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RESEARCH

Heavy metals assessment of ecosystem polluted with wastewaters and taxonomic profiling of multi-resistant bacteria with potential for petroleum hydrocarbon catabolism in nitrogen-limiting medium

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Abstract
The coexistence of heavy metals (HMs) and petroleum hydrocarbons (PHs) exacerbates ecotoxicity and impair the drivers of eco-functionalities that stimulate essential nutrients for the productivity of the impacted environment. Profiling the bacteria that stem the ecological impact via HMs sequestration and PHs catabolism with nitrogen fixation is imperative to bioremediation of the polluted sites. The sediment of site that was consistently contaminated with industrial wastewaters was analysed for ecological toxicants and the bacterial strains that combined HMs resistance with PHs catabolism in a nitrogen-limiting system were isolated from the sediment and characterized. The geochemistry of the samples revealed the co-occurrence of the above-benchmark concentrations of HMs with the derivatives of hydrocarbons. Notwithstanding, nickel and mercury (with 5% each of the total metal concentrations in the polluted site) exhibited probable effect concentrations on the biota and thus hazardous to the ecosystem. Approx. 31% of the bacterial community, comprising unclassified Planococcaceae, unclassified Bradyrhizobiaceae, *Rhodococcus*, and *Bacillus* species, resisted 160 µmol Hg^{2+} in the nitrogen-limiting system within 24 h post-inoculation. The bacterial strains adopt volatilization, and sometimes in combination with adsorption/bioaccumulation strategies to sequester Hg^{2+} toxicity while utilizing PHs as sources of carbon and energy. Efficient metabolism of petroleum biomarkers (> 87%) and Hg^{2+} sequestration (≥ 75% of 40 µmol Hg^{2+}) displayed by the selected bacterial strains portend the potential applicability of the bacilli for biotechnological restoration of the polluted site.

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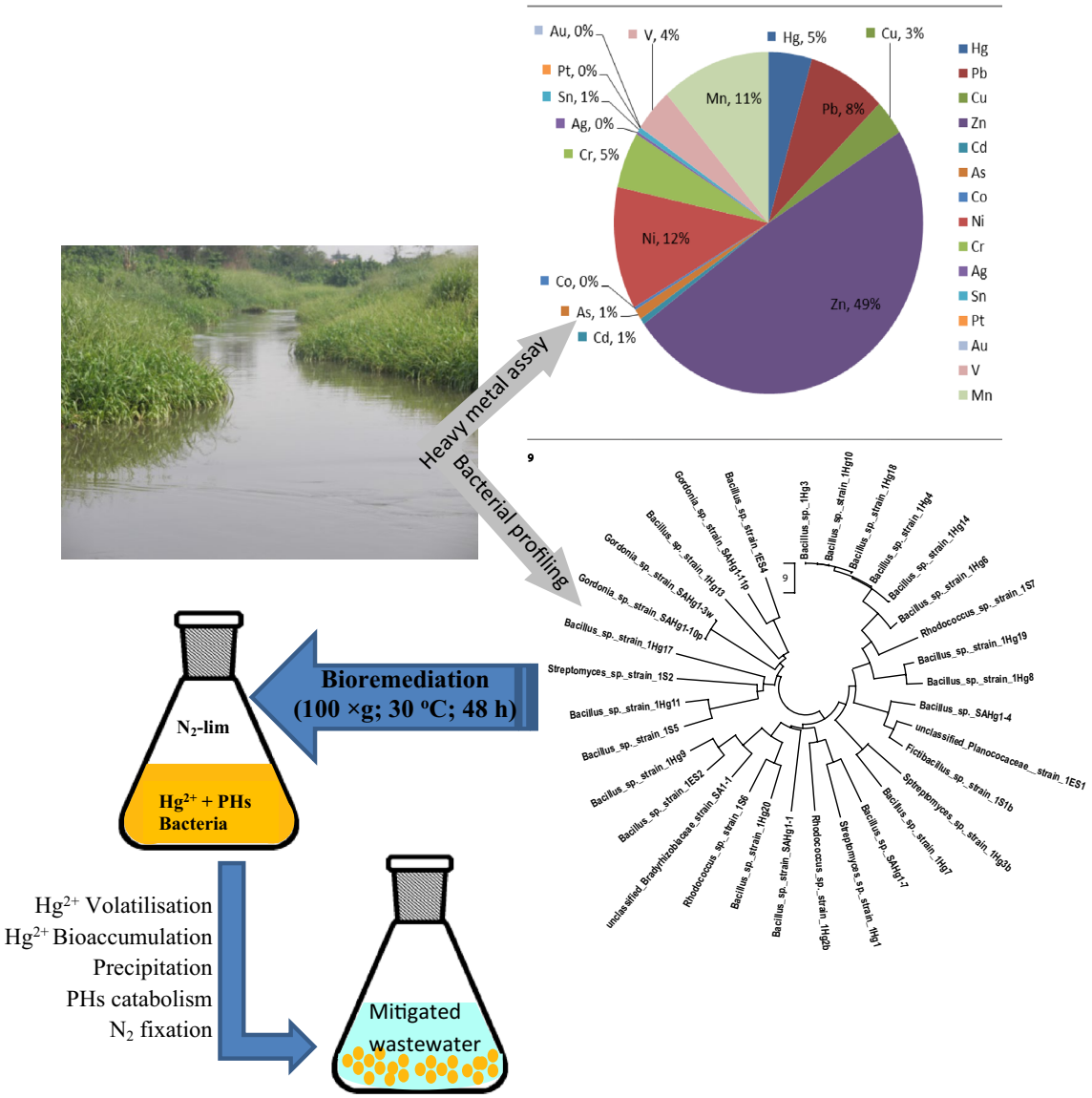
Graphical Abstract



Keywords Mercury sequestration · Wastewater · Heavy metal · Petroleum hydrocarbon catabolism · Bioremediation · Freshwater pollution

Introduction

Heavy metals (HMs) have recently gained global awareness for being major pollutants of environments. They are naturally introduced into the environment via earth crust. However, anthropogenic sources are widely reported to be the major means by which various species of HMs contaminate the ecosystem along with other organic pollutants like petroleum hydrocarbons (PHs) (Oyetibo et al. 2017). Emerging sources by which HMs and micro-organic compounds co-contaminate environmental matrixes include discharges of wastewaters from hospitals (Ogwugwa et al. 2021), textile industries (Odubanjo et al. 2021), and midstream petroleum operations (Oyetibo et al. 2021; Parus et al. 2023; Zhang et al. 2022). Concurrent pollution of HMs and PHs has negative impact on the environment, affecting agricultural productivity and socio-economic wellbeing of the people via deterioration of soil fertility and public health. Pollution of

agricultural soil with concomitant HMs and PHs, for example, deplete microbial diversities, resulting to impeding ecosystem functionality and productivity (Egbe et al. 2021; Mustafa et al. 2013; Oka and Uchida 2018). Whereby, the existing "fixed" forms of nitrogen are constantly converted to gaseous nitrogen and become unavailable to crops (Canfield et al. 2010; Oka and Uchida 2018). The widespread diazotrophs in such affected agricultural soil decreases, and their hydrolase/nitrogenase activities are inhibited, causing loss of fertility, and agricultural yield losses (An and Kim 2009; Egbe et al. 2021; Oka and Uchida 2018). Also, co-existence of HMs with organic pollutants constrained bioremediation strategies of the affected ecosystems (Dell'Anno et al. 2020; Mustafa et al. 2013; Wong et al. 2005).

Among the HMs, mercury (Hg), lead (Pb) and cadmium (Cd) are among the best-known toxic metals to life, without any known metabolic relevance and with low solubility potential in the environment. In 2019, arsenic (As), Pb, Hg and Cd ranked 1st, 2nd, 3rd and 7th, respectively, in the priority list for hazardous substances (ATSDR 2020). Upon transport of Cd^{2+} into the cytoplasm, for example, it causes thiol-binding and protein denaturation; interaction with calcium metabolism and membrane damage; and interaction with zinc metabolism, or loss of protective function of the cell (Nouairi et al. 2019). Cadmium was reported to be less preferred by organisms to other HMs in terms of biosorption, which has contributed to its persistence in the environment (Oyetibo et al. 2014). Upon exposure to toxic metals, bacteria adopt various mechanisms to circumvent, immobilize, detoxify, or sequester the metals into innocuous forms. These mechanisms include physicochemical adsorption to cell surfaces via non-active binding to functional groups (Oyetibo et al. 2014, 2016a), extracellular partitioning to form micro-precipitates (Oyetibo et al. 2016b), and activation of various genes leading to efflux pumping (Chaturvedi et al. 2021), intracellular compartmentalization (Singh et al. 2018), and reduction (Chien et al. 2010; Sanjay et al. 2020; Song et al. 2021) of metal ions. Diverse genetic-based metal resistance/detoxification mechanisms displayed by bacteria have been widely reported for mercury (Narita et al. 2003; Chien et al. 2010; Song et al. 2021).

Toxicity impact of HMs on many drivers of bioremediation of PHs requires substitution with novel microbial taxa that exhibit divergent abilities to circumvent HMs in nutrient-limiting environmental matrixes while utilizing the PHs as sources of carbon and energy. Previously, the challenges of HMs toxicity on PHs biodegradation were approached with selection of competent bacterial strains that showcased resistance to toxic concentrations of HMs (Mustafa et al. 2013) without demonstrating the competence of the strains in a system where HMs and PHs co-exist. Moreover, some bacterial strains were reportedly able to simultaneously metabolise hydrocarbons and sequester toxic metals in a multi-polluted environment (Dell'Anno et al. 2020) as demonstrated in simulated culture system supplemented with HMs and PHs (Oyetibo et al. 2013, 2017). Interestingly, these interventions did not consider the strong negative effect of PHs on nitrification (Oka and Uchida 2018; Scott et al. 2014) that would limit the applicability of such competent strains during in situ bioremediation campaign. However, the works of Cai et al. (2021) recommended biostimulation strategy via addition of extra nutrients into polluted sites for effective biodegradation of PHs in polluted ecosystem since the bacterial strain was not known to be active in nitrogen-limiting system. Nevertheless, the genetic features of a novel metagenome belonging to the family Bradyrhizobiacea revealed capabilities for hydrocarbons utilisation, nitrogen fixation and HMs resistance (Tikariha and Purohit. 2019), but this remains a prospective tool for bioremediation under nitrogen stress environment until the bacterium is cultivated. Consequently, report on bacterial cultures demonstrating combinational capabilities of HM resistance, PH catabolism and nitrogen fixation in a nitrogen-limiting system remains desirable. Therefore, this study seeks to profile bacterial strains exhibiting HM (with emphasis on Hg^{2+}) resistance and PH catabolism in a nitrogen-limiting culture system. The bacterial strains would be an excellent candidate for biotechnological decommissioning of nitrogen-limiting environments where toxic metals and PHs co-exist.

Materials and methods

Chemicals and preparation of culture media

Phenanthrene, $ZnCl_2$, $MnCl_2$, $HgCl_2$, CrO_3, $PbCl_2$ and $NiCl_2$ were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA); Escravos crude oil (see Table S1 in Supplementary document for its characteristics) was obtained from 'Chevron Nigeria Limited', and all other chemicals were of analytical reagent grade. Phenanthrene (100 mg) was dissolved in hexane (100 ml), shared into 10 Erlenmeyer flasks (500 ml) and nitrogen-free mineral salts medium (N_2-free MSM) was added to each flasks containing 10 mg hexane-free phenanthrene to make up 100 mg l^{-1}, final concentration, before sterilization as earlier reported (Oyetibo et al. 2013, 2017). Similarly, a 1% (v/v) final concentration of crude oil-MSM whereby crude oil formed an immiscible oily layer on aqueous MSM in Erlenmeyer flasks was prepared as earlier explained (Oyetibo et al. 2017). The N-free MSM contained (g l^{-1}) Na_2HPO_4, 2.13 g; KH_2PO_4, 1.30 g; NaCl (instead of NH_4Cl), 0.50 g and $MgSO_4.7H_2O$, 0.20 g; pH was adjusted to 6.9. Sterile trace elements solution, SL-6 (1.0 ml l^{-1}) (Atlas 2005) was aseptically added to the medium after sterilization. All sterilization processes were by moist heat in autoclave at 121 °C for 15 min unless otherwise stated.

Site description and sampling

Ikeja Industrial Estate, Lagos, Nigeria was established in the mid-1960s and industrial activities of the estate were peaked in the late-1980 until Nigeria's economy began to experience downturn. Despite that, the estate still housed plethora of diverse industries that discharge their multi-component wastewaters into the environment, forming "Odo-Iyalaro" stream, which empties into Lagos lagoon and finally into the Atlantic Ocean. Composite samples (10) of sediments were randomly collected from the site and GPS coordinates were as presented in the supplementary document (Table S2).

Geochemical and physical parameters analyses

Physico-geochemical parameters including pH, chlorides, total dissolved solids (TDS), cation-exchange capacity (CEC), acidity, alkalinity, chemical oxygen demand (COD), and biochemical oxygen demand (BOD) were determined as previously reported (Oyetibo et al. 2017, 2019). A total of 15 HMs/metalloids were quantified in the sediment via inductively coupled plasma mass spectrometry (ICP-MS) (ELAN 9000, Perkin Elmer SCIEX, Boston, MA, USA) after digesting dry sample (0.1 g) with HNO_3/HCl (4:1, v/v) in a microwave oven (Multiwave 3000, Anton Paar, Graz, Austria) as described in previous report (Oyetibo et al. 2019). Extraction of total hydrocarbons was with *n*-hexane and quantified using standard gravimetric method coupled with GC-FID as previously reported (Obayori et al. 2009).

Isolation of heavy metals resistant bacteria with potential to fix free nitrogen

Isolation of multi-resistant bacteria was based on culture enrichment procedure where samples were inoculated into N_2-free MSM amended with sterile HMs solutions (1%) and glucose (10 g l^{-1}). The HMs solutions consisted of $ZnCl_2$ (0.5 mmol l^{-1}), $MnCl_2$ (0.5 mmol l^{-1}), $HgCl_2$ (0.01 mmol l^{-1}), CrO_3 (0.1 mmol l^{-1}), $PbCl_2$ (0.5 mmol l^{-1}), and $NiCl_2$ (0.5 mmol l^{-1}). After incubation (30 °C; 96 h; 50 × g), the culture was diluted appropriately, and plated onto N_2-free MSM containing 1.5% bacteriological agar (Wako Pure Chemicals Ltd., Japan) and supplemented with 2% HMs solutions. The choice of Zn^{2+}, Mn^{2+}, Hg^{2+}, Cr^{6+}, Pb^{2+} and Ni^{2+} in this study was because they were individually predominant (≥ 5% [33.4 mg $kgdw^{-1}$]) in the sediment. Pure cultures were further screened for resistance to higher concentrations of the $HgCl_2$ to select those colonies that showed high resistance.

Determination of mercury resistance and sequestration pattern of bacterial isolates

Mercury resistance and sequestration pattern was chosen for further analyses in this study because of its recent global attention towards public health. Isolates grown in Luria Bertani (LB) broth (containing per litre: 10 g tryptone, 5 g yeast extract, and 5 g NaCl) for 18 h at 30 °C were harvested by centrifugation (7,000 × g, 10 min), washed twice with sterile buffered phosphate solution, and re-suspended in the same buffer solution. The cell density of bacterial suspensions was determined by measuring the absorbance at 600 nm in relation to a calibration curve (10^{10} cfu l^{-1} = 1 OD unit). LB broth amended with $HgCl_2$ (10—240 µmol l^{-1}) in aliquots (5 ml) into test tubes were inoculated with 50 µl inoculums (OD_{600}, 0.1). Growth media without HMs but inoculated with test organisms, and growth media amended with $HgCl_2$ but inoculated with dead cell mass (boiled thrice at 100 °C, 10 min. at 12 h interval to totally kill sporogenous bacteria) served as positive and negative controls, respectively.

Growth of the bacteria was measured using absorbance at 600 nm ($OD_{600\ nm}$) with extrapolated viable counts, and plating out to confirm bacterial purity and viability. Resistance was tested as minimum tolerance concentrations (MTCs) for the isolated strains after incubation (36 h; 30 °C). The MTC was defined as the highest concentration of Hg^{2+} which did not affect the viability of organisms (OD_{600} > 0.1). The total mercury in LB broth before inoculation, culture and supernatant (100 µl) were quantified directly without any pre-treatment using a fully automated thermal vaporization mercury analysis system, Mercury/MA-3000 (Nippon Instrument Corp., Osaka, Japan) as earlier reported (Oyetibo et al. 2015, 2016a, 2016b). Nevertheless, capabilities of bacterial strains to sequester Hg^{2+} were determined as volatilisation, bioaccumulation/adsorption, and bio-removal efficiencies using $VE = \frac{MQM-CQ}{MQM} \times 100$, $AE = \frac{CQ-SQ}{MQM} \times 100$ and $RE = \frac{MQM-SQ}{MQM} \times 100$, respectively. Where VE is volatilisation efficiency, MQM is the quantity of Hg in medium before inoculation with bacterial strain, CQ is the quantity of Hg in culture, AE is bioaccumulation/adsorption efficiency, SQ is the quantity of Hg in cell-free supernatant of centrifuged (14,000 × g, 2 min) culture, and RE is the removal efficiency.

Molecular characterization and identification of the selected bacteria

Pure colonies of overnight culture of selected bacteria were suspended in mix-solutions of Cica Geneus DNA Extraction Reagent (Kanto Chemical Co. Inc., Tokyo, Japan) and genomic DNA was extracted using two temperature regimes

(72 °C, 20 min; 93 °C, 3 min) according to manufacturer's instructions. The 16S rRNA genes in the purified genomic DNA samples were amplified by using Ex-Taq polymerase (TaKaRa, Ohtsu, Japan) according to the manufacturer's instructions. A domain bacteria-specific PCR primer set of 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGHTACCTTGTTACGACTT-3') (Weisburg et al. 1991) was used to amplify 16S rRNA genes (approximately 1,500 nucleotides). The PCR condition was: initial denaturation (94 °C, 5 min); followed by 35 cycles of denaturation (94 °C, 30 s); annealing (50 °C, 30 s), and extension (72 °C, 2 min). The final extension was at 72 °C for 7 min. The PCR products were confirmed by electrophoresis after staining with ethidium bromide. Clean amplicons (after elution through Sephacryl S-300 [GE Healthcare Bio-Sciences, Sweden]) was subjected to cycle (sequencing) PCR using each of the primer and a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster, CA, USA). Cycle (sequencing) PCR condition was: initial denaturation (96 °C, 1 min); followed by 25 cycles of denaturation (96 °C, 10 s); annealing (50 °C, 5 s), and extension (60 °C, 4 min). The cycle PCR products were purified (elution through Sephadex G-50 [Sigma-Aldrich, Germany]), vacuum-dried, resuspended with Formamide (~ 10 µl) and sequenced using the Applied Biosystems 3130xl Genetic Analyzer. Sequences were compared to those present in the Ribosomal Database Project (RDP) at Michigan State University (Sequence Match (msu.edu)), and aligned with the CLUSTALW program. Phylogenetic trees were obtained using the Mega Software (Mega 11; Tamura et al. 2021), for the neighbor-joining method. The confidence of the phylogenetic trees was analysed by the bootstrap method (resampling value was 1000). All 16S rRNA gene sequences were deposited in the DDBJ/EMBL/GenBank databases under accession numbers LC681799-LC681830.

Crude oil degradation in N_2-free chemically defined medium supplemented with Hg

Seven bacterial strains, identified as *Bacillus* sp., showing luxuriant growth in LB broth supplemented with 160 µmol l^{-1} $HgCl_2$, were selected and consorted together for crude oil degradation assay and Hg sequestration analysis. Triplicate 250-ml flasks containing 50 ml of N_2-free MSM supplemented with sterile $HgCl_2$ solution (40 µmol l^{-1}) and crude oil (1%) as sole source of carbon and energy were prepared to mimic a N_2-limiting ecosystem that is co-contaminated with HMs and PHs. The flasks were inoculated with washed bacterial cells (1 ml) and incubated at 30 (± 2) °C with shaking (50 × g; 18 d). Growth-associated depletion of crude oil was determined by gas chromatography equipped with flame ionization detection (GC-FID) analysis of residual crude oil upon extraction with hexane (Obayori et al. 2009; Oyetibo et al. 2017). Control experiments and Hg sequestration assay were as explained in the earlier Section.

Statistical analyses

All experiments, readings and analyses were in triplicates. All values represented mean of triplicate experimental data. Column statistics, row statistics, and one-way ANOVA at 95% confidence were determined using GraphPad Prism 5 (GraphPad Software, Inc., Avenida de la Playa La Jolla, CA., USA).

Results

Geochemical and physical parameters analyses

Geochemical data of the understudied site were as presented in Fig. 1. While Zn predominate the HMs/metalloids composition in the sediment by 49% (360 mg kg^{-1} dry weight); Ni, Pb, and Hg constituted at least 5% components of HMs/metalloid consistently endangering the ecosystem (Fig. 1). Nevertheless, observed concentrations of Hg and Ni in the sediments were extremely higher than benchmarks for freshwater sediments (see Supplementary Table S3). Also, representative fingerprints of the GC-FID spectra (see Fig. S1) revealed the components of the total petroleum hydrocarbon (0.105 mg kg^{-1}; Table S4) were present in the freshwater. Values of total solids (1595 mg l^{-1}), COD (780 mg l^{-1}), and BOD (185 mg l^{-1}) among other parameters of water column of the freshwater were higher than recommended limits in freshwater environments (see Table S4).

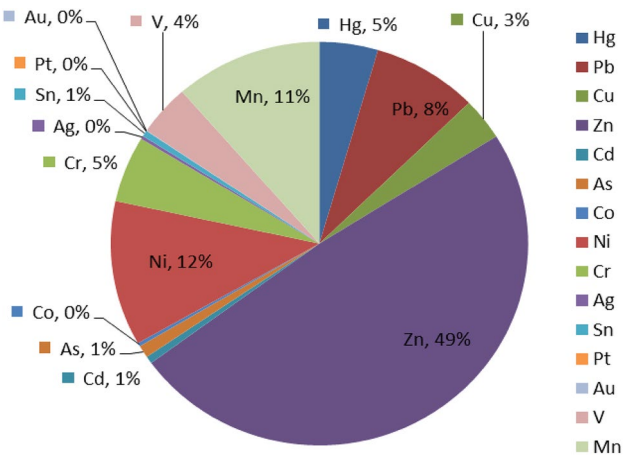


Fig. 1 Pie chart representing the mean distribution of toxic metals in the sediments of polluted sites

Table 1 Putative identities and mercury resistance pattern of bacterial strains isolated from ecosystem receiving industrial wastewaters, exhibiting multiple resistance to heavy metals and growth in nitrogen limiting growth medium

Identity	Accession number	Mercury resistance pattern within 24 h post-inoculation (μmol)
Streptomyces_sp_1Hg1	LC681799	40
Bacillus_sp_SAHg1-1	LC681800	40
Gordonia_sp_SAHg1-3w	LC681801	40
Bacillus_sp_SAHg1-4	LC681802	40
Bacillus_sp_SAHg1-7	LC681803	40
Bacillus_sp_1Hg3	LC681804	80
Bacillus_sp_1Hg4	LC681805	160
Bacillus_sp_1Hg6	LC681806	160
Bacillus_sp_1Hg7	LC681807	160
Bacillus_sp_1Hg9	LC681808	160
Bacillus_sp_1Hg10	LC681809	80
Bacillus_sp_1Hg11	LC681810	40
Bacillus_sp_1Hg13	LC681811	160
Bacillus_sp_1Hg14	LC681812	40
Bacillus_sp_1Hg17	LC681813	80
Bacillus_sp_1Hg18	LC681814	40
Bacillus_sp_1Hg19	LC681815	80
Bacillus_sp_1Hg20	LC681816	160
Bacillus_sp_1S5	LC681817	80
Rhodococcus_sp_1S6	LC681818	80
Rhodococcus_sp_1S7	LC681819	80
Gordonia_sp_SAHg1-10p	LC681820	40
Gordonia_sp_SAHg1-11p	LC681821	40
Planococaceae_1ES1	LC681822	160
Bacillus_sp_1ES2	LC681823	20
Bacillus_sp_1ES4	LC681824	160
Bacillus_sp_1Hg8	LC681825	80
Streptomyces_sp_1S2	LC681826	10
Bradyrhizobiaceae_SA1-1	LC681827	160
Rhodococcus_sp_1Hg2b	LC681828	160
Sptreptomyces_sp_1Hg3b	LC681829	10
Fictibacillus_sp_1S1b	LC681830	10

Characteristics and identities of isolated resistant bacteria strains with capability to grow in nitrogen-limiting system

Putative identities of the 32 bacterial strains showing multiple resistances to Zn, Mn, Hg, Cr, Pb and Ni in a nitrogen-limiting chemically defined medium were as presented in Table 1 with respect to at least 97% homology to 16S rRNA genes in the GenBank. However, the phylogeny of the bacterial strains revealed poor diversity based on evolutionary history was deduced via Neighbor-joining method as an unrooted phylogenetic tree after using Maximum Composite Likelihood to compute the evolutionary distances (Fig. 2). The highest concentration of mercuric ions at which the bacteria exhibited luxuriant growth in N_2-limiting growth medium at 24 h post-inoculation as evidence of possessing Hg-resistance traits are shown in Table 1. While 12.5% of the bacterial strains barely resisted ≤ 20 μmol Hg^{2+}, 32.25% and 25% of the strains resisted 40 μmol Hg^{2+} and 80 μmol Hg^{2+}, respectively. Nevertheless, 31.25% of the bacterial strains comprising unclassified Planococaceae (1 strain), unclassified Bradyrhizobiaceae (1 strain), *Rhodococcus* species (1 strain) and *Bacillus* species (7 strains) exhibited growth in nitrogen-limiting medium amended with 160 μmol Hg^{2+} at 24 h post-inoculation.

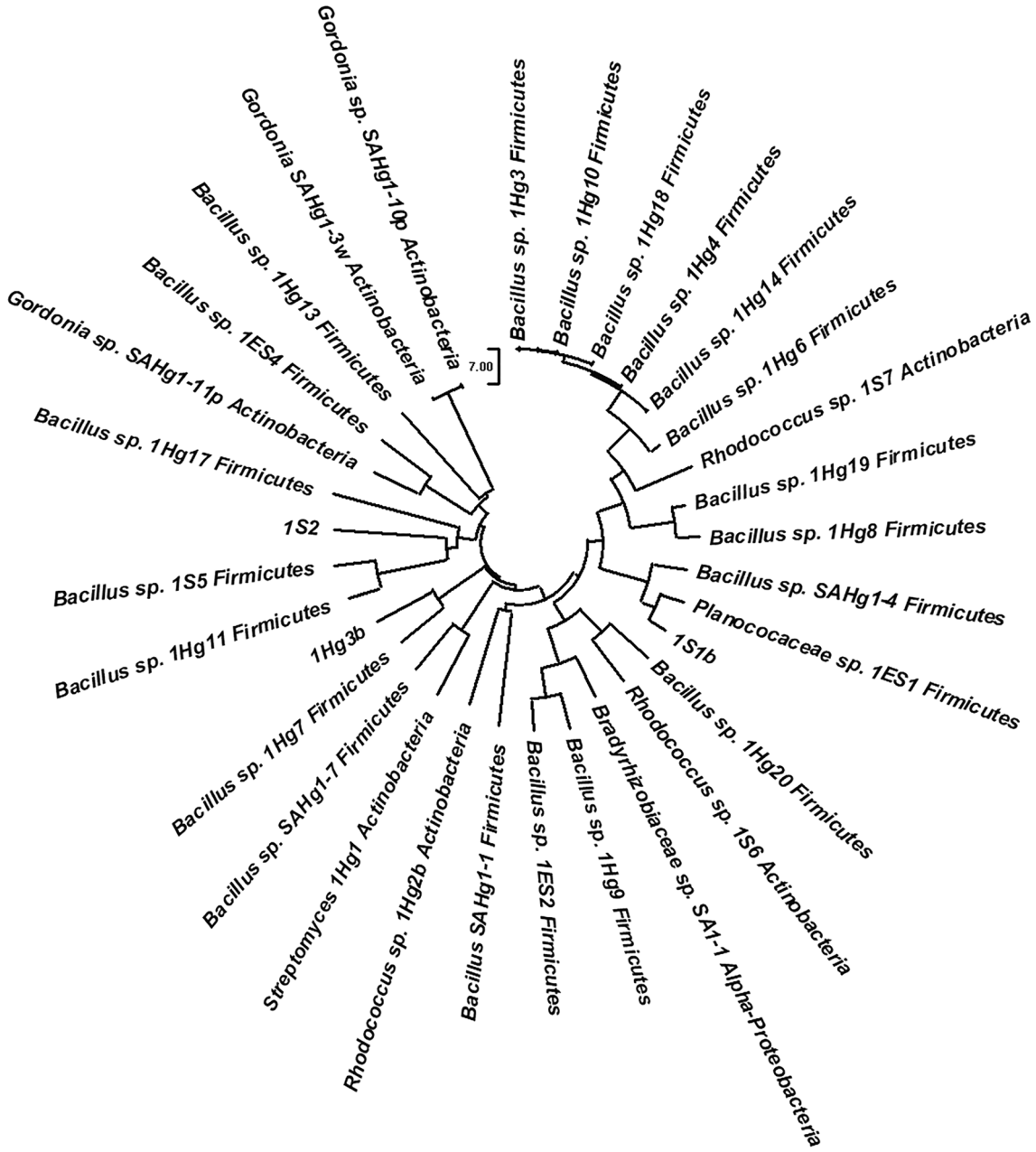


Fig. 2 Phylogeny based on evolutionary relationships of bacterial taxa exhibiting heavy metal resistant in nitrogen-limiting growth medium. The evolutionary history was inferred using the Neighbor-Joining method displayed as optimal unrooted phylogenic tree drawn to scale, with branch lengths in the same units as those of the evolutionary distances. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 32 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1451 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al. 2021)

Mercury sequestration pattern of bacterial isolates

The Hg-removal efficiency exhibited by the bacterial strains was apparently biased towards Hg concentrations in the growth medium within the incubation period (Fig. 3). Not less than 90% Hg-removal efficiency was observed with majority of the bacteria that grew in nitrogen-free medium supplemented with 20 µmol $HgCl_2$ for 24 h, with the exception of *Bacillus*_sp_1Hg10, *Bacillus*_sp_1Hg11, *Bacillus*_sp_1Hg14, *Bacillus*_sp_1Hg20, *Gordonia*_sp_SAHg1-11p, Planococcaceae_1ES1, and *Bacillus*_sp_1Hg8. Interestingly, *Bacillus*_sp_1Hg13 and *Bacillus*_sp_1Hg9

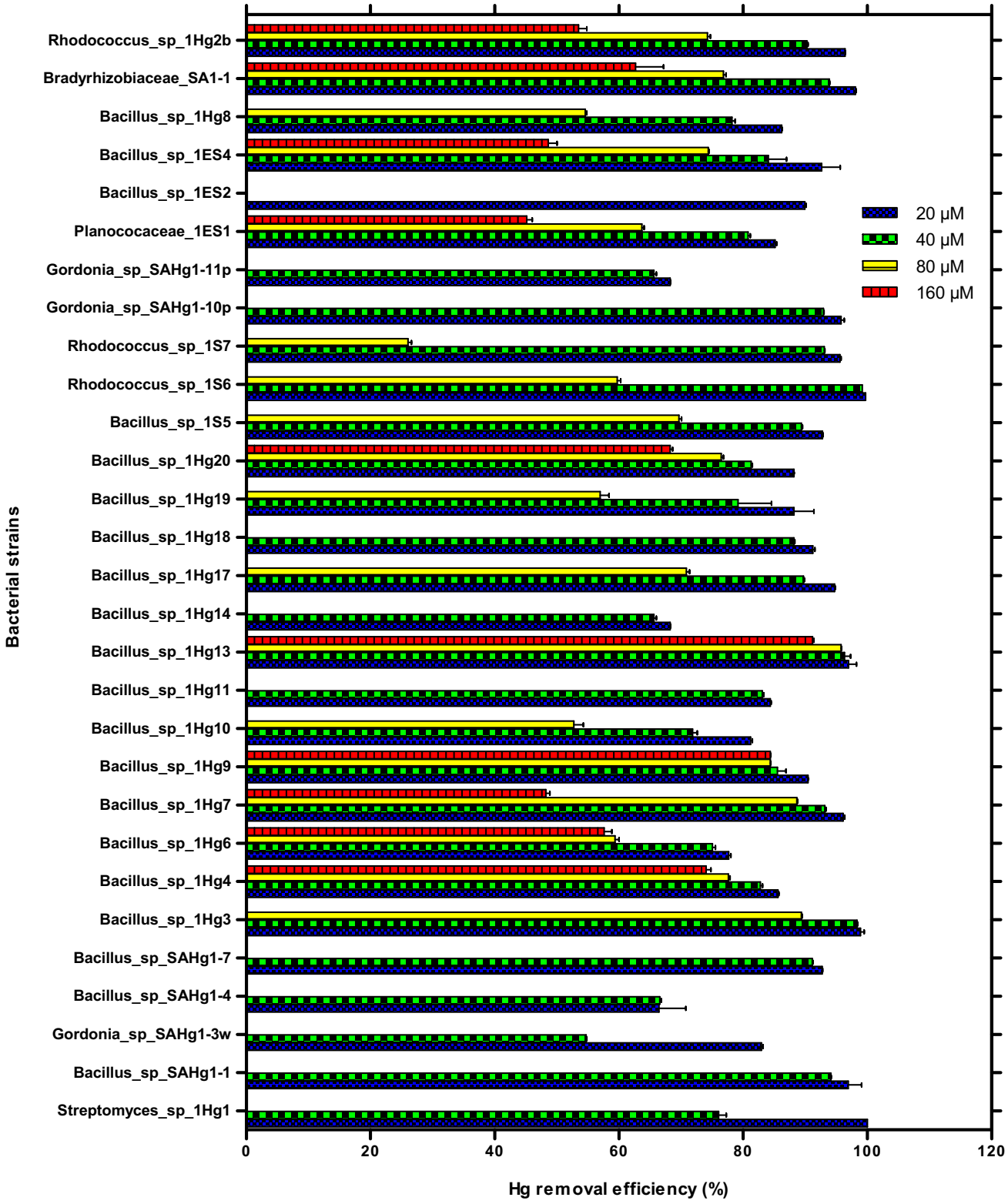


Fig. 3 Mercury removal efficiency of the isolated bacterial strains in nitrogen-limiting growth medium

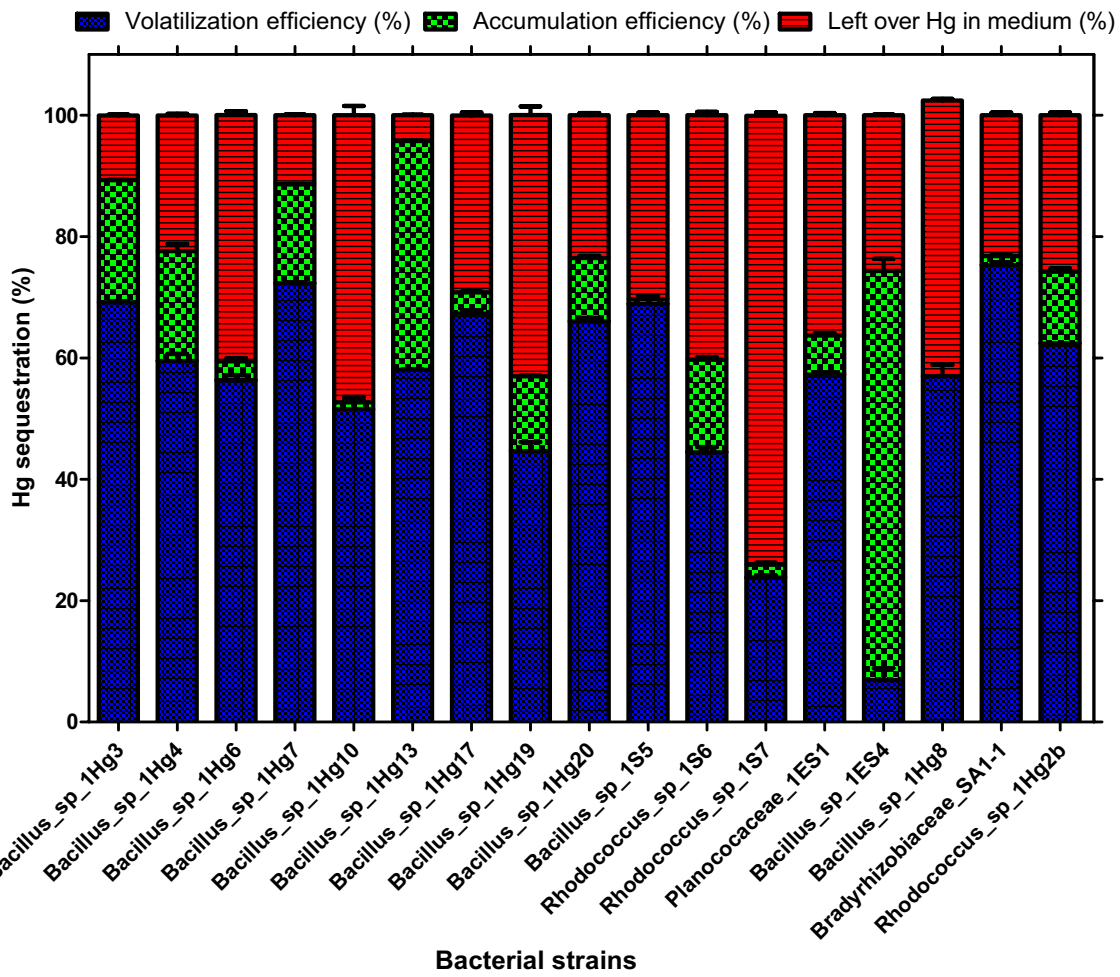


Fig. 4 Bacterial sequestration of mercury (80 µmol l^{-1}) in nitrogen-limiting medium

efficiently removed more that 80% of the mercuric ions in the N_2-free medium amended with 160 µmol l^{-1} $HgCl_2$. On the contrary, 19 bacterial strains were unable to grow in medium supplemented with 160 µmol l^{-1} $HgCl_2$, while *Bacillus*_sp_1ES2 could only grew in medium supplemented with 20 µmol l^{-1} $HgCl_2$. Furthermore, palpable sequestration mechanisms of mercuric ions in the batch culture system supplemented with 80 µmol l^{-1} $HgCl_2$ is shown in Fig. 4. Volatilization of mercuric ions (80 µmol l^{-1}) to elemental mercury ($Hg^{2+} \rightarrow Hg^0$) apparently dominated the mechanisms used by the bacterial strains. Contrarily, *Bacillus*_sp_1ES4 seemingly immobilise the 80 µmol l^{-1} mercuric ions majorly via adsorption and/or intracellular accumulation (67.4 ± 2.01%) but meagrely through volatilisation (6.93 ± 1.95%). Of note are Bradyrhizobiaceae_SA1-1, *Bacillus*_sp_1Hg8, and *Bacillus*_sp_1S5 that appear to mainly sequester 80 µmol l^{-1} mercuric ion through volatilisation (75.3 ± 0.47%, 57.0 ± 1.88%, and 68.9 ± 1.04%, respectively) with little or no evidence of intracellular accumulation (1.5 ± 0.153%, 0%, and 0.762 ± 0.671%, respectively). Similarly, *Bacillus*_sp_1Hg10 demonstrated preference for volatilisation (51.6 ± 1.44%) to adsorption and/or intracellular accumulation (1.15 ± 0.836%) during sequestration of 80 µmol l^{-1} Hg^{2+} in growth medium at 30 °C, 24 h incubation. Nevertheless, *Bacillus*_sp_1Hg3 and *Bacillus*_sp_1Hg13 exhibited combinational excellent volatilisation with good adsorption and/or intracellular accumulation in sequestering 80 µmol l^{-1} Hg^{2+} in growth medium at 30 °C, 24 h incubation.

Crude oil degradation in N_2-free chemically defined medium supplemented with Hg

The extent of biodegradation of crude oil as determined by total extractable hydrocarbon concentrations, hydrocarbon class distribution, and C17/pristane vis-à-vis C18/phytane ratios through 18 d degradation studies were reflected in GC-FID fingerprints (Fig. 5). The populations of the selected bacteria showed excellent growth magnitude within 10 d incubation in chemically defined nitrogen-free medium

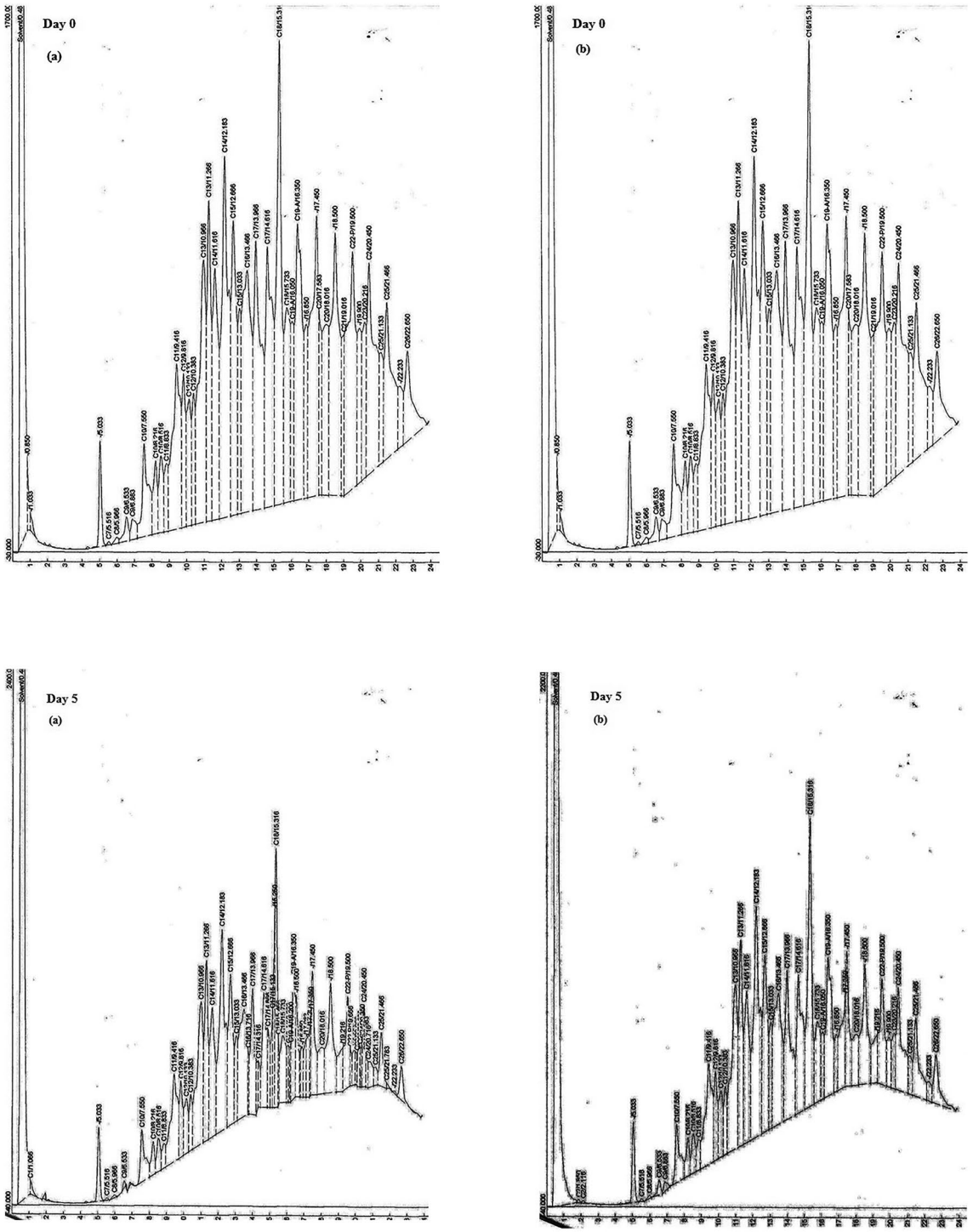


Fig. 5 GC-FID fingerprints of crude oil recovered from cultures of selected bacteria **a** *Bacillus* sp. 1Hg4, and **b** *Rhodococcus* sp. 1Hg2b in chemically defined, nitrogen-free medium amended with 40 µmol l^{-1} $HgCl_2$ at day 0, 5, and 10, while the experiment lasted 18 d. There was overall 98.19% (±1.7), and 94.41% (±1.5) crude oil degradation for *Bacillus* sp. 1Hg4, and **b** *Rhodococcus* sp. 1Hg2b, respectively, at the end of experiment

containing 40 µmol l^{-1} $HgCl_2$, and crude oil as sole source of carbon. At this period, the bacterial cell density had increased from 10^6 to 3.9×10^9 cfu ml^{-1} (approx.) in all the bacterial strains. Consistent decrease in the residual extractable crude oil hydrocarbons corresponded with increase in populations of the bacteria, whereby not less than 45%

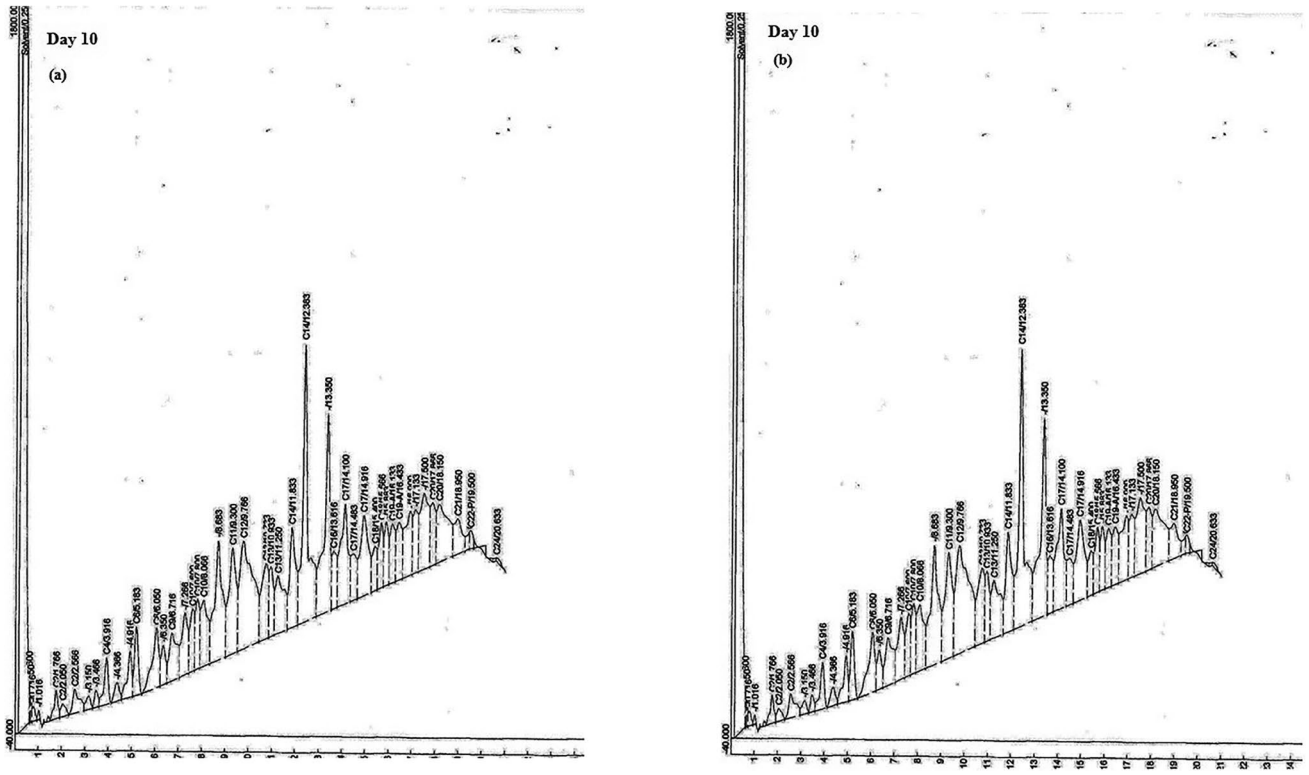


Fig. 5 (continued)

and 80% degradation were observed at day 5 and 10 post-incubation, respectively. Luxuriant growth of the bacteria was observed within 14 d of the experiment with sequential disappearances of the hydrocarbons as evident of being used as source of carbon for cell mass increase. Afterwards, the cell densities assumed gradual decrease with characteristic colour changes in the medium from yellow to colourless. Taken as a whole, at least 87.1% (±0.4) of petroleum biomarkers, in the chemically defined, nitrogen-free medium supplemented with 40 µmol l^{-1} $HgCl_2$, were utilized for growth by the bacteria strains.

Discussion

Ikeja is the capital city of Lagos State that is the commercial nerve centre of Nigeria. The city houses a number of industries and artisanal cottages as evidence of emerging urban centre. Urbanization and artisanal practices are accompanied with attendant consequences on the environment and humanity via the release of untreated or partially treated wastewaters. Comparing the metal concentrations obtained from the sediment with the Sediment Quality Guidelines (SQG) revealed that mercury (Hg), lead (Pb), zinc (Zn), cadmium (Cd), nickel (Ni), and silver (Ag) exceeded the Threshold Effect Concentrations (TEC) stipulated by United States of America Environmental Protection Agency (EPA Region III BTAG—Freshwater Sediment Screening Benchmarks—8/2006), below which harmful effects are unlikely to be observed. Meanwhile, copper (Cu), arsenic (As), cobalt (Co), chromium (Cr), and manganese (Mn) have concentrations in the sediment that were below the benchmark. Whereas, tin (Sn), platinum (Pt), gold (Au) and vanadium (V) concentrations in the sediment could not be compared since there is no existing guideline. Overall, all other metals measured in the sediment, except Hg and Ni, have fewer concentrations than the probable effect concentrations and are deduced non-hazardous to the ecosystem based on global (Burton 2002) and consensus (MacDonald et al. 2000) criteria. Unregulated release of untreated and/or partially treated wastewaters could be linked to the spread of elevated concentrations of heavy metals in the understudied site. More importantly, Hg concentration that severely exceeded the probable effect concentrations with likely observation of severe effects on the sediment biota confirmed accessible industrialization of the site (MacDonald et al. 2000). Similarly, the concentrations of Ni, above such that would likely cause harmful effects in the sediment ecosystem, could be adjudged that it will exert less severity (49 fold-change) when compared to Hg. Consequently, the high threshold concentrations possibly exert harmful impacts on the

aesthetic quality of the freshwater and the autochthonous biota as previously reported (Oyetibo et al. 2019).

Nevertheless, arrays of petroleum hydrocarbons as evident with total petroleum hydrocarbons and gas chromatographic fingerprints could infer presence of persistent organic pollutants (POPs) including a number of species of poly-aromatic hydrocarbons, aliphatic hydrocarbons, and halogenated hydrocarbons in the site. The detection of quantifiable spectra of PHs in the freshwater is an evidence of contamination of the ecosystem with organic compounds. Furthermore, the measurable chemical elements of the water column as shown via elevated values of total solids, COD, and BOD pointed at escalated pollution levels of the samples above safe level for aesthetic environmental media and it is of public health concern. Taken as a whole, high concentrations of heavy metals and detection of arrays of petroleum hydrocarbons along with their derivatives depict co-contamination of environmental matrixes around the site. An indiscriminate discharge of industrial wastewaters into sewerage that finally empties into the lagoons was responsible for pollution of coastal waters.

Robust microbial diversity is usually used as indicator of pristine environment. On the contrary, the bacterial isolates in the understudied sediments were predominantly Firmicutes, which were mostly species of *Bacillus,* while few strains belong to Actinobacteria and only a strain of Bradyrhizobiaceae belonging to Proteobacteria was identified. The skewed diversity of bacteria in the polluted systems towards *Bacillus* species demonstrated negative impact of pollutants on the biodiversity of the hydrosphere as a consequence of decades of exposure to matrix of bio-hazardous compounds (Oyetibo et al. 2010, 2019; Wang et al. 2021). This implies that the toxic pollutants might have narrowed the bacterial community to those that have developed survival strategies while the vulnerable strains could have gone extinct (Wang et al. 2021). As such, there is ecological shift in the polluted site that could consequently endanger public health.

The multiple resistance ability of the bacterial strains to Zn, Mn, Hg, Cr, Pb and Ni in nitrogen limiting growth medium indicated they are capable of fixing free atmospheric nitrogen to make-up for the deficient nitrogen in the medium. Up till now, information on bacteria exhibiting HM-resistance in a N_2-limiting system is few. It is noteworthy that volatilisation of Hg^{2+} among all the bacterial strains were growth-associated as earlier reported for strains of *Yarrowia* species isolated from multi-polluted estuarine ecosystem (Oyetibo et al. 2015, 2016a). The volatilization of mercuric ions by the bacteria might be extracellular reduction via activities of sulfhydryl exopolymers (Oyetibo et al. 2016b), and/or intracellular via reductive actions of proteins synthesized by *merA* genes in the bacterial genomes (Narita et al. 2003). Adsorption of Hg^{2+} during active growth of microorganisms, leading to intracellular accumulation and compartmentation have been observed in *Yarrowia* species in previous studies (Oyetibo et al. 2015, 2016a). Activities of intracellular and/or extracellular biomolecules of the bacterial strains must be connected to sequestration mechanisms observed in this study as previously discussed in other microorganisms (Oyetibo et al. 2016b).

A clear understanding of the response of any organism used for decommissioning of hydrocarbon polluted sites to hidden environmental factors such as antibiosis due to the presence of HMs is crucial to the success of bioremediation efforts. Hitherto, literatures describing bacteria that sequester toxic doses of Hg^{2+} while degrading petroleum hydrocarbons in nitrogen-limiting chemically-defined media are scarce. Various indirect interventions to overcome the burdens of HMs toxicity and N_2-limitation include, but not limited to, applications of biosurfactants to spur HMs partitioning (Chrzanowski et al. 2012; Lopes et al. 2021; Parus et al. 2023), and nutrient fortification to pave for nitrogen stress (Cai et al. 2021) in the environment. Exploring the selected bacterial strains for mineralisation of petroleum hydrocarbons in N_2-deficient medium amended with $HgCl_2$ as panacea to multi-polluted matrices is innovative and sustainable for environmental management. Basic adaptation and evolution principles must have contributed to the unique physiology of the multiple-resistant bacterial strains with traits of hydrocarbon catabolism and nitrogen fixation. For the fact that these isolates could be related to those bacteria reported in other parts of the world, the uniqueness of the isolated bacteria cannot be ignored. Recently, microbial catabolism of organic compounds with simultaneous reduction/removal of HMs was discussed (Oyetibo et al. 2013, 2017). Luxuriant growth of the bacterial strains in chemically defined medium where hydrocarbons serve as sole source of carbon and energy indicated that the bacteria mineralized the hydrocarbon to synthesize macromolecules that translates to increase in biomass. Unlike in the previous studies where fate of the HMs could not be established (Oyetibo et al. 2013, 2017), mercuric ions were apparently reduced to gaseous elemental mercury. Consequently, the bacterial strains were assumed to be diazotrophs capable of fixing atmospheric nitrogen via activities of nitrogenases under microaerophilic conditions (Desnoues et al. 2003) upon expression and regulation of nitrogen-fixation (*Nif*) genes (Gupta et al. 2019) in order to thrive in the nitrogen-limiting culture system.

Conclusion

In this study, bacteria diversity in a multi-polluted site skewed towards Firmicutes. The bacilli simultaneously volatilise bioavailable Hg^{2+} concentration in the systems

while degrading hydrocarbons. It was demonstrated that the selected bacteria strains did simultaneously sequester mercuric ions via volatilisation and utilize petroleum hydrocarbons as source of carbon and energy in a system devoid of nitrogen source. The bacterial strains practically relied of sources of nitrogen out of the culture system, most likely via fixing atmospheric nitrogen to circumvent the N_2-limiting environment without need for nutrient fortification. Clearly, such culture of autochthonous bacilli, exhibiting HM sequestration and nitrogen fixation during degradation of petroleum hydrocarbons in a N_2-deficient system constitutes an invaluable resource for bioremediation of nitrogen-depleted environmental matrixes contaminated with mercuric ions and petroleum hydrocarbons. The findings in this study are therefore, doubtlessly relevant to restoring the aesthetic state of polluted environmental compartments, where anti-bioremediation forces like metal toxicity and nitrogen stress abound.

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Data availability The 16S rRNA gene sequence data in this study were deposited in the DDBJ/EMBL/GenBank databases under accession numbers LC681799-LC681830.

Declarations

Conflict of interest Ganiyu O. Oyetibo, Sunday A. Adebusoye, Matthew O. Ilori, Olukayode O. Amund, Authors has no conflict of interest.

Ethical approval Neither animal nor human was used in part or whole during this study, as regards the procedures performed during the present investigation. Therefore, this article does not contain any studies with human participants or animals performed by any of the authors.

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