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The growth of the *Rhodococcus* sp. on diesel fuel under the effect of heavy metals and different concentrations of zinc

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Abstract Co-contamination of diesel fuel and heavy metals can be challenging for microbial remediation due to the complex composition of the fuel and the inhibitory effect of heavy metals. There is an urgent need to study this interaction to improve the pollutant removal efficiency in the Polar Regions. The growth of an Antarctic bacterium, *Rhodococcus* sp. was studied by comparing the growth at the logarithmic phase under the effect of selected heavy metals (Pb, Cr, As, Cd, Cu, Zn, Ni, Hg and Co). The selected heavy metals inhibited the growth of the *Rhodococcus* sp. on diesel fuel in an order from highest to lowest of: Hg > Zn > Cd > Cu > Co > Ni > As >Pb> Cr. Growth on diesel fuel co-contaminated with Hg and Zn were 2.95% and 5.71%, respectively compared to the no-metal control. A further experiment with various Zn concentrations was conducted. The specific growth rate of *Rhodococcus* sp. co-contaminated with different concentrations of Zn showed a correlation coefficient (*r*) of 0.916, and was modelled with an exponential decay model. Additional investigation is needed to determine the effect of low concentration of Zn on hydrocarbon degradation. It is important to understand the relationships between microbes, hydrocarbons and heavy metals, especially in the Polar Regions because this interaction might be promising in treating hydrocarbon-polluted sites containing heavy metals. The data and results also provide baseline tools of bioremediation processes at low temperatures and the knowledge of the ecological roles of *Rhodococcus* sp. in Antarctica.

Keywords **co-contamination, diesel, heavy metals, *Rhodococcus* sp., exponential decline model**

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1 Introduction

In this modern era with increasing industrial activities, the demand for petroleum hydrocarbon has increased drastically. In Antarctica, petroleum hydrocarbon such as diesel fuel is generally used for heating, transportation and power generation (Aislabie et al., 2006). Even the storage of petroleum-derived fuels can cause soil contamination (Aislabie and Foght, 2009). Occasionally, during transport or use, diesel fuel may be accidentally released to the soils and the groundwater which then causes a significant threat

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to the environmental quality (Stark et al., 2017). Rapid migration of the diesel fuel compound from the contaminated sites can worsen problem and negatively affect the terrestrial and aquatic ecosystems and humans (Abha and Singh, 2012).

Degradation of diesel fuel in a sensitive environment such as Antarctica can be very expensive using physical methods, and dangerous using chemical methods which have a risk of additional environmental impacts (Delille, 2000; Cury et al., 2015). The Antarctic ecosystem is considered one of the last remaining pristine zones on Earth and remains uncontaminated by anthropogenic hydrocarbons (Hughes et al., 2013). Diesel fuel contamination of the Antarctic ecosystem can be stressful to environmental quality due to a lack of full understanding of the fate and transport of the fuel, technological restrictions and also cost-efficiency concerns.

Co-contamination by diesel fuel and heavy metals is a complex problem. This is because these two pollutants have different characteristics and usually have to be treated separately. Co-contamination of petroleum hydrocarbons, polycyclic aromatic hydrocarbons and metals such as Co, Pb and Zn in Antarctica have been reported at Thala Valley and Casey Station (Deprez et al., 1999). At a site near Marambio Station, Seymour Island, the baseline levels of soil heavy metals were 6.1 $mg \cdot kg^{-1}$ Cu, 10.2 $mg \cdot kg^{-1}$ Pb and 36 $mg \cdot kg^{-1}$ Zn (Chaparro et al., 2007). The presence of several potentially toxic metals was also detected near an Antarctic refuge on Robert Island (de Lima Neto et al., 2017). The presence of these heavy metals not only contaminates the soil, but microbial activities are also restricted at the contaminated sites (Chu et al., 2019). As a result, the degradation of other organic pollutants, in this case, diesel fuel, will be impacted (Mittal and Ratra, 2000). In general, heavy metals can block essential functional groups, displace essential metal ions or transform the active conformations of biomolecules; and thus exert an inhibitory effect on microorganisms (Nies, 1999). It is known that high levels of metals in the hydrocarbon-contaminated environment reduce the rate of oil degradation (Mattina et al., 2003). Bioremediation can be very difficult due to the adverse effects of heavy metals on the degradation rate of organic matter (Mitsch and Jørgensen, 2004).

The use of bacteria such as *Rhodococci* for co-contamination remediation may be promising. Some *Rhodococci* can degrade a variety of organic compounds, including xenobiotic compounds such as chlorinated compounds, while others can degrade numerous aliphatic or aromatic hydrocarbons (Iwabuchi et al., 2000). Some of the *Rhodococcus* strains are even psychrotrophic, which is very important for bioremediation in cold climates, such as pristine Antarctic regions (Bell et al., 1998; Habib et al., 2018). It is important to study the effects and efficiency of biodegradation of diesel fuel by a *Rhodococcus* sp. in the presence of heavy metals. Therefore, this study aims to investigate the growth of a *Rhodococcus* sp. isolated in a previous study (Habib et al., 2018) under the effect of various heavy metals (Pb, Cr, As, Cd, Cu, Zn, Ni, Hg and Co). Its growth under different concentrations of Zn was also studied and the maximum specific growths of the strain were also modelled. Zn was selected in this study due to its dual roles in the growth of microorganisms. At certain levels, it can be essential to microbial growth but otherwise, toxic to the microbes (Lo Giudice et al., 2013).

2 Materials and methods

2.1 Media and bacterial culture

Bushnell-Hass Mineral (BH) Medium (Bushnell and Hass, 1941) (0.2 $g \cdot L^{-1}$ $MgSO_4$; 0.02 $g \cdot L^{-1}$ $CaCl_2$; 1.0 $g \cdot L^{-1}$ KH_2PO_4; 1.0 $g \cdot L^{-1}$ K_2HPO_4; 1.0 $g \cdot L^{-1}$ NH_4NO_3; 0.05 $g \cdot L^{-1}$ $FeCl_3$; and 7.0 pH) was used to grow *Rhodococcus* sp. on diesel fuel. All media were sterilised and all chemicals were of analytical grade.

The *Rhodococcus* sp. was cultured from glycerol stocks and retained on the nutrient agar plate at 10 ℃ for 14 d (Habib et al., 2018). A single colony was then inoculated into the sterile nutrient broth and incubated at 10 ℃ for 24 h with shaking at 160 rpm. The culture was then adjusted to optical density (OD) at 600 nm of 1 by adding nutrient broth. The $OD_{600\ nm}$ values were converted to the number of colony-forming unit (CFU) per milliliter of sample ($OD_{600\ nm}$ of 1 = $1 \times 10^{7.5}$ $CFU \cdot mL^{-1}$).

2.2 Growth on selected heavy metals

The culture flasks (100 mL) containing 50 mL BH supplemented with diesel fuel (1% (v/v)) and selected heavy metals (10 $mg \cdot L^{-1}$) were inoculated with 100 μL of *Rhodococcus* sp. that was adjusted to $OD_{600\ nm}$ = 1. All the inoculated flasks and controls (in triplicate) were incubated at 10 ℃ for 24 h with shaking at 160 rpm. In this experiment, a range of selected heavy metals of 10 $mg \cdot L^{-1}$ concentration was incorporated into the samples in the form of sterilized salt solutions i.e. $Pb(NO_3)_2$, $Cr(NO_3)_3$, H_3AsO_4, $Cd(NO_3)_2$, $Cu(NO_3)_2$, $Zn(NO_3)_2$, $Hg(NO_3)_2$, $Ni(NO_3)_2$ and $Co(NO_3)_2$. The bacterial growth was monitored by measuring the $OD_{600\ nm}$ of 1. To measure the growth, 1 mL of culture was extracted and measured directly on the spectrophotometer on the logarithmic phase (day 4).

2.3 Growth on different concentrations of Zn

Culture tubes containing BH medium supplemented with diesel fuel (1% (v/v)) and different concentrations of Zn (1, 2, 5, 10, and 20 $mg \cdot L^{-1}$) were inoculated with *Rhodococcus* sp. culture that was adjusted to $OD_{600\ nm}$ = 1. All the inoculated flasks and controls (in triplicate) were incubated at 10 ℃ for 24 h with shaking at 200 rpm. The bacterial growth was then monitored. To measure the growth, a culture tube was read and measured directly on a HACH

spectrophotometer every 24 h.

2.4 Modeling the specific growth rate at different concentrations of Zn

The samples were serially diluted accordingly and plated onto nutrient agar (Figure 1). The data was then fitted logarithmically.

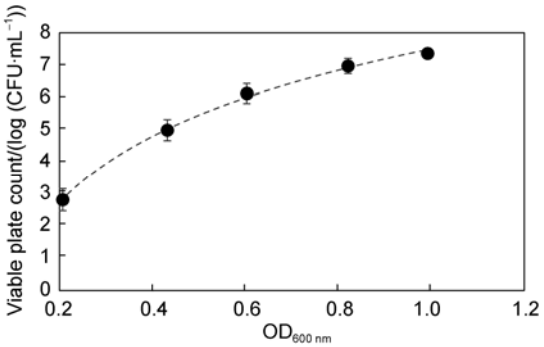

Figure 1 Standard curve for the determination of viable plate count from $OD_{600\ nm}$.

The maximum specific growth rates on diesel fuel in the presence of different concentrations of Zn were determined from the slope of the exponential growth. The values obtained from this was then used to model the effect of the metal. An exponential decay model, which was used to fit the data, is as follows:

$$\mu = \mu_{max} \exp(-kC) ,$$

where μ is the specific growth rate (h^{-1}), μ_{max} is the maximum specific growth rate (h^{-1}), k is a constant ($L \cdot mg^{-1}$) and C is the inhibitor concentration (Zn, $mg \cdot L^{-1}$).

3 Results and discussion

3.1 Growth of *Rhodococcus* sp. under the effect of selected heavy metals

The bacterial growth on diesel fuel had a lag phase from day 1 to day 3 and a logarithmic phase starting from day 3 to 4. During the logarithmic phase, the growth rate is constant, and a steady state is established. Thus, making exponential growth rate measurements is a general and sensitive physiological test that can be used for the study of a wide variety of effects.

The study was performed in a conical flask of heavy metals under sterile conditions and the growth of bacteria on day 4 of each sample was studied. The growth rates of -*Rhodococcus* sp. on diesel fuel supplemented with different heavy metals were compared with the no-metal control in terms of relative percentage (Figure 2).

From Figure 2, it is clear that Hg and Zn, which showed only 2.95% and 5.71% growth respectively, were the heavy metals that most inhibited the growth of the strain. Whereas, growth was not very much affected by Cr. The selected heavy metals inhibited the growth of the *Rhodococcus* sp. on diesel fuel in an order from highest to lowest of: Hg > Zn > Cd > Cu > Co > Ni > As > Pb > Cr.

Most cells have two types of uptake systems for heavy metal ions: The first type is fast, unspecific and constitutively expressed. The second type of uptake system requires a higher substrate specificity, is slower and often involves hydrolysis of ATP. Once the heavy metal ions in

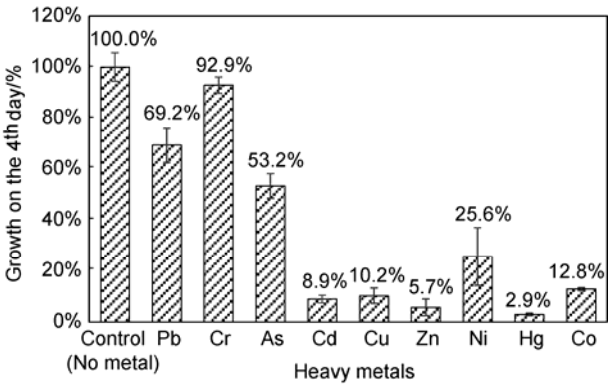

Figure 2 The growth of *Rhodococcus* sp. on day 4 (logarithmic phase) on 1% diesel fuel in BH media incorporated with different types of selected heavy metals (10 $mg \cdot L^{-1}$). The control was no-metal.

the cell, there are three possible mechanisms for a heavy metal resistance system. First, efflux (active extrusion of the heavy metal ion) can diminish the accumulation of the ion. Second, thiol-containing molecules can segregate cations, especially "sulphur lovers", into complex compounds. Third, some metal ions may be reduced or oxidized to a less toxic oxidation state (Nies, 1999).

In this study, the growth of *Rhodococcus* sp. in the presence of Hg was most inhibited. The affinity of Hg^{2+} to thiol (SH) groups is very strong, with a solubility product of HgS i.e. 6.38×10^{-53} (Nies, 1999). The thiol group, which is an organosulfur compound that contains a carbon-bonded sulfhydryl group, acts as the functional group of proteins and enzymes, such as amino acid cysteine. By binding to SH groups, mercury may inhibit the activity of sensitive enzymes, which may disrupt enzymatic activity, translation and transcription (Colombo Fleck et al., 2000).

Zn is needed by a variety of enzymes and DNA-binding proteins, and is a crucial element to life. However, Zn may interfere with a process that involves enzyme magnesium-transporting ATPase (MgtA) (Nies, 1999). The interruption by Zn to enzyme MgtA might be the reason that Zn inhibits the growth of *Rhodococcus* sp., which only showed a growth rate of 5.71% at the logarithmic phase compared to the control.

Chromium mostly occurs as Cr(VI) in the divalent oxyanion chromate and as Cr(III), the trivalent cation. Chromate is more toxic than Cr^{3+}, thus, only Cr^{3+} can perform beneficial functions. However, even if toxic chromate has entered the cell of a microorganism, chromate resistant bacteria can overcome the situation through the

interaction of chromate reduction and chromate efflux (Nies, 1999). Thus, in the case of *Rhodococcus* sp., this might be the reason that the growth with Cr co-contamination can still 92.86% at the logarithmic phase.

For some lead-tolerant bacteria, Pb is mediated by a P-type ATPase (a type of enzyme), proving that lead resistance may also be based predominantly on metal ion efflux. In this case of *Rhodococcus* sp., the growth in the presence of Pb was relatively high (69.18%) compare to other heavy metals. Thus, there is a good possibility that this *Rhodococcus* sp. is a lead-tolerant bacterium.

Arsenic (As) has well-known toxicity to cells that results from its interference with the metabolism of the major bio-element phosphorus. Despite this, the growth of the *Rhodococcus* sp. in the arsenic-present sample was still 53.22% relative to the control. This might be due to export of the arsenic by efflux. In the experiment, the arsenic occurred as As(V) in AsO_4^{3-}, arsenate. However, it is hard to export arsenate effectively due to its high concentration and structural similarity to phosphate. The arsenate detoxification process can differentiate arsenate from phosphate by reducing the arsenate to arsenite. The arsenite then leaves the bacterial cell by chemiosmotic gradient and simple arsenite efflux. The results suggest that 53.22% of the *Rhodococcus* sp. is able to differentiate arsenate from phosphate and export arsenic out of the cell (Nies, 1999).

Thus, various types of heavy metals might pose different types of inhibitory effects based on various cell mechanisms of cell nutritional uptake. The effect of different heavy metals on the microbial growth may be different based on the composition of the heavy metals and also the uptake mechanism.

3.2 Growth of *Rhodococcus* sp. under different concentrations of Zn

Zinc has dual roles in the growth of microorganisms. At certain levels, Zn is essential to the growth; but on the other hand, Zn is a metal that is highly toxic, following after Hg and Cd according to its position on the periodic table (Hellawell, 1986). Hence, further tests at different concentrations of Zn were conducted on the growth of *Rhodococcus* sp.

At low concentrations, Zn^{2+} is used by cells to protect their integrated property (Yao et al. (2005). In addition, it is essential to the DNA and RNA syntheses in the cells and to maintain the regular growth and the biological activity of the cells. Figure 3 shows that the presence of 2 $mg \cdot L^{-1}$ of Zn in the media is better than the presence of 1 $mg \cdot L^{-1}$. However, when the amount of zinc was increased to 5 $mg \cdot L^{-1}$, a small drop in growth started to occur, and for 10 and 20 $mg \cdot L^{-1}$ of Zn, significant drops were observed.

The results shown parallel the results of Cabrero et al. (1998) which studied the biomass yield on activated sludge. At high concentrations, Zn^{2+} has an inhibitory action on growth. One of the inhibitory factors of Zn is that Zn^{2+} combines with sulfhydryl groups on membrane proteins, resulting in changing cell membrane permeability and disrupting transport of nutrients and waste across the membrane (Yao et al., 2005). This shows that the presence of Zn^{2+} can have both positive and negative effects on the growth of *Rhodococcus* sp., depending on the concentration of the $Zn(NO_3)_2$.

3.3 Modelling the specific growth rate as a function of different concentrations of Zn

The specific growth rates at various concentrations of Zn was modelled using an exponential decay model (Figure 3). The correlation coefficient (r) was 0.916 and the coefficient of determination (r^2) was 0.839. The parameters μ_{max} and k obtained from the fitting data were 0.0311 ± 0.006 h^{-1} and 1.175 ± 0.490 $L \cdot mg^{-1}$, respectively. Further investigations are required to discern the effect of a low amount of Zn (preferably below 2 $mg \cdot L^{-1}$). Despite the significance of the co-contamination study, the use of a model involving metals as co-contamination is poorly studied and represented in literature (Shukor et al., 2018). The data from the model presented here can be used as baseline data for future study involving the co-contamination of diesel fuel and heavy metals, such as the effects of pH, temperature, varying concentrations of hydrocarbons and many more. The comparison of kinetic data will allow researchers to predict the critical heavy metals concentration which can completely inhibit bacterial growth. The results from this study will be important for field trials where bioremediation of hydrocarbons is conducted in areas co-contaminated with heavy metals (Deprez et al., 1999).

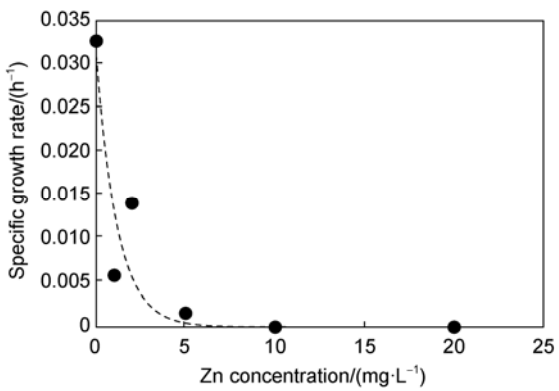

Figure 3 The specific growth rate (h^{-1}) at 10 ℃ against different concentrations of Zn. The values from the experiments are depicted as a solid markers and the model is depicted as a dashed line.

4 Conclusion

The growth of the *Rhodococcus* sp. on diesel fuel co-contaminated with heavy metals and under different concentrations of zinc has been observed and compared in this study. It was found that the selected heavy metals

inhibited the growth of the *Rhodococcus* sp. on diesel fuel in an order from highest to lowest of: Hg > Zn > Cd > Cu > Co > Ni > As > Pb > Cr. A recommendation from this study is that further similar experiments should be undertaken to understand why the *Rhodococcus* sp. can tolerate the presence of 10 $mg \cdot L^{-1}$ of Cr and Pb. It is important to understand the relationships between microbes, hydrocarbons and heavy metals, especially in the Polar Regions because this interaction might be promising in treating hydrocarbon-polluted wastewater containing heavy metals. The data and results also provide baseline tools of bioremediation processes at low temperatures and knowledge of the ecological roles of the *Rhodococcus* sp. in Antarctica.

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