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ORIGINAL ARTICLE

Bacteria from spent engine-oil-contaminated soils possess dual tolerance to hydrocarbon and heavy metals, and degrade spent oil in the presence of copper, lead, zinc and combinations thereof

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Abstract We studied the behaviour of 12 metal-resistant bacteria isolated from spent engine oil (SEO)-contaminated soil in Ibadan, Nigeria, during their growth on SEO in mineral salts medium amended singly or in combination with varying concentrations of Cu, Zn and Pb. *Kocuria varians* PbB3, *Kocuria varians* MB2 and *Achromobacter xylosoxidans* CuE2 showed no appreciable growth on SEO even in the absence of metals. *Achromobacter xylosoxidans* MC1, *Achromobacter xylosoxidans* CuC3 and *Arthrobacter* sp. ZnC1 were inhibited by the tested metals in a dose-dependent manner. However, while *Acinetobacter* sp. ZnB2 and *Acinetobacter* sp. ZnE3 were similarly inhibited, both degraded appreciable quantities of SEO in the presence of toxic concentrations of Zn^{2+}. Cu^{2+} at 100 μg mL^{-1} has no significant effect on the growth of *Acinetobacter calcoaceticus* CuD2, which degraded 34.7 % and 33.0 % spent oil, respectively, at 0 and 100 μg mL^{-1} Cu^{2+}. SEO degradation by *Pseudomonas* sp. PbC4 and *Burkholderia cepacia* MD1 reduced by 34.8 % and 19.8 %, respectively, at the lowest concentration of Pb^{2+} (200 μg mL^{-1}) and metal combination (25 μg mL^{-1}) tested. While the activity of strain PbC4 was inhibited at 600 μg mL^{-1} Pb^{2+}, there was no significant difference ($P<0.05$) in the quantity of SEO degraded by isolate MD1 at all concentrations of the combined metals tested. Our results revealed these bacterial strains to be potential candidates for bioremediation of sites co-contaminated with metals and hydrocarbons and, more importantly, provided evidence that some patterns of metal inhibition previously attributed to community effects may also be linked to the physiology of individual degrading bacterial strains.

Keywords Spent engine oil · Hydrocarbon-degrading bacteria · Heavy metals · Pollution

Introduction

The extensive use of petroleum hydrocarbons has resulted in the Nigerian environment being heavily contaminated with petroleum hydrocarbon pollutants that enter the environment through various routes (Adelowo et al. 2006). One important route presently receiving very little attention is the discharge of spent engine oil (SEO) from automobile repair workshops (ARWs), which are a common feature of the urban landscape in Nigeria. The operations of ARWs in Nigeria are not well regulated and a large number are sited within residential areas. The ceaseless discharge of heavy-metal-laden SEO from these facilities is a frequent source of metal- and hydrocarbon-pollution in surrounding environments. A study by Bamiro and Osibanjo (2004) reported that 14,344 L used oil was generated per week from 22 selected sources within the transport and industry sectors in southwestern Nigeria. The spent oil generated within these facilities is released carelessly into the environment, where it causes serious damage and health concerns (Andrysik et al. 2006). SEO in particular contains aliphatic hydrocarbons, and polycyclic aromatic hydrocarbons (PAHs) distilled from crude oil, solvents and detergents added during the blending process, and metals from engine wear that are either toxic themselves or can combine with products of combustion to generate carcinogens and endocrine disrupters (USEPA 1996; ATSDR 1997). Thus, it is

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often necessary to apply remedial measures for the restoration of SEO-contaminated ecosystems.

As a result of the presence of metals and heavy PAHs in spent oil (Boonchan et al. 2000; Abioye et al. 2010), ecosystems contaminated with spent oil are usually also contaminated with toxic heavy metals, which may be inhibitory to the growth of potential remediating bacterial species. Exposure to heavy metals has been reported to affect the growth and survival of microorganisms (Bååth 1989; Franco et al. 2009) and, by extension, biodegradation of organic pollutants in the environment (Sokhn et al. 2001; Al-Saleh and Obuekwe 2005; Pepi et al. 2008). Therefore, effective remediation of such co-contaminated ecosystems will require that the remediating organisms be able to grow and metabolise the contaminating hydrocarbon in the presence of inhibitory heavy metals. A combination of genetic systems for degradation of organic pollutants and heavy metal resistance is one of the approaches suggested to create polyfunctional strains for bioremediation of co-contaminated ecosystems (Ali et al. 2011). However, tight regulatory control and the difficulty of introducing genetically modified organisms into natural ecosystems is a compelling reason why special attention must be paid to indigenous strains with multiple tolerance mechanisms for the bioremediation of co-contaminated natural ecosystems.

Very often, polluted sites in many urban areas are frequently co-contaminated by organic pollutants and toxic heavy metals (Sandrin and Maier 2003) such that the allochthonous microorganisms are exposed simultaneously to a variety of pollutants through multiple exposure routes (Feron and Groten 2002). Such organisms, confronted with the toxicity of different contaminants, will most likely develop multiple tolerance mechanisms (Wetzel and van Vleet 2003) necessary for survival in the presence of the pollutant mix. Microorganisms capable of interacting with more than one type of pollutant through the evolution of multiple tolerance mechanisms are of particular interest in bioremediation of sites contaminated with mixtures of pollutants. However, bioremediation studies have been carried out mostly using single pollutants. Although studies based on single pollutants enable us to gain insight into the behaviour of individual pollutants under carefully controlled conditions, they do not reflect real-world exposure scenarios (Shen et al. 2006). Thus, there is a need for more extensive studies on the nature of combined pollution in natural ecosystems (Shen et al. 2006). Particularly important is the effect of combined selection pressure exerted by multiple pollutants on the allochthonous microflora of natural ecosystems exposed to multiple contaminants. These effects may vary and will depend on the constituents of the pollutant mixture (Jensen and Sverdrup 2002) and the tolerance of the allochthones to the different components of the pollutant.

Metal-tolerance is commonly reported among microorganisms inhabiting metal-polluted natural ecosystems (Malik and Jaiswal 2000; Matyar et al. 2010; Sevgi et al. 2010). Similarly, there have been reports on the biodegradation of spent oil by bacteria isolated from the Nigerian environment (Adelowo et al. 2006; Abioye et al. 2009; 2010), but studies evaluating the growth of the reported organisms on spent oil in the presence of inhibitory heavy metals are still very few. The few studies that have investigated biodegradation of hydrocarbons in the presence of inhibitory concentration of metals typically used non-viscous hydrocarbon substrates such as crude oil, and commonly one metal ion (Benka-Coker and Ekundayo 1998; Ali et al. 2011; Oyetibo et al. 2013). Evaluations using single metal ions may be far from the ideal situation in natural ecosystems where there is often a mix of metals at concentrations that vary from one site to the other. A possible limitation of these studies is that the reported organisms may not be of wide application in bioremediation of co-contaminated natural ecosystems. Thus, indigenous microorganisms able to degrade hydrocarbons in the presence of a mixture of metals may be more desirable.

We assumed that ecosystems contaminated by SEO, such as the ARWs selected for this study, are ideal systems with which to study the proliferation of bacterial species capable of tolerating the combined toxicity of metals and SEO. Thus, the main objective of this study was to validate this assumption and to study the effect of three metals and their mixture on the degradation of SEO by these bacterial species in mineral salts medium containing SEO as carbon and energy source. Bacterial degradation of SEO in the presence of toxic heavy metal ions is an area that is yet to be well explored. Our study, therefore, investigated the degradation of SEO by metal-tolerant bacteria strains isolated from SEO-polluted soils in Ibadan, southwestern Nigeria using model systems containing SEO and toxic concentrations of Cu^{2+}, Pb^{2+} and Zn^{2+} and their combination. The three metals are frequently reported in association with SEO (Randles et al. 2007). This model system will present at least two limitations to the growth of the test bacteria species: the viscosity of the hydrocarbon substrate, which has been reported previously as a limiting factor in the biodegradation of lubricating oils (Amund and Adebiyi 1991), and the presence of toxic heavy metal. This will be an important step in the development of processes for the enhanced remediation of sites co-contaminated by SEO and metals.

Materials and methods

Sample collection and heavy metal analysis

Subsurface soil samples were collected in sterile polythene bags from five different ARWs located in the Bodija, Orogun (n=2) and Ojoo areas in Ibadan, the Capital of Oyo State, Southwestern Nigeria. The samples were collected from three spots selected randomly in each workshop and pooled to form composite samples. Control soil samples were similarly collected from the Teaching and Research Farm and the relatively protected Botanical Garden, which are two areas selected to reflect two extremes of anthropogenic impact within the campus of the University of Ibadan. Spent motor oil was collected from one of the workshops in sterile 4 L plastic gallon containers. The soil samples (including the controls) and spent oil were digested as described by US EPA method 3050A (USEPA 1990) and the digests analysed by Atomic Absorption Spectrophotometry (UNICAM SOLAAR 32) to determine the total concentration of Cu, Pb and Zn.

Determination of total heterotrophic bacteria count and isolation of metal-resistant bacteria strains

The population of heterotrophic bacteria of the soil samples were determined on Mueller Hinton agar plates as an index of the effect of spent oil contamination on the microbiota of the polluted samples. Similarly, Mueller Hinton agar (MHA) plates supplemented separately with 50 μg mL^{-1} of filter-sterilized Cu^{2+}, Pb^{2+}, Zn^{2+} and a mixture of the three metals (15 μg mL^{-1} each) were used to isolate metal-resistant bacteria from the soil samples. Nystatin (50 μg mL^{-1}) was added to the isolation medium to prevent the growth of fungi. The metal ions were incorporated into the medium as soluble salts viz zinc sulphate ($ZnSO_4$), copper sulphate ($CuSO_4 \cdot 5H_2O$) and lead acetate [$Pb(CH_3COO)_2$]. The plates were incubated at 30 °C for 72 h and distinct colonies growing on each metal supplemented plate were selected and purified on fresh MHA plates before storage in 20 % glycerol at −20 °C. The identities of the isolates were determined by morphological and biochemical tests (Sneath 1996).

Metal and hydrocarbon tolerance assay

The tolerance of the isolated bacteria to Cu^{2+}, Pb^{2+}, Zn^{2+} and their combinations was determined as the minimum inhibitory concentration (MIC) on MHA plates as described by Aleem and co-workers (2003). The MHA plates were supplemented with graded concentrations of the metals; 100 μg mL^{-1} was used as the starting concentration for Cu^{2+}, Pb^{2+}, Zn^{2+} while the starting concentration for the metal mix was 20 μg mL^{-1}. Similarly, isolates were screened for spent oil degradation by spot inoculation on Bushnell and Haas (BH) agar (Bushnell and Haas 1941) plates supplemented with the test metals and 5 % tyndallized SEO, respectively. The plates were incubated at 30 °C and observed for growth for 7 days.

Spent oil degradation

Duplicate flasks containing liquid BH medium (pH 7.0±0.2) supplemented with 5 % tyndallized SEO as sole source of carbon and energy were used to investigate the effect of the individual metal ions and their combination on the utilization of SEO by selected metal resistant bacteria species. The effect of Cu^{2+} on SEO utilization was assayed at 100 μg mL^{-1}, 150 μg mL^{-1} and 200 μg mL^{-1}, while Pb^{2+}, Zn^{2+} and the metal combinations were assayed at 200 μg mL^{-1}, 400 μg mL^{-1} and 600 μg mL^{-1}; 100 μg mL^{-1}, 200 μg mL^{-1} and 300 μg mL^{-1}; and 25 μg mL^{-1}, 50 μg mL^{-1} and 75 μg mL^{-1}, respectively. The metals were filter-sterilized (0.22 μL pore size, Millipore Nucleopore, Pleasanton, CA) before incorporation into the growth medium. One millilitre of a standardised suspension (OD_{600}=1) of each tested isolate in saline was used as inoculum in each flask to bring the final volume of the test medium to 50 mL. The flasks were incubated at 30 °C and the growth of the isolates monitored every 24 h by UV visible spectrophotometry at 600 nm (OD_{600}) for 7 days. Liquid BH medium without oil was used to blank the spectrophotometer. Controls were set up similarly but without added metals. At the end of the experimental period of 7 days, residual oils in control and experimental flasks were extracted with chloroform (1:1 v/v). After solvent evaporation, the quantities of oil utilised by the metal-resistant bacterial strains were determined by gravimetry with reference to uninoculated controls (Chaillan et al. 2004). Growth and SEO degradation by the isolates at the different metal concentrations were compared by one-way analysis of variance (ANOVA) at $P<0.005$.

Results

Heavy metal analysis and total heterotrophic bacteria count

As expected, the concentration of Cu, Pb and Zn in the soil samples from the five ARWs was higher than that of the garden soil samples used as control (Table 1). The concentration of the

Table 1 Concentration of Cu, Pb and Zn and total heterotrophic bacteria count (THBC) of the soil samples. The metal concentration and bacteria count are per fresh weight of soil. *SEO* Spent engine oil

Sample	Metal concentration (mg kg^{-1})			THBC [a] (log CFU g^{-1})
	Cu	Pb	Zn	
Orogun I	290.6	93.1	799.5	4.48 (1.00)
Bodija	100.9	83.0	550.0	4.67 (0 .48)
Orogun II	80.0	67.6	426.7	5.02 (0.55)
Ojoo	76.2	70.6	741.6	4.98 (1.00)
Agbowo	71.7	62.2	901.1	4.92 (0.50)
Research farm	19.9	11.6	85.0	5.17 (0 .89)
Botanical garden	10.2	0.00	50.8	5.09 (0.50)
SEO[b]	6.2	843.0	147.0	ND[c]

[a] Each count is a mean of two replicates with the standard error in bracket

[b] Spent oil is not a soil sample

[c] Not determined

three metals is in the order Zn>Cu>Pb. However, in terms of the sample sites, the order of metal contamination is Orogun I> Bodija>Orogun II>Ojoo>Agbowo>Teaching and research farm>Botanical garden. These results suggested that operations of the ARWs contributed to the contamination of the surrounding soil ecosystem with toxic heavy metals. Further evidence pointing in this direction is the equally high concentration of these metals in the spent oil collected from one of the workshops (Table 1). Similarly, the total heterotrophic bacteria count (THBC) of the control soil samples was higher than that of the ARWs (Table 1). Exposure to the combined toxicity of SEO and heavy metals is likely to have contributed to the reduction in the abundance of heterotrophs in the contaminated soils.

Metal-resistant bacteria isolates

A total of 68 bacterial strains were isolated on metal-supplemented MHA plates. The number was reduced to 28 bacterial isolates able to tolerate the lowest concentration (100 μg mL^{-1}) of Cu^{2+} (n=6), Pb^{2+} (n=10), Zn^{2+} (n=7), and the metal mixture (20 μg mL^{-1} each) (n=5) used for the metal tolerance assay. A total of 28 isolates was selected for hydrocarbon degradation assay on BH agar supplemented with 5 % SEO. Among these 28 isolates, 12 (3 for each metal and 3 for metals combination) showing the highest level of tolerance to the metals and their combination, and capable of growth in the presence of 5 % SEO within 72 h were selected to investigate the effect of metals on degradation of SEO. The metal and SEO tolerance profiles of the selected bacteria are shown in Table 2. The three bacteria selected for Cu^{2+} tolerance were identified as *Acinetobacter calcoaceticus* CuD2 (MIC_{Cu} 400 μg mL^{-1}), *Achromobacter xylosoxidans* CuC3 (MIC_{Cu} 250 μg mL^{-1}) and *Achromobacter xylosoxidans* CuE2 (MIC_{Cu} 250 μg mL^{-1}). Isolates selected for Pb^{2+} tolerance were identified as *Kocuria varians* PbB3 (MIC_{Pb} 1,100 μg mL^{-1}), *Acinetobacter calcoaceticus* PbD1 (MIC_{Pb} 1,100 μg mL^{-1}) and *Pseudomonas* sp. PbC4 (MIC_{Pb} 900 μg mL^{-1}); while those selected for Zn^{2+} tolerance were identified as *Acinetobacter* sp. ZnE3 (MIC_{Zn} 400 μg mL^{-1}), *Acinetobacter* sp. ZnB2 (MIC_{Zn} 350 μg mL^{-1}), and *Arthrobacter* sp. ZnC1 (MIC_{Zn} 350 μg mL^{-1}). *Burkholderia cepacia* MD1 ($MIC_{Cu,Pb,Zn}$ 200 μg mL^{-1}), *K. varians* MB2 ($MIC_{Cu,Pb,Zn}$ 200 μg mL^{-1}) and *Achromobacter xylosoxidans* MC1 ($MIC_{Cu,Pb,Zn}$ 200 μg mL^{-1}) were selected for their tolerance to the three metals combination at the stated MICs.

Table 2 Heavy metal and SEO tolerance profile of the bacterial isolates. *MIC* Minimal inhibitory concentration

Isolate[a]	Metal MIC (μg mL^{-1})[b]	Time of growth in 5 % SEO (h)
Acinetobacter calcoaceticus CuD2	400	48
Achromobacter xylosoxidans CuC3	250	72
Achromobacter xylosooxidans CUE2	250	72
Acinetobacter sp. ZnE3	400	24
Acinetobacter sp. ZnB2	350	48
Arthrobacter sp. ZnC1	350	72
Acinetobacter calcoaceticus PbD1	1,100	48
Kocuria varians PbB3	1,100	72
Pseudomonas sp. PbC1	900	48
Burkholderia cepacia MD1	200	72
Kocuria varians MB2	200	48
Achromobacter xylosooxidans MC1	100	72

[a] Cu, Pb, Zn and M in the strain number denotes isolates showing resistance to Cu, Zn, Pb and the three metals combined

[b] Values are MIC for the corresponding metals to which isolates showed resistance

SEO utilization potentials in the presence of metals

The effects of metals on the growth and degradation of SEO by the selected isolates varied. OD_{600} measurements and residual oil analysis by gravimetry showed that increasing concentration of Cu^{2+} inhibited the growth and degradation of SEO by *Achromobacter xylosoxidans* CuC3 and CuE2, while the pattern for *Acinetobacter calcoaceticus* CuD2 was slightly different (Fig. 1a,b). Cu^{2+} at 100 μg mL^{-1} appears to have little or no effect on growth and SEO degradation by *Acinetobacter calcoaceticus* CuD2 (Fig. 1c). For this isolate, there was no significant difference (P<0.05) in growth and SEO degradation between 0 and 100 μg mL^{-1} and between 150 and 200 μg mL^{-1}. In the presence of 0 μg mL^{-1}, 100 μg mL^{-1}, 150 μg mL^{-1} and 200 μg mL^{-1} of Cu^{2+}, isolate CuC3 utilized 40.3 %, 12.6 %, 5.9 % and 3.8 % of the SEO supplied in the growth medium at degradation rates of 0.14 mL, 0.045 mL, 0.045 mL and 0.0125 mL, respectively, per day. Isolate CuE2 degraded 12.4 %, 7.3 %, 6.2 %, and 5.5 % SEO corresponding to degradation rates of 0.045 mL, 0.025 mL, 0.023 mL and 0.02 mL day^{-1}, respectively. The respective percentage degradation and degradation rates per day in the presence of the same concentration of Cu^{2+} for strain CuD2 are 34.7 %, 33.0 %, 15.7 %, 15.3 % and degradation rates of 0.125 mL, 0.118 mL, 0.055 mL and 0.055 mL day^{-1} (Fig. 1d).

Kocuria varians PbB3 showed no significant growth on SEO even in the absence of Pb^{2+} despite its high MIC_{Pb} (1,100 μg mL^{-1}) and growth on BH agar supplemented with 5 % SEO within 72 h during the screening for metal and hydrocarbon tolerance (Fig. 2a). The growth and SEO utilization of *Acinetobacter calcoaceticus* PbD1 increased gradually with increasing Pb^{2+} concentration up to 600 μg mL^{-1} when there was a marked inhibition of growth and SEO utilization (Fig. 2b). However, for *Pseudomonas* sp. PbC4, there was significant difference (P<0.05) in growth and SEO degradation at 0 and 200 μg/mL, which is most inhibitory to

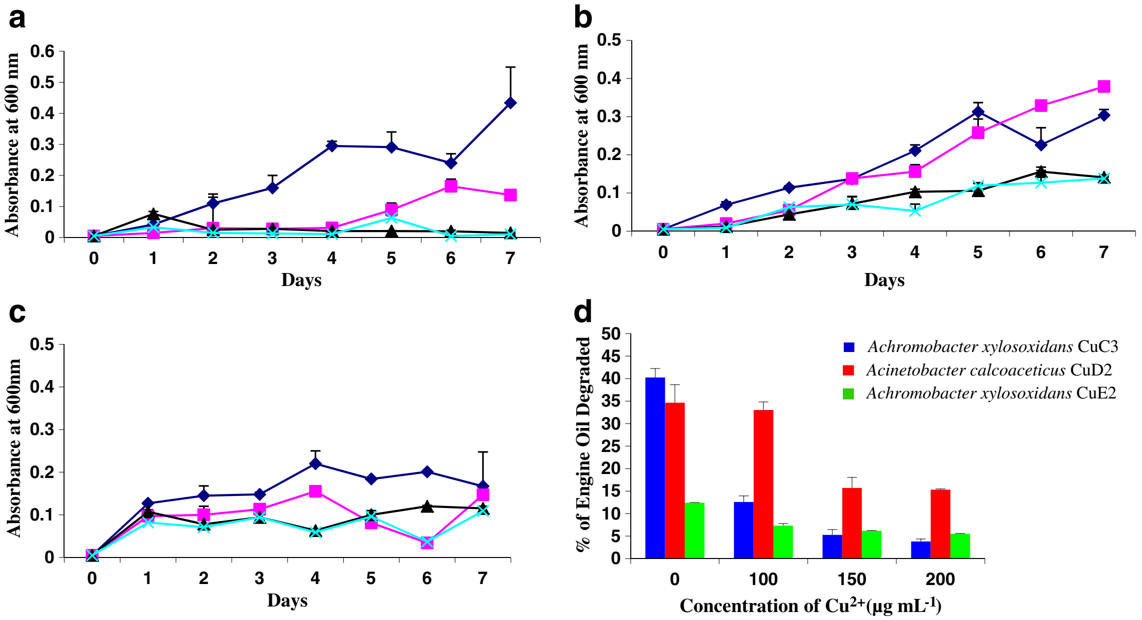

Fig. 1 Growth pattern of **a** *Achromobacter xylosoxidans* CuC3, **b** *Acinetobacter calcoaceticus* CuD2, **c** *Achromobacter xylosoxidans* CuE2 on SEO in the presence of different concentrations of Cu^{2+} (◆ No metal, ■ 100 μg mL^{-1}, ▲ 150 μg mL^{-1}, ✳ 200 μg mL^{-1}) and **d** quantity of SEO degraded over a period of 7 days

the growth of the isolate (Fig. 2c). However, the isolate showed a recovery of its ability to utilize SEO at higher concentrations of the metal ion. Isolate PbC4 degraded 45.4 %, 29.6 %, 39.9 % and 32.1 % of the SEO supplied in the growth medium at 0, 200, 400 and 600 μg mL^{-1} of Pb^{2+} corresponding to degradation rates of 0.165 mL 0.105 mL, 0.1425 mL and 0.115 mL day^{-1} respectively, as against 18.2 %, 20.0 % 23.7 % and 10 % and degradation rates of 0.065 mL, 0.0725 mL, 0.085 mL and 0.035 mL day^{-1} by *Acinetobacter calcoaceticus* PbD1 (Fig. 2d).

Zinc ion was inhibitory to the growth of *Arthrobacter* sp. ZnC1 and *Acinetobacter* sp. ZnB2. Growth and SEO

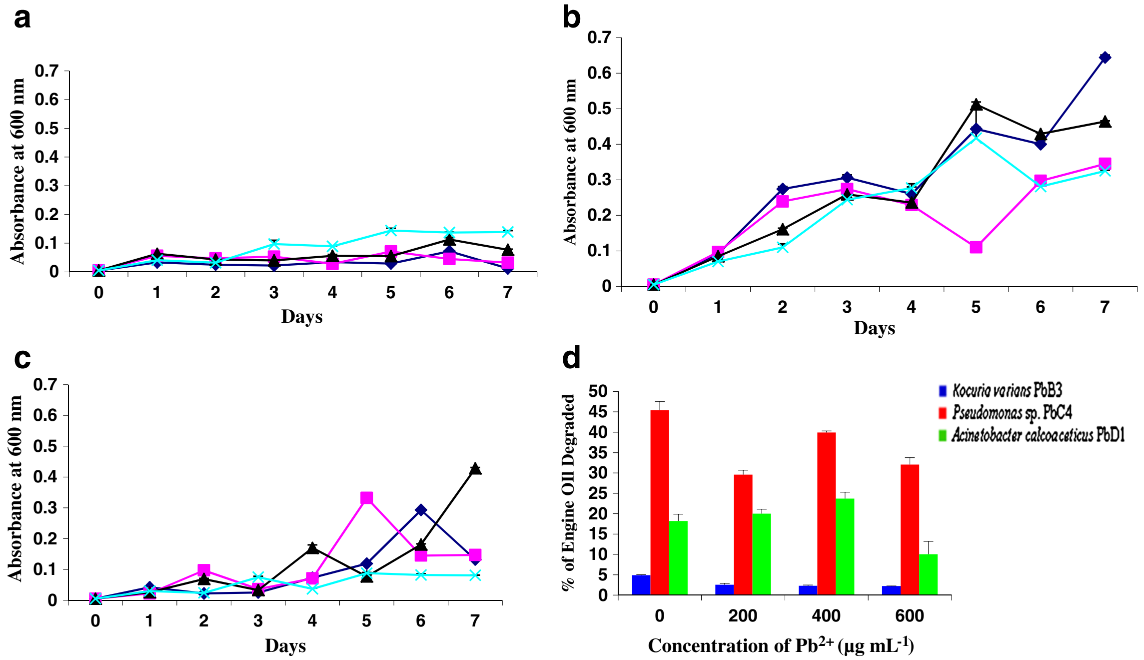

Fig. 2 Growth pattern of **a** *Kocuria varians* PbB3, **b** *Pseudomonas* sp. PbC4, **c** *Acinetobacter calcoaceticus* PbD1 on SEO in the presence of different concentrations of Pb^{2+} (◆ No metal, ■ 200 μg mL^{-1}, ▲ 400 μg mL^{-1}, ✳ 600 μg mL^{-1}), and **d** quantity of SEO degraded over a period of 7 days

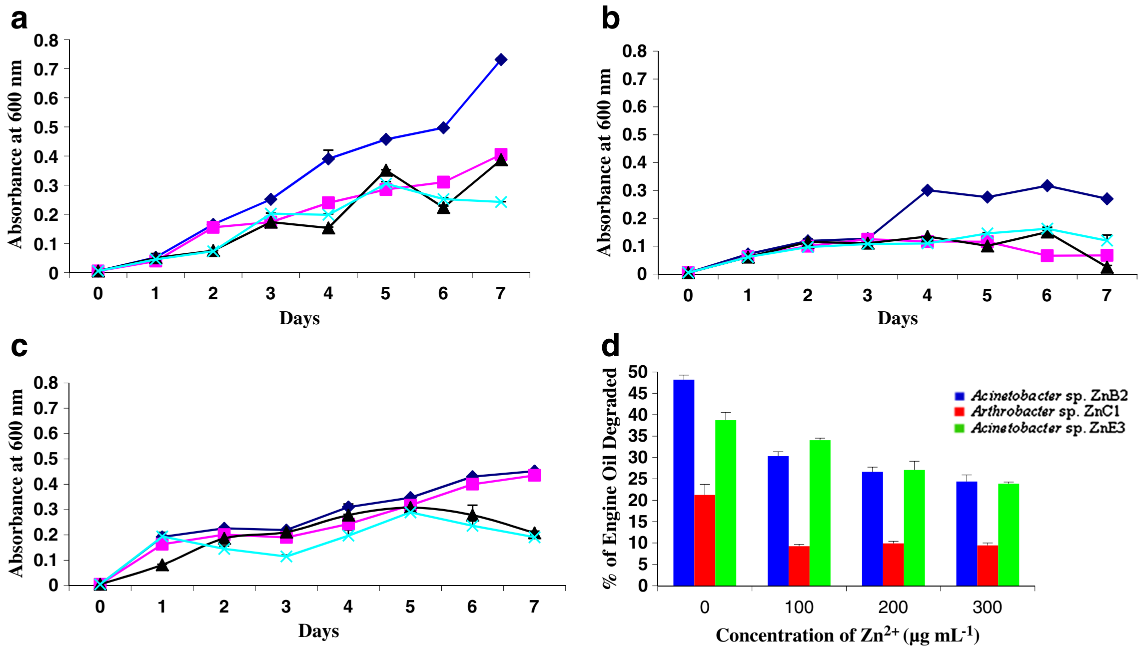

Fig. 3 Growth pattern of **a** *Acinetobacter* sp. ZnB2, **b** *Arthrobacter* sp. ZnC1, **c** *Acinetobacter* sp. ZnE3 on SEO in the presence of different concentrations of Zn^{2+} (◆ No metal, ■ 100 µg mL^{-1}, ▲ 600 µg mL^{-1}, ✳ 300 µg mL^{-1}) and, **d** quantity of SEO degraded over a period of 7 days

degradation was reduced significantly by the introduction of Zn^{2+} into the growth medium. The difference in the growth and degradation of SEO by these isolates was significant in the presence and absence of Zn^{2+} ($P<0.05$) (Fig. 3a). However, despite the inhibitory effect of the metal, strain ZnB2 still utilized an appreciable quantity of the SEO supplied in the growth medium. Strains ZnC1 and ZnB2 degraded 21.3 %, 9.3 %, 9.9 %, 9.5 %; and 48.2 %, 30.3 %, 26.7 %, and 24.4 % of the SEO corresponding to degradation rates of 0.075 mL, 0.0325 mL, 0.035 mL, 0.035 mL; and 0.1525 mL, 0.1075 mL, 0.095 mL, and 0.0875 mL, respectively per day in the presence of 0, 100, 200 and 300 µg mL^{-1} Zn^{2+}. There was only a slight difference in the growth and degradation of SEO by *Acinetobacter* sp. ZnE3 at 0 and 100 µg mL^{-1} and at 200 and 300 µg mL^{-1} (Fig. 3c). However, these differences were not statistically significant at $P<0.05$. ZnE3 degraded 37.7 %, 34.0 %, 27.0 % and 23.9 % SEO (Fig. 3d) with degradation rates of 0.135 mL, 0.1225 mL, 0.0975 mL and 0.085 mL day^{-1} at the stated test concentration.

The mixture of Cu^{2+}, Pb^{2+} and Zn^{2+} at all tested concentration (25, 50, 75 µg mL^{-1}) was toxic for *K. varians* MB2 and *Achromobacter xylosoxidans* MC1 (Fig. 4a, b). While *K. varians* MB2 showed little or no growth even in the absence of the metals, showing that it is not a strong primary utilizer of SEO, *Achromobacter xylosoxidans* MC1 showed appreciable growth and degradation of SEO (30.2 %) in the absence of the metals, showing that the presence of the metals inhibited its growth and degradation activities in a dose-dependent manner. There was a slight reduction in growth and SEO utilization by *B. cepacia* MD1 at 25 µg mL^{-1} of the metal mix (Fig. 4c); the difference was, however, significant at $P<0.05$ when compared to growth and SEO degradation in the absence of the metals. Thereafter, increasing concentration of the metal mix did not have significant effect ($P<0.05$) on the growth and degradation of SEO by this isolate, which degraded 36.3 %, 29.1 %, 30.6 % and 31.7 % of the SEO supplied in the medium as sole carbon and energy source. These corresponded to degradation rates of 0.13 mL, 0.105 mL, 0.11 mL, and 0.1125 mL day^{-1}, respectively in the presence of 0, 25, 50 and 75 µg mL^{-1} of the metals combination (Fig. 4d).

Discussion

Indiscriminate discharge of heavy metal-laden SEO into the environment from ARWs is an important source of metal and hydrocarbon pollution in Nigeria that poses a significant threat to soil ecosystems and public health. The soil surfaces in all the workshops were covered with spent oil slicks and sparsely populated with plant species. The few plants growing in the ARWs of the present study are stunted and their leaves are covered with black oil slicks. This physical evidence of contamination is supported by the higher concentration of Cu, Pb and Zn recorded in soil samples and spent oil collected from the ARWs in comparison to the control soil samples. Previous studies in Nigeria and elsewhere have reported elevated concentrations of metals in hydrocarbon-impacted natural ecosystems (Benka-Coker and Ekundayo 1995; Adeniyi and Afolabi 2002; Muniz et al. 2004; Adesodun and Mbagwu 2008;

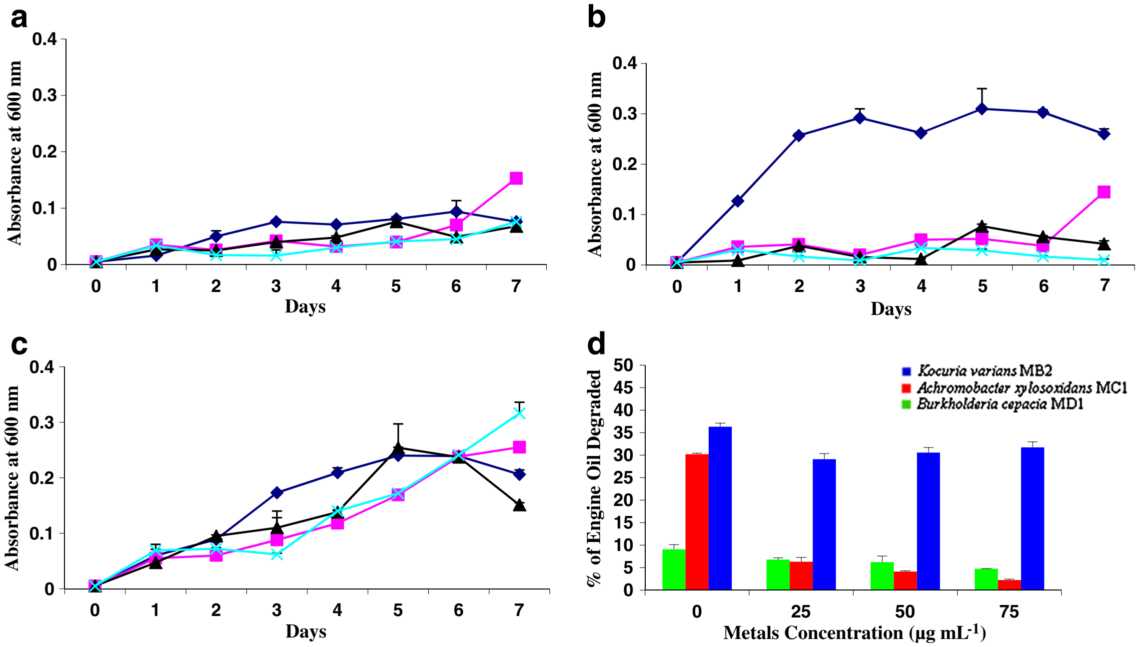

Fig. 4 Growth pattern of **a** *K. varians* MB2, **b** *Achromobacter* xylosoxidans MC1, **c** *Burkholderia cepacia* MD1 on SEO in the presence of different concentrations of the mixture of Cu $^{2+}$, Pb^{2+}, and Zn^{2+} (—◆— No metal, —■— 250 µg mL^{-1}, —▲—, 50 µg mL^{-1} —✳— 75 µg mL^{-1}), and **d** quantity of SEO degraded over a period of 7 days

Adeniyi and Owoade 2010; Abdullah et al 2011). As expected, the microbial population of the polluted soils is generally lower than that of the control soil samples. This may be due to a shift in the ecological balance of the biota in favour of metal-tolerant strains (Jiang et al. 2008); or may result from the sparse population of plants in the polluted soils, which deprives the soil of the organic matter inputs necessary to support the growth of the microbial population (Kuperman and Carreiro 1997).

It is expected that microbial processes will play an important role in the restoration of these and other similar polluted ecosystems. Bacterial degradation of SEO has been reported previously in the scientific literature (Mandri and Lin 2007; Abioye et al. 2009, 2010; Thenmozhi et al. 2011). However, studies on the degradation of SEO by bacteria in the presence of toxic concentrations of heavy metals are not very common, despite the commonly reported association between heavy metals and SEO (Boonchan et al. 2000). Particularly scarce are studies investigating growth of hydrocarbon-degrading bacterial species in the presence of a mixture of metals. In this study, we studied the degradation of SEO by 12 bacterial strains in the presence of Cu^{2+}, Pb^{2+}, Zn^{2+} and a mixture of the three metals. The 12 bacterial strains were selected after tests to establish their dual adaptive tolerance to metals and SEO. While 4 of the 23 metal-tolerant bacteria are from Research Farm soil samples, none of the isolates from the relatively protected Botanical Garden were able to grow beyond the initial concentration of metals used for isolation. However, despite their high metal MICs (MIC$_{Cu}$ 150 µg mL^{-1}, MIC$_{Pb}$ 1,100 µg mL^{-1} and MIC$_{Cu,Pb,Zn}$ 25 µg mL^{-1}), the four metal-tolerant strains from the Research Farm were unable to grow on 5 % SEO after 72 h indicating that, while they showed tolerance to toxic concentrations of metals, they are not primary utilizers of hydrocarbon as substrates for growth. In contrast, all the strains capable of utilizing SEO in the presence of the toxic metal ions did so without any lag phase, indicating that they are strong primary utilizers of hydrocarbon.

All the patterns of inhibition reported previously in the literature (Sandrin and Maier 2003) were observed among isolates in this study with some interesting variations. Three of the isolates, *K. varians* PbB3, *K. varians* MB2 and *Achromobacter xylosoxidans* CuE2 showed no appreciable growth on SEO even in the absence of metals. It is possible that while these organisms are tolerant to metals, colony growth on the agar media used in screening for hydrocarbon utilization protected them against SEO toxicity, leading to a false positive result (Sandrin and Maier 2003). The degradation of SEO by *Achromobacter xylosoxidans* MC1, *Achromobacter xylosoxidans* CuC3 and *Arthrobacter* sp. ZnC1 was inhibited by the tested metals in a dose-dependent manner. The quantity of SEO degraded reduced progressively with increasing metal concentration, showing that while these isolates are tolerant to metals and SEO separately, they are unable to tolerate the combined toxicity of the metal + hydrocarbon mixture.

This is similar to the pattern of inhibition previously reported during the degradation of toluene (Amor et al. 2001), methyl tert-butyl ether (Lin et al. 2007) and hydrocarbons (AL-Saleh and Obuekwe 2005). A similar pattern of inhibition was observed for *Acinetobacter* sp. ZnB2 and *Acinetobacter* sp. ZnE3. However, compared to the metal-free controls, there is appreciable degradation of SEO by these two isolates in the presence of toxic concentrations of Zn^{2+}, suggesting a decrease in activity rather than complete inhibition of SEO metabolism. Sokhn and co-workers (2001) similarly reported continued degradation of phenanthrene by indigenous soil microorganisms in the presence of toxic concentrations of Cu, but with a reduction in CO_2 emissions, which, according to the authors, indicated a reduction in microbial activity. However, our study used single isolates in pure cultures; hence while the continued degradation of phenanthrene in the presence of toxic concentrations of Cu observed by Sokhn and co-workers was attributed to the effect of the metal on community dynamics, our own observation is likely linked to metal-induced physiological adaptation of the bacterial strains under consideration.

Higher concentrations of Pb^{2+} are less inhibitory to growth and degradation of SEO by *Acinetobacter calcoaceticus* PbD1. SEO degradation increased with increasing metal concentration until 600 $\mu g\ mL^{-1}$ when there was a marked inhibition of growth and degradation. This metal inhibition pattern has been observed previously during methanogenesis (Capone et al. 1993), and the degradation of phenol, 2-chlorophenol, 3-chlorophenol and benzoate (Kuo and Genthner 1996). However, it is important to note that these studies used a consortia, and not pure cultures as used in this study. Thus, the observed patterns of inhibition were attributed to the effects of the metals on populations rather than a single organism carrying out the degradation. However, our results suggest that, in addition to the population effect, this pattern of inhibition may also be linked to the physiology of a single degrading organism. This observation warrants further investigation.

The lowest concentration of Pb^{2+} (200 $\mu g\ mL^{-1}$) and the metal combinations (25 $\mu g\ mL^{-1}$) showed a stronger inhibitory effect on the degradation of SEO by *Pseudomonas* sp. PbC4 and *B. cepacia* MD1. Thereafter, SEO degradation by isolate PbC4 increased at 400 $\mu g\ mL^{-1}$ before reducing at 600 $\mu g\ mL^{-1}$. Interestingly, there was no significant difference ($P<0.05$) in the quantity of SEO degraded by isolate MD1 at all concentrations of the combined metal tested. Similar to the behaviour of *B. cepacia* MD1, Benka-Coker and Ekundayo (1998) reported that addition of 0.5 $mg L^{-1}$ Cu, Pb and Mn to the growth medium reduced the toxicity of Zn (0.5 $mg\ L^{-1}$) to crude oil degrading *Pseudomonas* sp. and *Micrococcus* sp. However, isolate MD1 was able to degrade SEO at a concentration of the metal mixture higher than that reported by the latter authors, showing that it is more tolerant to the toxicity of the metals and thus may be a potential candidate for the decontamination of sites polluted with hydrocarbons and a mixture of toxic metals. It is important to report that this strain was isolated from Orogun I—the study site with the highest level of metal contamination.

Conclusion

In this study, we established that dual adaptive tolerance to hydrocarbon and metals are common among bacteria isolated from soil ecosystems that are heavily contaminated with metal-laden SEO. Such dual tolerance was not found in bacteria from ecosystems not exposed to SEO contamination included in the study as controls. In addition, we found evidence that some metal-inhibition patterns previously attributed to the effect of metals on community dynamics may also be linked to the physiology of single isolates degrading hydrocarbons in pure cultures. The isolated strains utilized SEO as sole source of carbon and energy and more importantly, some of the tested bacteria consumed appreciable quantities of the SEO (>30 %) within 1 week in the presence of Cu^{2+}, Pb^{2+}, Zn^{2+} and their mixtures. This is a satisfactory rate, considering the viscosity of the SEO and the high concentration of metal ions used in this study. Particularly outstanding was the continued degradation of SEO by isolate MD1 at all concentrations of the mixtures of the three metal ions tested. These findings suggested that these bacteria strains may be useful for the cleanup of ecosystems contaminated with a mixture of toxic heavy metals and hydrocarbons.

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