# Variant effects of arsenic compounds on crude oil bioremediation by crude oil-degrading bacteria in Kuwait

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## Abstract

Crude oil spills into the environment such as that of Kuwait cause health and ecological problems which necessitates the cleanup of such pollution by efficient methods such as bioremediation. However, the associated heavy metals such as arsenic with crude oil pollution exert inhibitory effects on bioremediating agents for instance soil microbiota leading to hindered bioremediation. In the current study, the tolerance of two dominant crude oil-degrading bacteria in Kuwaiti soil, *Acinetobacter spp.* a Gram-negative bacteria and *Nocardia spp.* a Gram-positive bacteria, to varying concentrations of arsenate and arsenite was investigated. Results showed the higher potentials of *Nocardia spp.* to resist the inhibitory effects of added arsenic compounds. Also, arsenite demonstrated significantly higher inhibitory effects on bacterial growth and activity compared to those of arsenate. Additionally, determination of the crude oil mineralization potentials of isolated bacteria demonstrated the significantly higher potentials of *Nocardia spp.* to mineralize crude oil in presence of arsenic compounds. Moreover, the phylogenetic assessment of isolated strains of *Acinetobacter* and *Nocardia* using 16S-RFLP analyses showed the higher diversity of *Nocardia* strains compared to *Acinetobacter*. Thus, this study demonstrated the variant effects of arsenic species on the growth and activity of different crude oil-degrading bacteria. Also, results indicated that, the potential of crude oil-degrading bacteria to tolerate the inhibitory effects of heavy metals, such as arsenic, could accelerate the bioremediation of crude oil pollution.
*Keywords: Crude oil-degrading bacteria, Gram-negative bacteria, Gram-positive bacteria, Acinetobacter spp., Nocardia spp., arsenite, arsenate.*

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# 1 Introduction

Crude oil contamination is a worldwide continuous problem especially to oil-exporting countries such as Kuwait. Oil spills both deliberate and accidental [1] have the tendency to affect human health [2] and ecological niches [3]. Thus, bioremediation is highly required for such polluted environments and considered an excellent approach for cleaning-up crude oil pollution [4] via the indigenous microbiota that in general are sequentially exposed to a variety of pollutants [5]. Usually crude oil pollution is associated with heavy metals contamination, in particular arsenic [6–8]. In nature arsenic occurs in four oxidation states: (-3) in the form of arsenides, arsine ($AsH_3$) and arsenic chloride ($AsCl_3$); (0) in the form of native arsenic; (+3) in the form of oxides, sulfides, sulfo-salts, and arsenite, and (+5) in the form of arsenate [9]. Arsenic exerts severe toxic effects to all forms of organisms [10], and as a result it was ranked as number one on the Environmental Protection Agency (EPA) list of toxic metals [11]. However, scarce studies are available on the effects of arsenic compounds on hydrocarbon biodegradation potentials of the soil microbiota [12]. Therefore, the growth, activity, diversity and tolerance of two dominant crude oil-degrading bacteria (CODB) in Kuwaiti soil, *Acinetobacter spp.* [13, 14] a Gram-negative bacteria and *Nocardia spp.* [15, 16] a Gram-positive bacteria to varying concentrations of arsenate and arsenite were investigated in the current study.

# 2 Materials and methods

## 2.1 Effect of arsenate and arsenite on the growth of the CODB *Acinetobacter spp.* and *Nocardia spp.*

The potential inhibitory effects of arsenic compounds (arsenate and arsenite) on the growth of the CODB *Acinetobacter spp.* and *Nocardia spp.* were determined using the well diffusion method and optical density measurements.

### 2.1.1 Agar well diffusion method

The method of (Lertcanawanichakul and Sawangnop [17]) was adopted. Stocked bacterial cultures were streaked on NA plates and incubated at 30°C for 24 hours. Grown bacterial cultures were transferred into sterile 15 ml Falcon tubes containing sterile 0.85% NaCl solution followed by adjusting the number of bacterial cells to 1.2 x $10^9$ $cells^{-1}$ ml using McFarland No.0.5 as a standard solution. Then, aliquots (100 µl) of the prepared bacterial suspension were spread on the surface of NA plates, spread evenly and left for 30 min at room temperature. Then, wells (10 mm in diameter) were punched using sterile stainless steel cork borer. Prepared metal ($As^{+5}$, 50 $mg^{-1}$ ml and $As^{+3}$, 5 $mg^{-1}$ ml) solutions were directly filled (50 µl) into the wells, plates incubated at 30°C for 24 hours and the diameter of the inhibition zones were measured in millimeter [17].

### 2.1.2 Optical density measurements

Prepared bacterial suspensions used for the well diffusion method mentioned previously were used simultaneously for the optical density measurement method. For this purpose, bacterial suspension (50µl), sterile nutrient broth (50µl) and different concentrations of arsenate and arsenite solutions (300 µl) were transferred to 100-well Honeycomb plates. Then, plates were incubated shaking at 30°C for 24 hours in automated optical density reader (600 nm). Determined optical density values were plotted against time followed by calculation of bacterial growth rates. Bacterial growth rates were used to determine the minimum inhibitory concentrations (MIC) for tested metals. MIC values were defined as the lowest concentration of tested metals that inhibited the growth of bacteria after 24 hours of incubation.

## 2.2 Effect of arsenic compounds on the activity of the CODB *Acinetobacter spp.* and *Nocardia spp.*

The inhibitory effects of arsenate and arsenic on the activities of the CODB *Acinetobacter spp.* and *Nocardia spp.* were determined. For this purpose, the activity of the dehydrogenase and the rate of carbon dioxide evolution were used as indicators for bacteria activity under different carbon sources and metal concentrations.

### 2.2.1 Determination of dehydrogenase activity

The method of (Alef [18]) was adopted. Overnight grown bacterial cultures on nutrient broth were harvested at 4200 xg in sterile 50 ml falcon tubes. Pellets were suspended in 0.85% NaCl and the optical density of the cultures was adjusted to 1.0 OD (620 nm). Then, bacterial suspension (1.5 ml), carbon source (crude oil, 100µl and 20 mM; nutrient broth as control) and metal solution (0.5 ml of arsenate and arsenite) were transferred into 50 ml sterile Falcon tubes followed by the addition of triphenyltetrazolium chloride (TTC) solution (5 ml) solution, contents mixed well and incubated shaking (200 rpm) at 30°C for 24 hours. Solution of TTC was prepared by dissolving 0.1 g TTC in 100 ml 100 mM Tris HCl buffer (pH 7.6). Following incubation, the reduction product of TTC, triphenyl formazan (TPF), was extracted with acetone (40 ml) by agitation in the dark. The extracts were membrane filtered (0.45 mm) and the presence of the red-coloured TPF was detected in the filtrate by a spectrophotometer (Genesis 5) at 546 nm. Concentration of formed TPF was determined using TPF calibration curve.

### 2.2.2 Mineralization of crude oil

The effects of the addition of different concentrations of arsenate (5, 10 and 20 $mg^{-1}$ ml) and arsenite (0.6, and 1.2 $mg^{-1}$ ml) in on the utilization of crude oil by crude oil-degrading bacteria were determined using respirometry. The amounts of carbon dioxide evolved were measured using a Micro-oxymax respirometer (Columbus Instruments, USA). The reaction vessels contained 1 ml of overnight bacterial cultures with optical density adjusted to 1.0 (600 nm) as mentioned previously, Hutner's minimal media (45 ml), crude oil (100µl), and

desired metal concentration (4 ml). Reaction vessels were incubated in a shaking water bath at 30°C for 24 hours. Three sets of controls were used. One set of controls constituted vessels contained sterile Hutner's minimal media and crude oil. Another set of controls included vessels contained Hutner's minimal media, crude oil and bacterial cultures. In addition, one set of controls constituted vessels contained sterile Hutner's minimal media and bacterial cultures. The amounts of evolved carbon dioxide determined were used as indicator of bacterial activity.

### 2.3 Fingerprinting of the CODB *Acinetobacter spp.* and *Nocardia spp.* using restriction fragment length polymorphism (RFLP) of 16S rRNA

Sequences of 16S rDNA genes of the CODB *Acinetobacter spp.* and *Nocardia spp.* strains were amplified from extracted DNA. Following PCR amplification, 10 µl of amplified 16S rDNA from each of isolates were digested separately with *BstUI* restriction endonuclease (New England Biolabs) in 15 µl reaction mixtures as recommended by the manufacturer. Digests were electrophoresed in 2% agarose gels, with TBE buffer. Gels were stained with ethidium bromide and then photographed under UV light. DNA fragment sizes were determined using Kodak Digital Image analysis software (Kodak, Rochester). The quality of the numerical data was checked by comparing the sum of fragment sizes in each restriction pattern with the original product size (approximately 1,499 bp). Cluster analyses of band patterns were carried out using GelCompar II software (Applied Maths). Profiles of isolated bacteria were compared based on Pearson coefficient with an optimization of 1.0% and a tolerance of 1.0%. Dendrograms were obtained using the hierarchical Dice correlation/unweighted pair group method with arithmetic mean (UPGMA) algorithm. Fragments smaller than 48.5 bp in length were not used in the analyses [19]. Each phylotype was defined as a group of sequences that have indistinguishable *BstUI* restriction patterns [20].

## 3 Results

### 3.1 3.1 Effects of Arsenic compounds on the growth of the CODB *Acinetobacter spp.* and *Nocardia spp.*

The effects of arsenate and arsenite on the growth of *Acinetobacter spp.* and *Nocardia spp.* strains were determined by the well diffusion method and by optical density measurements (Tables 1–3). Based on the values of diameter of inhibition zones, it was considered convenient to group the tested bacteria into three groups (Table 1). All tested bacteria showed zone of inhibition of $\leq 0.5$ cm when reacted with arsenate. On the other hand, 38.1%, 19% and 42% of tested Acinetobacter strains showed zones of inhibition of 0.5, $> 0.5 < 1$, and $\geq$ 1cm, respectively, also, 55.6% and 44.4% of tested *Nocardia spp.* showed zones of inhibition of 0.5 and $> 0.5 < 1$ when reacted with arsenite (Table 1). Moreover the MIC values of arsenic compounds determined from the optical density

measurements showed the varying reactions of tested bacteria against arsenic compounds (Tables 2). Higher MIC value was recorded for arsenate (30 mg $ml^{-1}$) compared to arsenite (1.5 mg $ml^{-1}$) among *Acinetobacter spp.* and *Nocardia spp.* strains, respectively.

Table 1: Effects of arsenate and arsenite on the growth of *Acinetobacter spp.* and *Nocardia spp.*

| **Bacteria** | **Number of tested bacteria** | **Inhibition zone (cm)** | | |
|---|---|---|---|---|
| | | ≤ 0.5 | > 0.5 < 1 | ≥ 1 |
| **Arsenate ($As^{+5}$)** | | | | |
| *Acinetobacter spp.* | 24 | 24 | Nil | Nil |
| *Nocardia ssp.* | 34 | 34 | Nil | Nil |
| **Arsenite ($As^{+3}$)** | | | | |
| *Acinetobacter spp.* | 42 | 16 | 8 | 18 |
| *Nocardia ssp.* | 27 | 15 | 12 | Nil |

Table 2: MIC values of arsenate and arsenite of *Acinetobacter spp.* and *Nocardia spp.*

| **Serial No.** | **Bacteria** | **MIC (mg $ml^{-1}$)** | |
|---|---|---|---|
| | | **Arsenate** | **Arsenite** |
| 1 | *Acinetobacter schindleri* | Nil* | 1.5 |
| 2 | *Acinetobacter schindleri* | 30 | 1.5 |
| 3 | *Acinetobacter schindleri* | 30 | 1.5 |
| 4 | *Acinetobacter schindleri* | Nil* | 1.5 |
| 5 | *Nocardia cummidelens* | 30 | 1.5 |
| 6 | *Nocardia fluminea* | Nil* | Nil* |
| 7 | *Nocardia ignorata* | Nil* | 1.5 |
| 8 | *Nocardia ignorata* | Nil* | 1.5 |
| 9 | *Nocardia sp.* | 30 | 1.5 |
| 10 | *Nocardia sp.* | 30 | Nil* |
| 11 | *Nocardia sp.* | 30 | 1.5 |
| 12 | *Nocardia cummidelens* | Nil* | 1.5 |
| 13 | *Nocardia cummidelens* | Nil* | Nil* |
| 14 | *Nocardia sp.* | Nil* | 0.15 |

*No MIC values were determined due to the ability of bacteria to grow at high concentrations of the arsenic compounds.

In addition, the abilities of these bacterial strains to grow at high concentrations of arsenic compounds were used to demonstrate the arsenic resistance phenotypes (Table 3). Results revealed that while 50% of *Acinetobacter spp.* strains showed resistance to arsenate, none of them were resistant to arsenite. In contrast, 60% and 30% of *Nocardia spp*. strains showed resistance to arsenate and arsenite respectively. Also, 20% of *Nocardia spp*. strains demonstrated resistance against both arsenate and arsenite (Table 3).

Table 3: Resistance phenotypes of *Acinetobacter spp.* and *Nocardia spp.* reacted with arsenic compounds.

| Serial No. | Bacteria | Resistant |
|---|---|---|
| 1 | *Acinetobacter schindleri* | $As^{+5}$ |
| 2 | *Acinetobacter schindleri* | Nil* |
| 3 | *Acinetobacter schindleri* | Nil* |
| 4 | *Acinetobacter schindleri* | $As^{+5}$ |
| 5 | *Nocardia cummidelens* | Nil* |
| 6 | *Nocardia fluminea* | $As^{+5}/As^{+3}$ |
| 7 | *Nocardia ignorata* | $As^{+5}$ |
| 8 | *Nocardia ignorata* | $As^{+5}$ |
| 9 | *Nocardia sp.* | Nil* |
| 10 | *Nocardia sp.* | $As^{+3}$ |
| 11 | *Nocardia sp.* | Nil* |
| 12 | *Nocardia cummidelens* | $As^{+5}$ |
| 13 | *Nocardia cummidelens* | $As^{+5}/As^{+3}$ |
| 14 | *Nocardia sp.* | $As^{+5}$ |

*No phenotypes were determined due to the un-ability of bacteria to grow at high concentrations of arsenic compounds.

### 3.2 Effects of arsenic compounds on the dehydrogenase activity of isolated strains of *Acinetobacter spp.* and *Nocardia spp.*

The affects of arsenate and arsenite on the dehydrogenase activity in the presence of crude oil were investigated. Both *Acinetobacter spp.* and *Nocardia spp.* strains showed significantly low dehydrogenase activity when supplied with crude oil compared to that of nutrient broth. Moreover the dehydrogenase activity of tested strains was more labile to arsenite compared to that in the presence of arsenate (Table 4).

### 3.3 Effects of arsenate and arsenite on crude oil mineralization by the *Acinetobacter spp.* and *Nocardia spp.*

Crude oil mineralization was determined by respirometry. Higher amounts of crude oil mineralization were determined for *Nocardia spp.* strains compared to that of *Acinetobacter spp*. strains (Table 5).

Table 4: Effects of arsenate and arsenite on the dehydrogenase activity of *Acinetobacter spp.* and *Nocardia ssp. bacteria* supplied with crude oil.

| **Bacteria** | **Nutrient Broth (Control)** | | | **Crude oil** | | |
|---|---|---|---|---|---|---|
| | | | | | | |
| **Arsenate (mg ml$^{-1}$)** | 5 | 10 | 20 | 5 | 10 | 20 |
| *Acinetobacter spp.* | 96-3.8 | 91-1.7 | 83.3-0.64 | 33.3-0 | 25-0 | 5.5-0 |
| *Nocardia spp.* | 82-7.5 | 78-3.3 | 62-2.1 | 96-60 | 91-40 | 79-5 |
| | | | | | | |
| **Arsenite (mg ml$^{-1}$)** | 0.6 | | 1.2 | 0.6 | | 1.2 |
| *Acinetobacter spp.* | 66.6-0.5 | | 50.8-0.2 | 25-0 | | 20-0 |
| *Nocardia spp.* | 62-31 | | 60-26 | 67-30 | | 51-25 |

Table 5: Effects of arsenate and arsenite on crude oil mineralization by *Acinetobacter spp.* and *Nocardia spp.* bacteria.

| Bacteria | **Arsenic compounds (mg ml$^{-1}$)** | | | | |
|---|---|---|---|---|---|
| | Arsenate ($As^{+5}$) | | | Arsenite ($As^{+3}$) | |
| | 5 | 10 | 20 | 0.6 | 1.2 |
| | **Amount of carbon dioxide evolved (%)** | | | | |
| *Acinetobacter spp.* | 60 | 55.6 | 5.8 | 68.3 | 6.6 |
| *Nocardia spp.* | 98 | 64.9 | 9.3 | 8.2 | 68.5 |

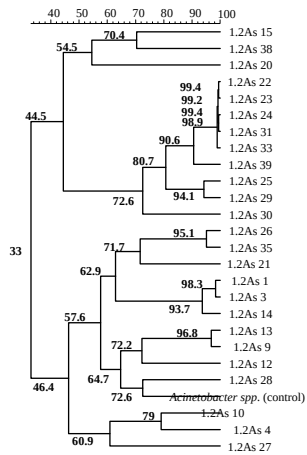


Figure 1: Dendrogram constructed by UPGMA cluster analysis of similarity coefficients derived from RFLP analysis of 16S rDNA of crude oil-degrading *Acinetobacter spp.*, isolated from soil amended with arsenite (1.2 mg$^{-1}$ ml) obtained with BstUI restriction enzyme. The levels of linkage representing the Dice correlation/UPGMA are expressed as percentages shown at each node.

### 3.4 Phylogenetic analysis of the CODB strains of *Acinetobacter spp.* and *Nocardia spp.* by RFLP

In order to evaluate the diversity of the *Acinetobacter spp.* and *Nocardia spp.* in the presence of arsenic compounds, the 16S rDNA sequences of different strains isolated from soil amended with 1.2 mg $ml^{-1}$ of arsenite were compared using the 16S-RFLP method. The assessment of the diversity of the *Nocardia* isolates by 16S-RFLP analysis demonstrated the presence of sixteen different phylogroups at similarity coefficient of 90% (Figure 2). Moreover, four different species belonging to the genus *Nocardia* were identified. On the other hand, 16S-RFLP analyses of *Acinetobacter* isolates showed the presence of fifteen different phylogroups at similarity coefficient of 90% (Figure 1). Also only two different species belonging to the genus *Acinetobacter* were identified.

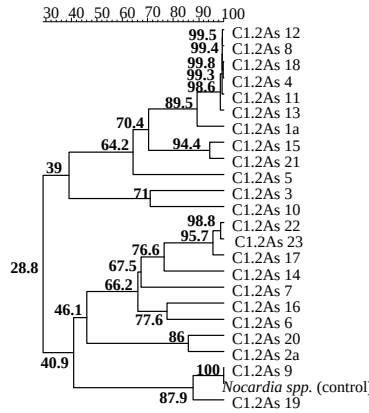


Figure 2: Dendrogram constructed by UPGMA cluster analysis of similarity coefficients derived from RFLP analysis of 16S rDNA of crude oil-degrading *Nocardia spp.*, isolated from soil amended with arsenite (1.2 $mg^{-1}$ ml) obtained with BstUI restriction enzyme. The levels of linkage representing the Dice correlation/UPGMA are expressed as percentages shown at each node.

## 4 Discussion

Differences in bacteria growth determined by well diffusion method were insignificant in the presence of arsenate which could be due to nature of the method or the potential of bacterial biofilms to resist heavy metals [21]. However, the addition of arsenite showed significant differences in bacterial growth (Table 1). This indicated the higher potentials of arsenite to inhibit bacterial growth which was further investigated by the optical-density measurement experiments that confirmed previous results (Tables 2 and 3). In

addition, higher MIC values were recorded for arsenate (30 mg $ml^{-1}$) compared to arsenite (1.5 mg $ml^{-1}$). Thus, based on well diffusion and optical density measurement experiments, it was concluded that arsenite possessed higher toxicity on bacterial growth compared to arsenate which was in agreement with previous studies [22, 23]. The effects of arsenic compounds on bacterial activity were further studied and results showed the higher inhibitory effects of arsenite on bacterial dehydrogenase activity compared to that of arsenate (Table 4). Also, the higher dehydrogenase activity determined in the presence of nutrient broth compared to that in the presence of crude oil indicated the bacterial preference to nutrient broth over that to crude oil. These results were in agreement with previous reports indicating that crude oil is typically non-preferred substrates for bacteria [24]. Furthermore, higher crude oil mineralization determined for the Gram-positive bacterium *Nocardia spp.* compared to that of the *Acinetobacter spp.* a Gram-negative bacterium. The potential of these bacteria to degrade hydrocarbons was documented previously [25–28] and have been isolated previously from Kuwait environment [13–16]. Thus, it was unsurprising to isolate them from crude oil-contaminated soils in Kuwait. Moreover, 16S-RFLP analyses showed the higher diversity of *Nocardia spp.* compared to *Acinetobacter spp.* strains which reflected the high adaptable nature of *Nocardia spp.* to the harsh environment of contaminated sites in Kuwait harboring high concentrations of hydrocarbons and heavy metals that reduced the diversity of less adaptable strains of *Acinetobacter spp.* These finding were in agreement with previous reports demonstrating the effects of hydrocarbons and heavy metals on bacterial diversity in soil [29, 30].

## 5 Conclusions

The bioremediation of crude oil in soil is a complex process that is affected by several factors explicitly the characteristics of soil indigenous microbiota and chemical composition of soil. Thus the assessment of the effects of arsenic compounds on crude oil bioremediation in soil for the predominant CODB in Kuwait *Acinetobacter spp.* a Gram-negative bacterium and *Nocardia spp.* a Gram-positive bacterium were determined. The potential inhibitory effects of arsenic compound on bacterial growth and activities showed higher toxicity of arsenite over that of arsenate. Moreover it was concluded that *Nocardia spp.* strains possessed higher adaptation potentials to the presence of arsenic compounds and crude oil compared to *Acinetobacter spp.* strains which most probably affected their diversity in soil. The current study was one of the first efforts to investigate the effects of arsenic compounds on soil microbiota in Kuwait, thus, more studies are required on more soil samples from other areas of Kuwait to investigate effects of arsenic compounds on crude oil bioremediation.

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