Investigating the simultaneous removal of hydrocarbons and heavy metals by highly adapted *Bacillus* and *Pseudomonas* strains

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ABSTRACT

The status, content and availability of heavy metals and hydrocarbons are highly affected by weathering processes – particularly – in areas characterized by harsh conditions. Their concomitant removal by highly adapted strains of *Bacillus* and *Pseudomonas* to weathered oil components was investigated. Indeed, weathered soil collected from Dukhan dumpsite was shown to contain 14 heavy metals with concentrations exceeding the US-EPA limits. Their distribution in such soil was not affected by the soil organics, which is important for the remediation processes. Most of them were strongly bonded to the iron–manganese oxide and the residual fractions, justifying their bioremediation. This study used 18 adapted bacterial strains isolated from extremely weathered oily soils, including the studied soil, all shown tolerant up to 5 mM and above of heavy metals. 4 *Bacillus* and 3 *Pseudomonas* strains exhibited the capability to remove 70%–80% of the heavy metals. Concomitantly, they removed up to 73% of the diesel-range organics. Using the PCA methodology with multivariate (bacterial type and adaptation and heavy metals properties), the efficiency of heavy metals removal was shown not related to the adapted bacteria, but to the heavy metal status. This means that the adaptation of these bacterial strains was at the level of the cells' structure and/or their exopolymeric substances which immobilize the heavy metals and reduce their toxicity allowing their growth and removing hydrocarbons. It is then, a double-adaptation route, leading to concomitant removal of organics and heavy metals, which is of high importance from the practical point of view.

1. Introduction

Anthropogenetic activities in the industrial sectors have been discharging hazardous pollutants into the environment (Arora, 2018). Major contaminants are hydrocarbons and heavy metals from fuels that accumulate into soil and water causing harmful impact on the environment and living organisms (Ayangbenro and Babalola, 2017; Ukaogo et al., 2020; Zaynab et al., 2022). Therefore, the demand for remediation methods that mitigate these issues with minimum destruction of the ecosystem is emerging. Physiochemical, thermal, and biological strategies can be implemented. However, biological approaches offer more environmentally friendly and cost-effective processes that have wider interest, such as microbial bioremediation (Usman et al., 2016). In principle, bioremediation is a process of using living organisms

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to degrade, immobilize or transform pollutants from their toxic form into innocuous structure (Juwarkar et al., 2010). Fuels hydrocarbons and heavy metals are chemicals found heavily in the environment and considered recalcitrant pollutants. However, it is now admitted that oil hydrocarbons can be partially or totally biodegraded by hydrocarbon-degrading bacteria (AlKaabi et al., 2020). Besides, it was shown that even weathered hydrocarbons could be degraded (Al-Kaabi et al., 2017, 2018; AlKaabi et al., 2020). Organic compounds can be biodegraded via specific biological pathways, although the type of pollutants and treatment conditions affect bioremediation (Peng et al., 2015). Heavy metals can be transformed from one form to another, through redox processes (Caporale and Violante, 2016). In addition, some microorganisms have been found to possess the ability to accumulate heavy metals by metabolism-dependent uptake (Timková et al., 2018; Zaynab et al., 2022). This can also be performed intracellularly (Usman et al., 2016). In nature, there are numerous hydrocarbon-degrading microbes, which are heavy metal-tolerant microorganisms as well (Dell'Anno et al., 2020). However, the tolerance to heavy metals complexed with weathered hydrocarbons was not reported. The most common hydrocarbons' bioremediators are *Achromobacter*, *Acinetobacter*, *Alkanindiges*, *Alteromonas*, *Arthrobacter*, *Burkholderia*, *Dietzia*, *Enterobacter*, *Kocuria*, *Marinobacter*, *Mycobacterium*, *Pandoraea*, *Pseudomonas*, *Staphylococcus*, *Streptobacillus*, *Streptococcus*, and *Rhodococcus* (Xu et al., 2018). Hydrocarbon-degrading bacteria own the ability to biodegrade unweathered alkanes and aromatic molecules in the presence of different soluble heavy metals (Ma et al., 2015). Unweathered naphthalene, anthracene, benzo[a]pyrene, phenanthrene, and pyrene can be biodegraded in the presence of heavy metals, such as Pb, Cu, Cd, and Cr (Liu et al., 2017). *Pseudomonas aeruginosa* is known to produce metal-chelating siderophores to make the metal bioavailable (Zaynab et al., 2022). They also produce biosurfactants (rhamnolipids) that improve the solubility and mobility of the organic compounds and heavy metals (Agnello et al., 2016). Biotreatment of heavy metals may be performed directly through surface binding of the metal to the extracellular substances or cell wall (Xu et al., 2018). It can also be indirect by cell uptake through metabolic processes (Gurav et al., 2017). Nonetheless, researchers are still investigating the conditions and adjusting the nutritional requirements of hydrocarbon-degrading bacteria that allow them to adapt and concomitantly degrade hydrocarbons and stabilize the heavy metals found in soils of industrial areas. The processes of concomitant biotreatment of organics and heavy metals are drawing increasing attention, aiming to provide the most appropriate approach for successful microbial remediation of oily-polluted soils. Heavy metals can also orient the fate of the hydrocarbons, as they can serve as electron acceptors (Gupta and Diwan, 2017).

On the other hand, weathering processes that are prominent in arid environments such as in Qatar are considered as additional limitations of bioremediation of oily-soils. Weathering contributes to the continuous alterations of the soil pollutants (Jiang et al., 2016; Biswas et al., 2018). Hence, hydrocarbons develop further recalcitrance to biodegradation. Moreover, the change of heavy metals status and their distribution in different forms, make them less bioavailable and more toxic (Al-Kaabi et al., 2017). Consequently, at such conditions, the endogenous hydrocarbon-degrading bacteria become less efficient because of the high level of toxicity. However, it was shown that several strains of the endogenous hydrocarbon-degrading bacteria may develop a specific adaptation to the weathered hydrocarbons and heavy metals and their bioavailability as well, since they exist in living forms even at low occurrence (Al Disi et al., 2017). Actually, the gap in the knowledge is related to the understanding of the tolerance to both heavy metals and hydrocarbons. As mentioned before, within fresh pollutants, microorganisms can exhibit mechanisms to overcome toxicity. However, the possibility that hydrocarbon-degrading bacteria can adapt to weathered hydrocarbons and heavy metals at the same time, both in complex mixtures and chemical status, is not evidenced. AlKaabi et al. (2020) demonstrated that the result of such weathering in an oil-wastes dumping site in Qatar is drastic to bacteria occurrence. Indeed, this study focused on using these endogenous hydrocarbon-degrading bacteria from the highly weathered oily soil in Dukhan (Qatar) to investigate their tolerance following their adaptation to weathered hydrocarbons and heavy metals. The current study would lead to a better understanding of the potential of these bacteria to concomitantly clean up the organics and heavy metals. In the industrial area of Dukhan in Qatar, the dumping site initially created to dispose the solid and liquid petroleum wastes from the oil industry becomes a site for study of the adaptation to weathered oil pollutants and diversity of bacteria. This study area represents an interesting model of highly weathered soil polluted with oil for more than three years, in which, hydrocarbons and heavy metals were subjected first to weathering processes due to Qatar weather conditions, second to self-purification processes and stabilization. As a consequence, it may be expected that they are adapted to complex residual oil pollutants. This makes the novelty of this approach. The objectives of this study were to (i) investigate and evidence the role of highly adapted bacterial strains isolated from weathered soils in Qatar to remove heavy metals when grown on hydrocarbons only, (ii) identify the most effective bacterial strains and their optimum conditions for both degrading oil hydrocarbons and removal of heavy metals, and (iii) investigate the relationship between the bacterial type, heavy metals proprieties and removal of hydrocarbons and heavy metals.

The findings clearly demonstrated the potential of highly adapted *Bacillus* and *Pseudomonas* strains due to their elevated tolerance to high concentrations of both hydrocarbons and heavy metals, to concomitantly biodegrade hydrocarbons and remove heavy metals. This makes them superbugs for the applications in remediation of weathered oil pollution.

2. Material and methods

2.1. Soil samples

The studied soil was sampled from a Dukhan dumping site, which was left for three years at open-air for self-purification after receiving solid and liquid oily wastes for three years. The soil was previously used for the isolation of some of the hydrocarbon-degrading bacteria used in this study (Table 1). The same soil sample was used to quantify the polluting heavy metals and their distribution in different forms.

Table 1
List of bacterial strains used in this study and their identification by ribotyping and/or MALD TOF.

Strain code	Strain identity	Accession number (Ribotyping)	MALDI TOF score
D12	*Bacillus cereus*	–	2.23
D1D2	*Bacillus licheniformis*	KY962349.1	2.06
Z3S1	*Bacillus licheniformis*	AF549498.1	2.04
Z4D1	*Bacillus licheniformis*	LN995452.1	2.05
D11	*Bacillus sonorensis*	–	1.97
D1D1	*Bacillus sp.*	MG855692.1	–
D1S1	*Bacillus sp.*	KY911251.1	–
D7D1	*Bacillus sp.*	KT945027.1	–
D2D2	*Bacillus subtilis*	MH071337.1	2.00
Z8D1	*Morganella morganii*	KU942503.1	2.10
D4D3	*Pantoea calida*	KX036541.1	2.07
Z6S1	*Providencia rettgeri*	CP027418.1	2.15
Z7S1	*Providencia rettgeri*	CP027418.1	2.10
D5D1	*Pseudomonas aeruginosa*	KY040017.1	2.21
D4S2	*Pseudomonas puteola*	NR114215.1	2.03
D13	*Pseudomonas stutzeri*	–	2.09
D9D1	*Pseudomonas stutzeri*	KY849415.1	2.20
D9S2	*Pseudomonas stutzeri*	KX180912.1	2.11

2.2. Analysis of heavy metals in Dukhan soils

The soil samples were first sieved with a 2 mm sieve and homogenized before use for analysis. The overall contents of each heavy metal in the soil samples were determined after digestion performed using 2 mL of (40% HF), 2 mL (65% HNO$_3$), and 6 mL (35% HCl) for each 0.25 g soil. Microwave Digestion System MARS 6 (CEM Corporation-USA) was used for samples digestion. Digestion was performed at 156 °C in a Teflon tube. The sequential extraction method was used to study the distribution of heavy metals in the soil. The sequential extraction was performed both with and without washing with dichloromethane (Migoni et al., 2021) (details with modifications provided in the supplementary online material). Regarding the analysis of the heavy metals in the bacterial cultures, 0.45 μm filter was used for filtering the liquid samples before analysis.

2.3. Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) of the heavy metals

Heavy metals were analyzed by the Inductively Coupled Plasma Optical Emission Spectroscopy using an ICP-OES PerkinElmer Optima 7300 DV (USA), [ESC-SOP-ICO15-01]. A calibration curve was prepared with a certified reference material (CRM) solution, containing 27 heavy metals. The calibration curve was performed using the blank and different concentrations of the CRM: 10 ppb, 50 ppb, 100 ppb, 500 ppb, and 1000 ppb. The standards and the fractions were analyzed in triplicates, and the average concentrations were calculated with standard deviations (for detailed ICP-OES method see Supplementary Online Data).

2.4. Hydrocarbon-degrading bacterial strains

In this study, 18 bacterial strains were previously isolated from soils polluted with weathered oil and sampled from oily wastes dumping areas in Dukhan and the beach of AlZubara in Qatar. All these strains were shown to be hydrocarbon-degrading bacteria (Al-Kaabi et al., 2018; Oualha et al., 2019; AlKaabi et al., 2020). Among these strains, 15 were identified using MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry) and confirmed by ribotyping (AlKaabi et al., 2020). The scores of similarities obtained by MALDI TOF are provided in Table 1. The strains were preserved at −80 °C in liquid Luria-Bertani (LB) medium supplemented with 30% glycerol. Before their use, each strain was first cultured in LB medium, then in Mineral Salts Medium (MSM) containing 5% oil medium to resuscitate its hydrocarbon-degrading activity.

2.5. Tolerance of the bacterial strains to nine heavy metals

Liquid LB and MSM-Diesel were used to evaluate the growth of the hydrocarbon-degrading bacterial isolates at several concentrations of the selected nine heavy metals based on the composition of the studied soil of Dukhan dumpsite. A stock solution of each heavy metal was prepared and then further supplemented to the medium to the final concentrations as indicated in the results. The heavy metals concentrations were 0 mM in the control and progressively increased up to 5 mM in the cultures. 1 mL diesel was added to 19 mL liquid MSM medium after inoculation with the strains. Diesel obtained from Mesaieed Refinery (though personal communication) at such a concentration of 5% was used as the sole carbon source for the growth of the inoculated strains. The cultures performed with diesel-MSM and LB media were inoculated starting with an initial optical density (OD) of 0.15 at 600 nm, using MSM-washed pellets collected

after centrifugation at 5000xg of overnight cultures in liquid LB medium. All the cultures, performed in triplicates, were incubated in a shaker set at 200 rpm and 30 °C. After 10 days of incubation, the OD_{600} of each culture was determined. The growth of each strain in the control (no heavy metals) was considered 100%. The relative growth of each strain at each heavy metal concentration was then calculated. A stock of diesel, stored in dark at room temperature, with a complete analysis of the contained hydrocarbons (n-C12-nC25) was provided from Um-Said refinery (Qatar). The diesel contained 75 g/L hydrocarbons. The C/N/P **ratio** in the MSM medium was adjusted to 100/10/1 as reported by Al Disi et al. (2017).

2.6. Growth kinetics at 1 mM heavy metals

Liquid MSM medium containing the heavy metal at 1 mM and supplemented with 5% Diesel (HM-MSM-5% Diesel) was inoculated by an inoculum prepared as described in Section 2.5, with an initial OD_{600} of 0.15. The initial concentration of the heavy metal in the culture was 1 mM. The cultures were then incubated at 30 °C in a shaker set at 200 rpm.

To estimate the number of viable bacterial cells in the liquid cultures, the colony-forming units (CFU) method was used. CFUs were measured by calculating the number of separate colonies formed on LB plates on which 100 μL of serial dilutions, in LB, were spread and incubated overnight at 30 °C. The CFUs are counted and the growth kinetics of each strain with each heavy metal **were** generated.

2.7. Diesel range organics (DRO) extraction and gas chromatography – Flame ionization detector (GC-FID) analysis

GC analysis was performed using a Perkin Elmer Clarus 680 GC-FID (USA) at a 150 °C injector temperature using Column Elite-1, with dimensions of 60 m, ID 0.25 mm, and DF 0.25 μm. After centrifugation of the cultures for 15 min at 5000 rpm, the fractions of the residual diesel were harvested. The diesel fraction was analyzed by GC-FID using hexane as a solvent and a 25 m silica column. U.S Environmental Protection Agency (EPA) method 8015 was employed for diesel analysis as reported by Al-Kaabi et al. (2018). The obtained chromatograms provided the total DRO concentrations. The removal of DRO by each bacterial strain was then calculated as the decreased percentage of their hydrocarbons content based on the control (non-inoculated).

2.8. Statistical analysis

Analysis of variance (ANOVA) were carried out at the 95% confidence level using the software IBM SPSS Statistics-Version 28.0.1.0. The Principal Component Analysis (PCA) was performed using (The Unscrambler X version 10.5.2).

3. Results and discussion

3.1. Analysis of heavy metals in the weathered polluted soil of the dumpsite of the oil industrial area of Dukhan

The overall content and distribution in fractions of heavy metals present in the polluted soil of the dumpsite in Dukhan (industrial area of Dukhan, Qatar), was determined using ICP-EOS after acidic digestion. In parallel, following a sequential fractionation, five different fractions of heavy metals were obtained from the same soil sample: exchangeable fraction (EXC), carbonate **fraction** (CA), iron–manganese oxide fraction (FMO), residual fraction (RES), and organic matter fraction (OM). The results are shown in Table 2.

The results indicate that 14 heavy metals were present at the detectable concentrations, in the Dukhan soil at concentrations higher than the EPA limits. The RES fraction was the hardest to remove from the insoluble components of the soil and showed the highest concentrations of the heavy metals compared to the other fractions. However, the sequential fractionation also showed that Fe, Mn, Ni, Zn, Ba, Mg, and Al were distributed in the RES and FMO fractions as well. Pb existed in the RES and EXC fractions, but not in the other forms. Mn, Zn, and Cd were the metals, which exist in the CA fraction in the washed soil with dichloromethane. Most of the metals were not associated with the CA fraction. Washing of the soil with dichloromethane allowed obtaining a CA fraction almost clean of heavy metals.

The procedure of the sequential fractionation was performed to determine the distribution of the different forms of the heavy metals in the soil. In addition, to study the effect of the soil-organic components on the distribution of the heavy metals in the different fractions, the fractionation was both performed with the raw soil and the washed soil. Indeed, the washing procedure with dichloromethane was performed to extract the oil organics bound to the soil components (Kwon and Hwang, 2017). Therefore, the impact of the oil organics and the weathering processes on the distribution of the metals into the different fractions were elucidated. The analysis of the different fractions obtained with and without soil washing revealed that the organic components in the soil did not strongly affect the forms and the distribution of the heavy metal in the soil. However, the majority of the detected heavy metals were strongly bonded to the residual and the iron–manganese oxide fractions. This would highlight the advantages of using microbial activities for the removal or immobilization of the heavy metals bonded to soil particles. Knowing that these hydrocarbons are highly weathered as shown by Al-Kaabi et al. (2018). However, reactions of sorption/desorption, in soils, through complexation with organic/inorganic ligands, as well as through redox reactions may occur abiotically (Caporale and Violante, 2016). Fundamentally, it is reported that the quasi-majority of the reactions are ensured biotically, allowing the control of their toxicity, bioavailability, and leaching (Violante et al., 2010).

Table 2
Analysis of heavy metals in the soil **and the** sequentially extracted fractions including carbonate fractions (CA), exchangeable fraction (EXC), iron–manganese oxide fraction (FMO), organic matter fraction (OM) and residual fraction (RES).

Heavy metals	Total (ppm)	Total (mM)	EPA limits (ppm)	Without washing with CH_2Cl_2					After washing with CH_2Cl_2				
				RES (ppm)	EXC (ppm)	CA (ppm)	FMO (ppm)	OM (ppm)	RES (ppm)	EXC (ppm)	CA (ppm)	FMO (ppm)	OM (ppm)
Cu	3.93	0.06	1.30	3.83	0.004	ND	0.002	ND	0.15	ND	ND	0.001	ND
Cr	24.76	0.54	0.10	22.2	0.06	0.2	0.9	0.09	2.7	ND	ND	0.9	0.08
Fe	642.8	11.46	–	284.4	0.06	2.1	372	1.5	254.8	1.5	ND	379	0.99
Mn	106.20	1.93	–	67.8	0.27	6.9	24.6	3.6	87.8	2.4	1.2	27	2.4
Ni	7.69	0.12	0.20	2.5	0.12	0.6	3.52	0.36	2.9	0.12	ND	3.77	0.3
Zn	39.92	0.61	0.50	16.5	1.32	2.4	20.3	0.052	17.1	2.6	0.9	21.1	1.2
Pb	9.96	0.05	15.00	7.2	2.15	ND	ND	ND	6.9	3.9	ND	ND	ND
Al	591	21.89	–	326.1	0.18	2.7	276	13.2	299	7.2	ND	275.5	8.7
Ba	601.8	4.39	0.002	477	3.1	4.2	119.3	3.3	468	2.9	ND	122.2	2.7
Mg	1188	51.55	–	953	2.6	2.1	128.7	1.9	998	1.9	ND	133.5	2.2
Ca	33.20	0.83	–	ND	ND	31.9	ND	ND	ND	ND	ND	34.1	ND
Cd	5	0.05	5	4.3	0.06	0.18	0.03	0.06	4.44	0.03	0.27	0.09	0.04
Co	ND	–	–	0.02	0.002	0.02	0.013	ND	0.015	0.001	0.02	0.014	ND
Ag	0.498	0.005	0.1	0.3	0.015	0.021	0.015	0.03	0.33	0.015	0.024	0.015	0.03
V	29.97	0.59	–	25.2	1.89	0.563	1.18	0.08	27.3	0.032	1.63	0.21	0.09

(ND): Not detected because below of detection, (–): not available.

3.2. Effect of the heavy metals on the growth of the bacterial isolates

The LB and diesel-MSM media were supplemented with one heavy metal in each liquid culture, at different concentrations. The Fig. 1 and Fig 1S illustrate the growth of the studied strains, on LB medium and on diesel-MSM medium at escalated concentrations of heavy metals (0–5 mM). The 18 bacterial strains were used in this study to explore the diversity of their adaptation to grow and tolerate the toxicity of different heavy metals in two culture media, LB as a rich medium composed mainly of organic compounds like peptones, and MSM-Diesel medium, which is fully composed of inorganic compounds, but supplemented with 5% diesel. The LB medium supplemented with heavy metals was used as previously reported by Govarthanan et al. (2016), Manasi Rajesh and Rajesh (2016), and Marzan et al. (2017). However, the use of inorganic media for this type of study was not previously reported. Since the objective of this study is to investigate the concomitant removal, by bacteria, of oil organics and heavy metals, the growth of the studied strains was also investigated when gown in MSM medium supplemented with 5% diesel as sole carbon source. The heavy metals used in this study were selected based on their occurrence at relatively high concentrations in the weathered oily soil of the studied area (Dukhan dumping site in Qatar). The growth of each bacterial strain was evaluated by measuring the optical density at 600 nm (OD_{600}) of the corresponding culture broth. Indeed, this technique allows estimating the density of cells, as viable and nonviable forms in the culture. The nonviable cells can also serve to adsorb heavy metals (Gupta and Diwan, 2017). All the 18 hydrocarbon-degrading bacterial strains showed growth in both media at the studied range of concentrations of each heavy metal. Although the tolerance to several heavy metals was higher in LB than in MSM-Diesel, a threshold of almost 1 mM of each heavy metal can be commonly observed in both media. A certain variability in tolerance to the heavy metals was also clear with several strains of *Pseudomonas* and *Bacillus* in the LB medium. However, in the MSM-Diesel medium the variability is less clear. These results confirm that these bacterial strains of *Pseudomonas* and *Bacillus* exhibit high potential of bioremediation of fresh and weathered oil as shown with other strains by Das and Chandran (2010) and AlKaabi et al. (2020). However, a growth of less than 20% would not be sufficient to correctly ensure a bacterial population dynamic (Allen and Waclaw, 2018).

3.3. Tolerance to toxicity

In general, Cr, Pb, Cu, and Fe were the least toxic heavy metals while the Mn, Zn, and Al exerted higher toxicity levels with a minimum inhibitory concentration of 3.5 mM and 2.5 mM in LB and MSM-Diesel media, respectively. Hence, Table 3 clearly shows that the 18 studied strains can tolerate the nine heavy metals at concentrations up to 3.5 mM with more than 20% growth. In addition, each group of strains (Fig. 1 & Fig. 1S) showed high similarity in their growth and behavior at each heavy metal concentration. The analysis of variance (ANOVA) performed at 95% confidence, showed that the differences between the strains of the same group were not significant.

The cultural conditions and the medium composition are known to affect the metabolism of the growing bacterial cells as well as the interaction between the bacterial cells and the microenvironment (Rosenthal et al., 2018). Consequently, these factors would affect differently the cell's tolerance to toxicity of heavy metals due to the diversity of bacterial metabolism, including hydrocarbon-degrading ones (Jallouli et al., 2010; Attar et al., 2017). This is mainly observed by comparing the tolerance of the strains in LB and MSM-Diesel media. All tested strains showed higher tolerance to the heavy metals when grown on LB compared with MSM-Diesel. Indeed, in the organic-complex media, organics may support absorbing heavy metals (Caporale and Violante, 2016). In addition, some heavy metals have a high affinity to bind to
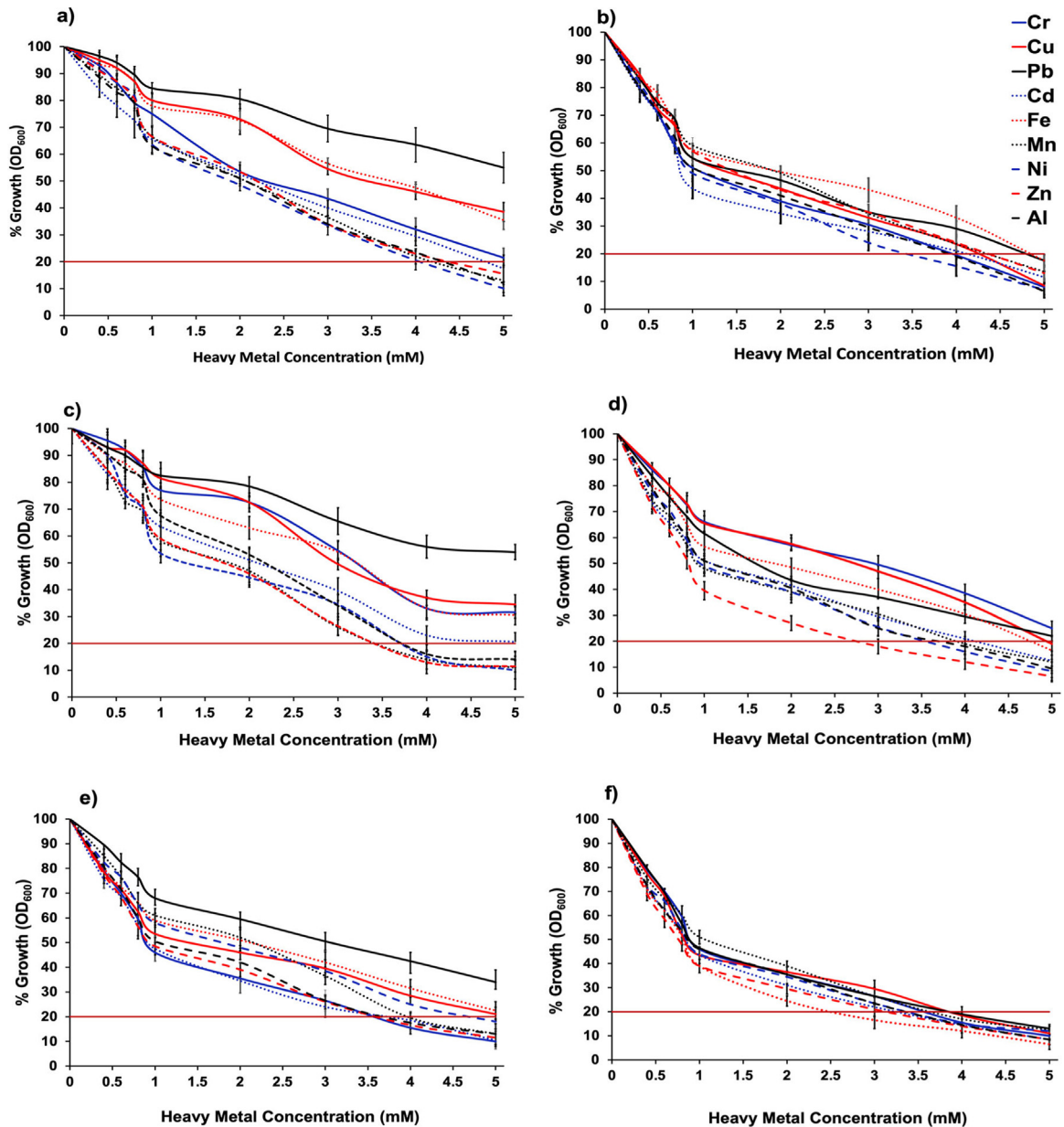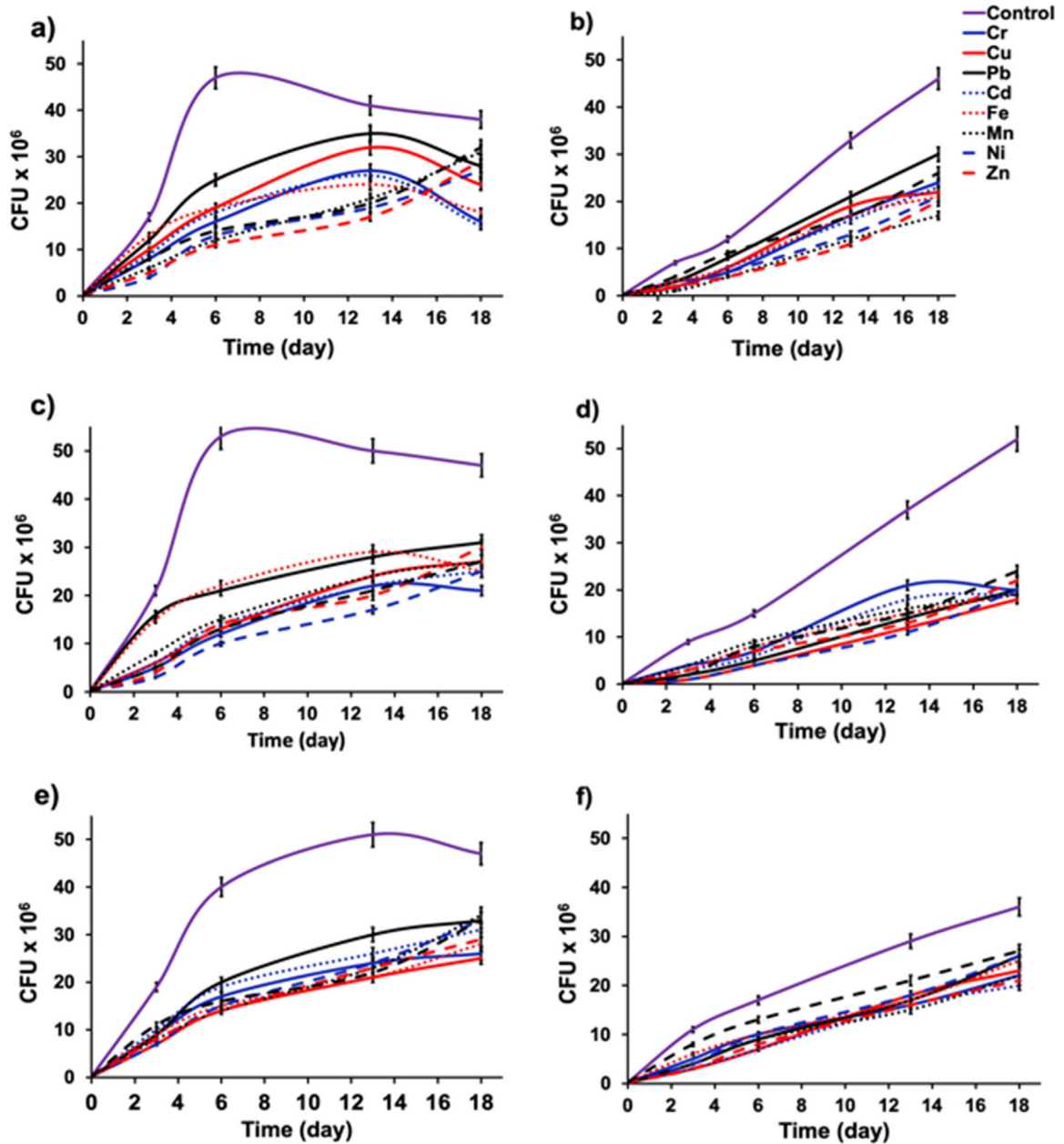
Fig. 1. Growth of the selected bacterial strains, each in LB medium or MSM-Diesel medium supplemented with different concentrations of heavy metals (0.4–5 mM); (a) *Pseudomonas* strains on LB, (b) *Pseudomonas* strains on MSM-Diesel, (c) *Bacillus* strains on LB, (d) *Bacillus* strains on MSM-Diesel, (e) *Pantoea calida* on LB and (f) *Pantoea calida* on MSM-diesel.

organic matter, which may cause a decrease in their bioavailability (Olaniran et al., 2013). Overall, *Pseudomonas* and *Bacillus* bacterial strains exhibited the highest tolerance to all the tested heavy metals. The threshold to cause less than 20% growth are much higher than those previous reported by Singh et al. (2019) and Ramírez et al. (2019) for these two types of bacteria. This is an additional indication of the high adaptation caused by the weathering processes occurring in the microenvironment of the studied strains in their natural habit from where they were isolated. In contrast, *Morganella morganii* and *Pantoea calida* showed the lowest tolerance.

3.4. Potential of the selected hydrocarbon-degrading bacterial strains to grow at 1 mM heavy metals

Based on their relative tolerance to heavy metals in a range of concentrations from 0 to 5 mM using both LB and MSM-Diesel media, seven strains were selected: *B. sonorensis* (D11), *Bacillus sp.* (D1D1), *B. subtilis* (D12), *B. licheniformis*

Fig. 2. Influence of the heavy metals on the growth of the bacterial strains cultured in LB medium and MSM-Diesel medium supplemented with each heavy metal (1 mM); (a) *B. sonorensis* (D11) grown in LB, (b) *B. sonorensis* (D11) grown in MSM-Diesel, (c) *Bacillus sp.* (D1D1) in LB, (d) *P. puteola* (D1D1) in MSM-Diesel, (e) *P. stutzeri* (D9D1) in LB and (f) *P. stutzeri* (D9D1) in MSM-Diesel.

(D1D2), *P. puteola* (D4S2), *P. aeruginosa* (D5D1) and *P. stutzeri* (D9D1). The concentration of the heavy metals was 1 mM, corresponding to the common threshold of 50% growth in MSM and LB cultures. The results are shown in Fig. 2 and Fig. 2S.

In this study, the evaluation of the growth was performed by counting the viable cells only. This was important to perform since the population dynamic, resulting in growth, is the result of the viability of cells, which in turn depends on the medium composition and the level of toxicity. Interestingly, the growth of almost all the strains on each of the nine heavy metals was continuous in both media (LB and MSM-Diesel) during the incubation period of 18 days. However, *B. sonorensis* D11 was an exception because it entered into the stationary and death phases after 12 days of incubation in LB supplemented with one of the following heavy metals: Pb, Cr, Zn, Cd, and Fe. This particular growth behavior of *B. sonorensis* D11 was not shown in the MSM-Diesel medium. In contrast, the stationary phase of *Bacillus sp.* D1D1 started

Table 3

Maximum tolerable concentration (MTC) of the nine metal ions tested against bacterial isolates. The values were extracted from the results of Fig. 1 and Fig 1S that correspond to 20% growth.

	Medium	Cr	Cu	Pb	Cd	Fe	Mn	Ni	Zn	Al
Pseudomonas sp.	LB	5	>5	>5	5	>5	4	4	4	4.5
	MSM	4	4.5	4	4.5	4	4	3.5	4	4
Bacillus sp.	LB	>5	>5	>5	>5	4	3.5	4	3.5	4
	MSM	5	5	5	3.5	4.5	3.5	3.5	3	4
Providencia rettgeri	LB	>5	>5	>5	4	5	4	4.5	4	4
	MSM	4	4.5	4	3.5	4	3.5	3.5	3.5	3.5
Morganella morganii	LB	>5	>5	>5	4	5	4.5	4.5	4	4.5
	MSM	3.5	4	4	3	2.5	3.5	3	3	3.5
Pantoea calida	LB	3.5	5	>5	3.5	5	4	4.5	3.5	3.5
	MSM	3.5	4	4	3	2.5	3.5	3	3	3

after 12 to 14 days in the cultures containing Cr or Cd in MSM-Diesel. The strain *P. stutzeri* D9D1 also started the stationary phase at around day 14 in MSM-Diesel medium containing Pb. It is clear from all the growth curves that the selected strains tolerate 1 mM with higher tolerance shown in the MSM-Diesel medium. Although a similar trend in the behavior of all the strains, several strains of *Bacillus* or *Pseudomonas* may show lower tolerance than others. In addition, since the study was performed using 1 mM, the strains were able to develop their growth as a response to the heavy metals' toxicity, which allowed the confirmation of the potential of adaptation of each of them. The adaptation of the strains in MSM-Diesel was interesting to consider because it occurs concomitantly with the degradation of diesel hydrocarbons as sole carbon sources for growth. In addition, the strains adapt and tolerate the combined toxicity of both the 5% diesel and 1 mM heavy metals. Here, it important to notice that since the diesel contained 75 g/L hydrocarbons (Al Disi et al., 2017), the 5% diesel provided 37.5 g/l hydrocarbons which should exhibit a high toxicity to the bacterial cells, growing in MSM-Diesel medium (Attar et al., 2017).

3.5. Potential of the selected strain to concomitantly remove heavy metals and hydrocarbons from diesel

The concentrations of the heavy metals were determined using ICP-OES at the inoculation time of the MSM-Diesel cultures with the bacterial cells, then after 10-days and 18-days incubation. The results are shown in Fig. 3.

The performance of each of the strains in the removal of each heavy metal at a soluble form was evaluated. In fact, in addition to the concentration of the nine supplemented heavy metals, K, Mg, Mn, Na, Ni, and Zn are also part of the MSM medium composition, although at low concentrations of (mM): Na, 0.016; K, 0.006; Mg, 0.0008; Mn, 1.18×10^{-6}; Ni, 7.75×10^{-7} and Zn, 6.21×10^{-7}. Besides, diesel, which was supplemented to the liquid cultures as sole carbon source, could supplement some heavy metals in the medium (Pulles et al., 2012). Therefore, the removal percentage in each culture was calculated based on the existing concentrations at the inoculation time. All the bacterial strains showed removal of all the heavy metals. Interestingly, higher removals of Pb and Fe, fluctuating between 75% and 89%, were obtained with all the strains. Removal of Cr and Ni did not exceed 55% with *Pseudomonas* strains and 50% with *Bacillus* strains. Removal of Al was not exceeding 40% although the moderate inhibition of growth. Removal of Pb by *P. stutzeri* D9D1was 79% after 18 days, although the stationary phase of growth started after 14 days. In addition, Cd and Cr exhibited the highest toxicity to *Bacillus sp.* D1D1 with removal efficiency reaching 46% and 54% respectively at 18 days of incubation (Fig. 2 and Fig 2S). These results may be due to the early entrance into stationary and death phases. It is observed that the removal efficiency was only 29% and 31%, respectively after 10 days, while the growth of *Bacillus sp.* D1D1 stopped on day 12. These findings indicate that the two heavy metals (Cd & Cr) can be continually transformed **to** insoluble forms by the bacterial cells or precipitated or trapped in biofilms, even if not associated with growth. During the stationary phase, the production of the exopolymeric substances may continue as a secondary metabolite (Cihak et al., 2017). Hence, the removal of the heavy metals with these bacterial strains was not only associated with their growth but also with the secreted molecules, which may also include biosurfactants. Hydrocarbon-degrading bacteria produce biosurfactants to enhance the bioavailability of hydrocarbons (Ma et al., 2015). Biosurfactants may play a role in their tolerance to heavy metals, by adsorbing them (Ayangbenro and Babalola, 2018). Variances in the mechanisms of heavy metals removal would be expected for *Bacillus* as a Gram-positive bacterium and *Pseudomonas* as a Gram-negative one.

The content of the DRO in the inoculated MSM-Diesel medium by each of the seven strains was reduced. The removal ranged from 29%–50% after 10 days and 37%–76% after 18 days of incubation as shown in (Table 4).

In order to attribute the removal of the heavy metals to the cell growth, the removal of diesel hydrocarbons, as the only carbon source was evaluated by analyzing the residual diesel fraction in each culture. The results show that the content of the DRO content in the inoculated MSM-Diesel medium of each of the studied strains was reduced. The removal efficiencies fluctuated from 29% to 50% after 10 days and 37% to 73% after 18 days of incubation. The differences between the strains were not statistically significant after the media analysis on the 18th day of incubation as confirmed by ANOVA at 95% confidence level. Some differences may be statistically significant with results obtained at 18 days of incubation.
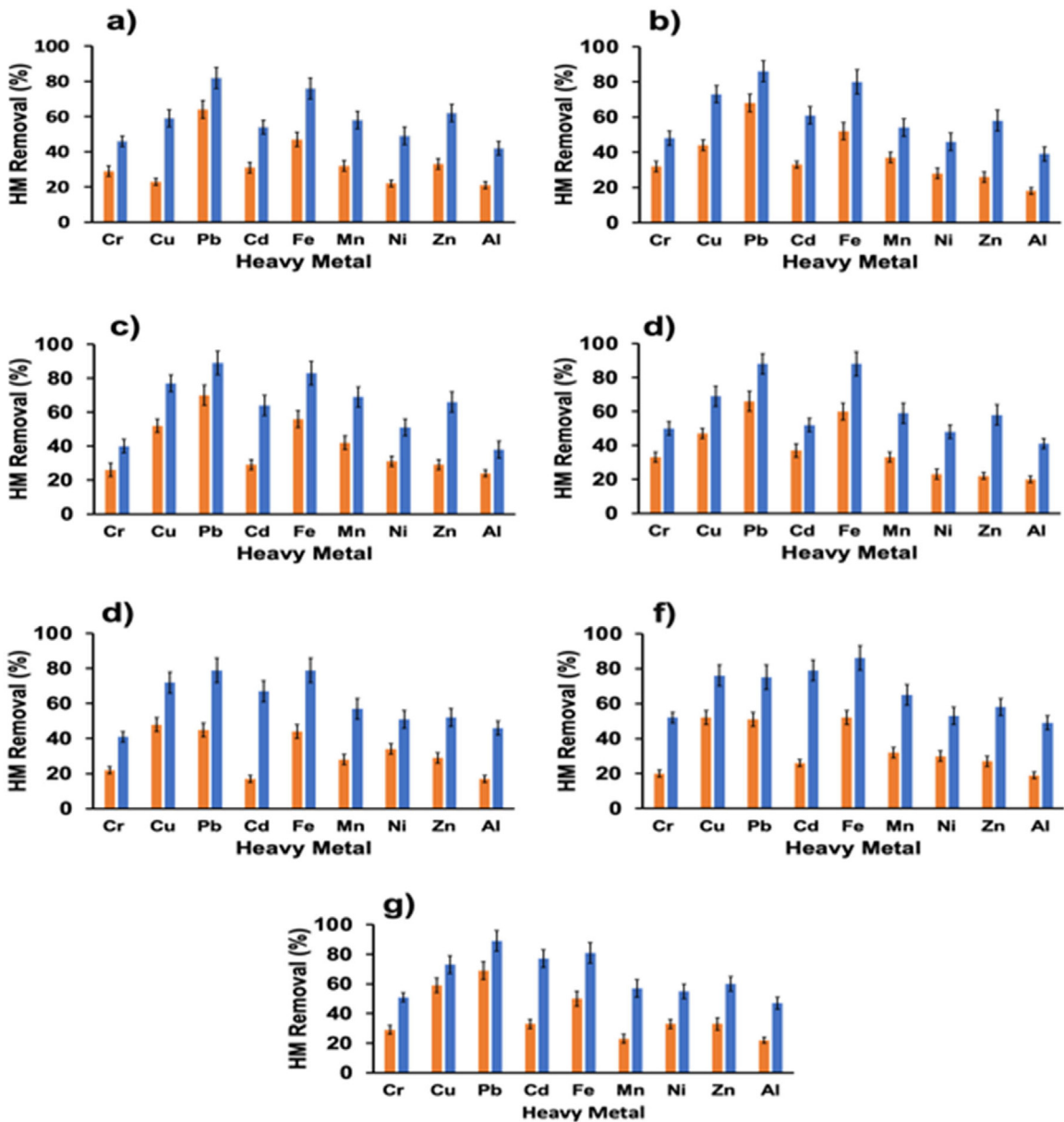
Fig. 3. Heavy metals removal efficiency (%) in each MSM-Diesel liquid culture of the studied strains after 10 days (orange bars) and 18 days (blue bar)s of incubation, (a) *B. sonorensis* (D11), (b) *B. subtilis* (D12), (c) *B. licheniformis* (D1D2), (d) *P. puteola* (D4S2), (e) *P. aeruginosa* (D5D1), (f) *P. stutzeri* (D9D1) and (g) *Bacillus sp.* (D1D1).. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The latter result confirms that the removal efficiency of some strains may continue at late growth phases. Overall, it may be concluded that the seven selected strains were able to remove hydrocarbons from diesel to serve as the sole carbon source, and concomitantly, remove heavy metals supplemented to the diesel medium at 1 mM. These strains showed adaptation and high tolerance to the high concentration of diesel (5%, providing 37.5 g/l hydrocarbons) and that of each heavy metal used at 1 mM.

Microbial degradation of hydrocarbons occurs through a series of catalytic reactions by intracellular and extracellular enzymes including uptake of pollutants and surfactants' synthesis to facilitate emulsification (Fayeulle et al., 2014). Consequently, the emulsified pollutants will be adsorbed on and eventually enter the cell membrane to go through particular enzymatic reactions organized in specific pathways for each hydrocarbon compound (Karlapudi et al., 2018).

Table 4
Removal (%) of Diesel Range Organics (DRO) in the cultures performed with the studied strains in 5% MSM-Diesel containing 1 mM heavy metals at incubation periods of 10 & 18 days.

HM	Incubation (d)	B. sonorensis D11	Bacillus sp. D1D1	B. subtilis D12	B. licheniformis D1D2	P. puteola D4S2	P. aeruginosa D5D1	P. stutzeri D9D1
Cr	10	38 ± 3	41 ± 3	37 ± 2	43 ± 4	37 ± 4	34 ± 3	37 ± 3
	18	66 ± 5	62 ± 5	61 ± 6	64 ± 5	61 ± 6	66 ± 6	67 ± 6
Cu	10	41 ± 3	47 ± 4	50 ± 5	43 ± 3	42 ± 4	44 ± 4	49 ± 5
	18	58 ± 5	63 ± 6	67 ± 6	71 ± 6	69 ± 6	70 ± 7	71 ± 6
Pb	10	41 ± 3	49 ± 4	47 ± 4	41 ± 3	40 ± 3	41 ± 3	49 ± 4
	18	67 ± 5	68 ± 6	72 ± 6	68 ± 6	69 ± 6	65 ± 5	69 ± 6
Cd	10	39 ± 3	37 ± 4	33 ± 3	31 ± 3	29 ± 3	35 ± 4	37 ± 3
	18	60 ± 5	63 ± 5	65 ± 6	59 ± 5	60 ± 5	59 ± 6	64 ± 6
Fe	10	46 ± 5	49 ± 4	50 ± 5	52 ± 5	49 ± 5	51 ± 5	49 ± 4
	18	69 ± 6	70 ± 6	70 ± 5	73 ± 6	66 ± 6	68 ± 5	71 ± 6
Mn	10	37 ± 4	39 ± 3	44 ± 4	39 ± 4	38 ± 4	40 ± 3	36 ± 3
	18	59 ± 4	64 ± 5	68 ± 6	58 ± 5	59 ± 6	62 ± 5	56 ± 4
Ni	10	29 ± 3	31 ± 3	30 ± 3	33 ± 4	32 ± 3	30 ± 4	33 ± 3
	18	51 ± 5	49 ± 5	54 ± 5	56 ± 5	56 ± 6	54 ± 5	59 ± 6
Zn	10	39 ± 4	36 ± 3	39 ± 3	40 ± 4	39 ± 3	37 ± 3	40 ± 3
	18	63 ± 5	58 ± 5	65 ± 5	62 ± 5	62 ± 5	68 ± 5	62 ± 5
Al	10	26 ± 2	28 ± 2	32 ± 3	30 ± 2	29 ± 3	33 ± 3	39 ± 4
	18	43 ± 3	44 ± 4	43 ± 3	47 ± 5	46 ± 5	48 ± 5	49 ± 5

Alkanes generally undergo terminal or subterminal oxidation. The oxidation products are further oxidized by alcohol and aldehyde dehydrogenases, while the resulting fatty acids enter the tricarboxylic acid (TCA) cycle (Khalid et al., 2021).

Diesel hydrocarbon compounds (long chain alkenes) are the most abundant components of DROs (Mohanakrishna et al., 2019). They undergo similar degradation in catabolic enzymatic processes, until they are mostly integrated as cell components during the anabolism.

Heavy metals cannot be biologically abolished but can be altered between oxidation states (Igiri et al., 2018; Briffa et al., 2020). Microorganisms develop their protection capability from heavy metal toxicity by various mechanisms, such as uptake, adsorption, oxidation/reduction and methylation (Lala et al., 2021). Bacteria can utilize metals as terminal electron acceptors for anaerobic respiration or may acquire reduction mechanisms that are not combined with respiration but meant to impart metal resistance (Joutey et al., 2013).

There are many metal tolerant-microbes which are capable of accumulating and transforming toxic metals and thus helps in detoxification processes. A number of processes are involved in metal removal by different tolerant microorganisms. These include (i) precipitation of heavy metals and radionuclides by production of extracellular substances (EPS) which interact with metal cations forming insoluble precipitates (Kuippers et al., 2021); (ii) biotransformation of metals and radionuclides either by oxidation, reduction or alkylation reactions (Filote et al., 2021); and (iii) intercellular or extracellular accumulation (Li et al., 2021).

3.6. PCA investigation of the interaction between the multi-variants influencing hydrocarbons and heavy metals removal

It is important to understand the type of interaction between the bacterial cell, in term of structure and metabolism, and the characteristics of the heavy metal, which lead to high or low removal of each heavy metal. This investigation within a so complex situation with many variables, was performed using PCA. The growth curves were further analyzed using principal component analysis. PCA allows the formation of groups that have similar variance characteristics (Abdel Samad et al., 2020). The slops of the growth curves on the LB (slop L1–L4) and on the MSM-diesel at different growth stages (slop M1–M4) were subjected to PCA analysis, in addition to ion radius, hydrated ionic radius, solubility product constant (Ksp) as reported by De Carvalho Izidoro et al. (2011), Ziyath et al. (2011) and Uysal et al. (2017) (Table 1S), heavy metals removal efficiency (HM RE%), and DRO removal (DRO RE%) at 10 days and 18 days incubation periods (Table 1S), As shown in Fig. 4a, PC1 and PC2 accounted for 82% and 8% variability, respectively. Hence, the first two components explained 90% of the variance of data, leading to sufficient discrimination of samples. The contribution of each variable to the differentiation of samples can be evaluated by computing the correlation loadings plot (Fig. 4b). The variables located in the outer ellipse have the greater impact on the specific pattern of each sample, representing 100% of the explained variance, while the variables with loading values near zero have similar values in all samples. Accordingly, the Ksp, ionic radius, and removal efficiencies (heavy metal RE% & DRO RE%) were found to contribute significantly to the discrimination of samples.

It is known that the hydrocarbon-degrading bacteria may involve secretion of extracellular substances and/or adsorption on the cell surface for removal of heavy metals (Karlapudi et al., 2018; Tarekegn et al., 2020). Four groups could be obtained from PCA (Fig. 4a). Group 1 is composed of all the studied strains grown in MSM-Diesel cultures supplemented
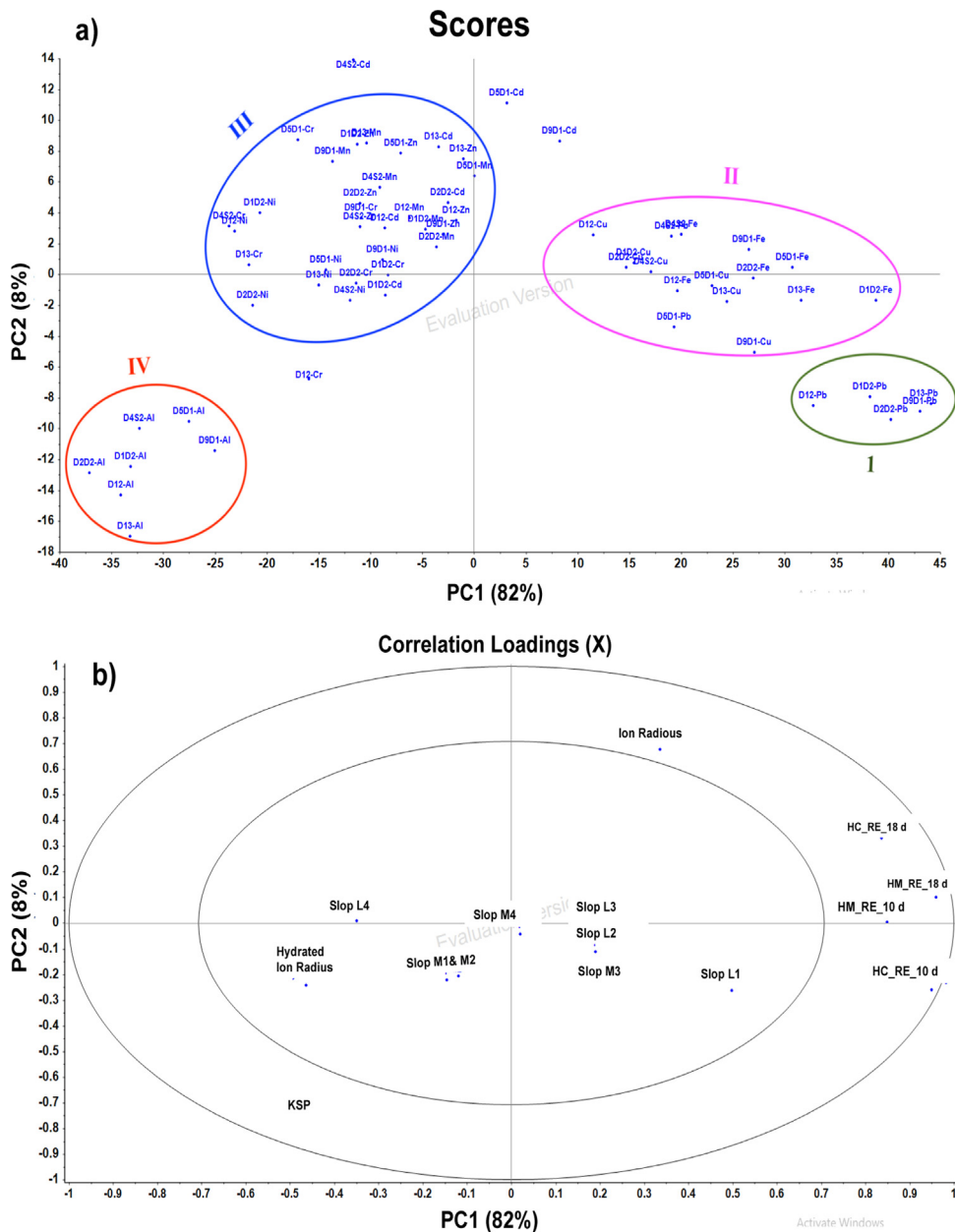
Fig. 4. Investigation of the role of bacteria type and growth and the characteristics of each heavy using PCA: (a) PCA grouping of the studied strains, (b) Correlation loading (X).

with Pb. Group II is represented by the strains grown in MSM-Diesel cultures supplemented with Cu and Fe. Group III is composed of the strains grown with Cr, Cd, Mn, Ni, Zn, except three *Pseudomonas* strains (D4S2, D5D1, and D9D1) with Cd, while group IV contained all the studied strains when grown in the presence of Al. The strain *B. sonorensis* (D11) grown with Cr was located at far distance from all the formed groups. Group I is characterized by high removal efficiencies in terms of DRO and heavy metals both have a positive correlation with PC1 and negative correlation with PC2. Group II can be subdivided into two sub-groups, for example, the strain *Bacillus sp.* (D1D2) exhibited high removal efficiencies at an incubation period of 10 days while the strain *P. aeruginosa* showed high removal efficiencies at an incubation period of 18 days. Group IV (all strains with Al) exhibited the lowest removal efficiencies as it correlates significantly negatively with removal efficiencies of heavy metals and DRO, but strongly positively correlated with Ksp. Hence, we can conclude that the removal efficiencies of heavy metals are highly dependent on the type and the status of chemical structure of the heavy metal.

4. Practical applications

Oil weathering processes change the properties and composition of oil. This is why petroleum-derived contamination events constitute a unique environmental issue. This represents the main origin of failure of bioremediation applications. Microbial ecology and adaptation provide solutions to regions characterized by over prolonged periods of weathering. However, the employed adapted bacterial strains should tolerate the toxicity and remove both weathered hydrocarbons and heavy metals. These types of strains were isolated and shown highly efficient on hydrocarbons. Here, it was clear that they can also remove concomitantly, the heavy metals detected in the weathered soil. This finding is of high importance for such situations. However, it is important to evaluate the performance of these strains on weathered soils, in biopiles as an example. Interestingly, it was shown in the laboratory that the endogenous bacteria of the studied weathered soil were able to remove up to 65% of the weathered hydrocarbons, if appropriately biostimulated. Actually, it will be interesting to combine the biostimulation to bioaugmentation using selected strains which showed high performance of concomitant removal of hydrocarbons and heavy metals, in soil and at non-sterile conditions.

5. Conclusions

In soils polluted with weathered petroleum components, the organic fraction does not strongly affect the distribution of the heavy metals which are strongly bonded to the iron–manganese oxide and the residual fractions. This fraction was the hardest to remove from the soil. This is in favor of further bioremediation. Among 18 highly adapted bacterial strains, several *Bacillus* and *Pseudomonas* strains were shown efficient for the concomitant removal of diesel range hydrocarbons and heavy metals. Using the PCA methodology, with multivariate components, the efficiency of heavy metals removal was shown depended on each heavy metal properties, not on the bacterial type. This means that the bacterial adaptation occurred at the level of the cells structure and/or their exopolymeric substances which immobilize the heavy metals and reduce their toxicity, allowing bacterial cells to grow and remove hydrocarbons. This is interesting for the bioremediation of highly weathered oil pollutants.

CRediT authorship contribution statement

Zulfa Al Disi: Conceptualized & designed the research, Performed the experiments, Analyzed the data, Wrote the manuscript. **Mohammad A. Al-Ghouti:** Helped with conceptualization, Formal analysis of the data. **Nabil Zouari:** Conceptualized & designed the research, Analyzed the data, Provided resources, Equipment and infrastructure, Reviewed & edited the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2022.102513.

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