs inhibited plant development, as evidenced by reduced plant height in the experimental group compared to the positive control, which exhibited slower growth.

Additionally, the phytotoxicity of the PAHs was further evaluated using *Artemia salina* (Table 9). This was performed each of the 10-brine shrimp was monitored hourly for mortality, providing an assessment of the potential toxic effects of the PAHs *A. salina* was used in toxicity assay because of its low cost, high reproducibility, simplicity, ability they can adapt to the environment, and they can remain usable for years if stored in dried place.[12] *M. paraoxydans and B. multivorans* strains mostly survived for the time intervals as stated but *S. haemolyticus* strain had a gradual decrease in the count with time.

4. Conclusion

This study identified bacterial strains capable of breaking down pyrene and anthracene and the percentage of degradation was assessed. These results provide credence to the potential effectiveness of bacterial bioremediation of anthracene and pyrene. As a result, these strains can be employed as possible biological agents to reduce soil pollution from pyrene and anthracene, enhance soil quality to encourage plant development and reduce the risk of pyrene and anthracene exposure to humans. These findings provide a number of opportunities for further research to investigate the application of these high-efficiency degraders for effective bioremediation techniques to purify the soil. Furthermore, the current study demonstrates that the novel isolates were able to eliminate a greater amount of pyrene and other chemicals than the isolates the scientists had previously studied.

References

1. P.D. Boehm. *Environmental Forensics*, 1964;**313**;37.
2. P. Chaudhary, M. Xu, L. Ahamad, A. Chaudhary, G. Kumar, B.S Adeleke, K.K Verma, D.M Hu, I. Širić, P. Kumar and S.M Popescu. *Agronomy*, 2023;**13**(3)643.
3. T.O. Elufisan, I.C Rodríguez-Luna, O.O Oyedara, A. Hernández and D. Gonzalez. *PeerJ*, 2020;**6**;8102.
4. G.J Udom, C. Frazzoli, O.C Ekhator, A.P Onyena, B. Bocca and O.E Orisakwe. *Environmental Research*, 2023;**1**;115620.
5. V. Kumar, N.C Kothiyal, Saruchi, P. Vikas and R. Sharma. *Journal of the Chinese advanced materials society*, 2016;**4**(4);302-21.
6. U. Samarajeewa. *Journal of the National Science Foundation of Sri Lanka*, 2023;**51**(2).
7. K. Ravindra, R. Sokhi and R.V Grieken. *Atmospheric environment*, 2008;**42**(13);2895-921.
8. A.K Haritash and C.P Kaushik. *Journal of hazardous materials*, 2009;**169**(1-3);1-5.
9. W. Wang, L. Wang and Z.Shao. *Applied and environmental microbiology*, 2018;**31**;84(21).
10. R.B Dharmasiri, L.J Undugoda, A.H Nilmini, N.N Nugara, P.M Manage and D. Udayanga. *International Journal of Environmental Science and Technology*, 2023;**20**(12):13359-72.
11. R.B Dharmasiri, A.H Nilmini, L.J Undugoda, N.N Nugara, D. Udayanga and P.M Manage. *In Proceedings of the 6th international conference on multidisciplinary approaches (iCMA)*, 2019;**25**.
12. N. Dharmasiri, S. Kannangara, L. Undugoda, J. Munasinghe, R. Madushika, K.M Thambugala, C. Gunathunga and D. Pavalakumar. *New Zealand Journal of Botany*, 2024;**6**;1-21.