MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society for Biochemistry & Molecular Biology (MSBMB)
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# BACTERIAL GROWTH AND DIESEL BIODEGRADATION IN THE PRESENCE OF As, Cu AND Pb BY ANTARCTIC MARINE BACTERIA

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History
Received: 19th November 2019
Accepted: 6th December 2019

Keywords:

*Antarctica, diesel, exponential growth, heavy metal, marine*

**Abstract**

Antarctica is considered to be low-impacted by anthropogenic activities despite the rising activities occurring in the Southern Ocean. Rising human activities from within Antarctica and external sources resulted in hydrocarbon and heavy metal pollution and create more and more evidence on how much anthropogenic pollution has influenced the southern polar region. Bioremediation possibilities in these parts are very limited in terms of maximum efficiency due to its unique climatic conditions. Furthermore, heavy metals come hand in hand with hydrocarbon pollution and there is a call to obtain. In the present study, diesel degradation was inhibited the most by Pb (31.75%), As (34.35%) and lastly Cu (48.91%) in comparison to control flask (65.19%). Bacterial growth was most inhibited by Cu. Pb had little effect to the growth of bacteria in comparison to As and Cu. Growth rates were obtained by fitting the Exponential mathematical model to the data and obtaining individual growth rates and measures of good fit. ANOVA analysis of the growth obtained P values of control, As and Pb to be <0.05 while Cu gave a P>0.05.

## INTRODUCTION

The growing concern over the extent of anthropogenic impacts on the far reaches of the Southern Hemisphere has been well documented over the years. Within the turn of the century, anthropogenic pollution has reached the Polar Regions through ocean currents and atmospheric circulation; among them heavy metals [1]. In the sub-zero temperatures of the continent, Antarctica houses global scientific communities all of whom depend on the consumption of fossil fuels to generate electricity at the research stations, many being situated by the coastline. Unsurprisingly, chronic to small scale spills have run risk in Antarctica, rendering slow natural attenuation due to chronic low temperatures [2-4]. With more and more handling of fossil fuels, diesel becomes the main source; as a result, heavy metals are contaminating the soil, snow and water of Antarctica, further causing damage to the last pristine habitat on Earth. Most activities are limited to the coastal areas of the continent. Heavy metals occur naturally within the Earth's crust. However, high industrial consumption of these metals has made it difficult to distinguish the source of heavy metal occurrence [5, 6].

The input of metals into the sea from either natural or anthropogenic origins mainly comes through atmospheric processes, whereas the contribution of some metals is greater than their natural sources [7]. In 1987, Antarctic seawater near Syowa station has detected measurable levels of Zn, Cu, Pb, Ni, Cd and Hg. Contamination by hydrocarbons poses a pervasive problem in the Antarctica region [8]. Combustion of fuels is a suspected source of heavy metals emitted into the environment with a magnitude of varying ppb ranges. It is thought that fuel combustion, oil spillage, waste incineration and sewage disposal in Antarctic scientific stations contribute most to the local

contamination of heavy metals in the Antarctic environment [10, 11].

Heavy metal contamination has become a growing concern on the marine ecosystem of Antarctica. Elevated levels of heavy metals have been detected from snow and aquatic ecosystems in Antarctica, as well as biotic components from both aquatic and terrestrial organisms [12]. Marine pollutants in Antarctica are derived from improper waste management, exhaust emissions and accidental oil spills [13]. In the Thalla Valley tip near Casey Station, high levels of Cu, Pb and Zn have been found at the waste disposal sites than non-impacted areas [14]. Various studies have documented lead (Pb), cadmium (Cd), copper (Cu) and arsenic (As) as among the contributing heavy metals polluting Antarctica [15]. Soil samples around Scott Base on Ross Island have revealed a contamination of silver (Ag) among Cd, Cu, Pb, Zn and As due to improper waste disposal and chemical and fuel spillage [16]. The same elevated level trace of heavy metals (Cu, Pb, Zn) with the addition of iron (Fe) and tin (Sn) have been found in marine sediments near Casey Station, Antarctica [17]. Sewage disposal, paint residues from buildings and/or petroleum are probably the sources of trace metals enrichment in sediments collected near research stations at Admiralty Bay.

Bioremediation in cold climates depends on the capability of microorganisms to degrade organic contaminants at different temperatures [18, 19]. Heavy metals are detrimental to most microorganisms at seemingly low concentrations even in natural waters [20-22]. Heavy metals bind to protein molecules and form complexes, which render them inactive while enzymes become inactive. This phenomenon has crucial implications for the microbial ecology in polluted ecosystems [23]. Heavy metals are elevated after the occurrence of oil spills, which have been proven lethal to the marine biota as they are bioaccumulated and work their way through the high trophic levels. There are several ongoing major remediation projects in Antarctica, with the largest being at McMurdo Station. However, information on bioaccumulation of pollutants in Antarctic species and the development of risk-assessment models is still limited [24]. In Antarctica, there is a very strict policy regarding the banning of introducing non-native microorganisms. For this reason alone, the efficiency of bioremediation relies heavily upon autochthonous microorganisms that can be optimised to increase the efficacy of bioremediation of hydrocarbons in the presence of heavy metals.

This current work proposes a kinetic study of marine psychrotolerant microorganisms that can degrade diesel in the presence of heavy metals. In biodegradation kinetics, substrates (diesel) are consumed through microbial enzymatic reactions; thus, the substrate degradation is directly proportional to the amount of microorganism and is dependent on the characteristic concentration of substrate saturation kinetics [25, 26]. Therefore, in the presence of heavy metals, the extent of the inhibition of microbial growth and correlation to the degradation of diesel become a question attempted to be answered from this work. Kinetic models can be used to study the performance of a biological process, in this case, the bacterial growth in the presence of a substrate and without heavy metals.

## MATERIAL AND METHODS

### Sample

Seawater samples were collected from the base station of Bernado O'Higgins, Antarctica during an expedition in 2018. Sample was then cryopreserved in glycerol solution at -80°C. Diesel degrading ability of the samples was already established in a preliminary study.

### Screening of bacteria growth on diesel and 1 ppm heavy metals

For screening, a standardised Bushnell–Haas (BH) salt medium [27] was used enriched with 1% (v/v) diesel in 50 mL media. BH media has a chemical composition of 1.0 g/L $NH_4NO_3$, 1.0 g/L $KH_2PO_4$, 1.0 g/L $K_2HPO_4$, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $FeCl_3$ and 0.02 g/L $CaCl_2$ in $dH_2O$, adjusted to pH 7.0 ± and further supplemented with 20 ppt (w/v) NaCl. Standard inoculum size with an absorbance reading of OD600 nm = 1.0 was applied throughout the study. A set of the samples was kept on a shaking incubator at 150 rpm for 7 d at 10°C. A set of media with the same chemical composition was also kept in the same conditions as abiotic control.

The most efficient diesel degrading sample was chosen for a further study to ascertain the optimum growth conditions in a modified BH media. The optimised BH media used for this study has similar chemical composition with 30 ppt (w/v) NaCl adjusted to pH 8.0 phosphate buffer. The media was aseptically inoculated with 2% (v/v) inoculum and 1 ppm of heavy metals namely As, Pb and Cu. The samples were kept on a shaking incubator at 150 rpm for 7 d at 10°C. A set of media with the same chemical and inoculum composition was also kept in the same conditions as a biotic control without the presence of heavy metals.

### Determination of diesel degradation

Residual diesel oil was quantified gravimetrically at the end of the 7 d incubation period. The degradation percentage of diesel was calculated following the formula proposed by Ganesh and Lin [28]. The growth of sample was determined by measuring the optical density of the media ($OD_{600}$) on a day-to-day basis. To assess the significance of the difference in bacterial growth in the presence of heavy metals, an analysis of variance (ANOVA) was performed using graph pad prism 5.0.

### Determination of kinetic parameters on bacteria growth in diesel with the presence of heavy metals

The growth profile of the bacterial growth was used to obtain kinetic parameters from batch studies. The values of the specific growth rate coefficient m every 24 h can be obtained by plotting ln x (bacterial dry weight) vs. time. These values can be plotted against time to plot a nonlinear curve. Taking the log of the growth curve, a linear regression was performed around the highest peak or maximum growth. An exponential growth model was also fitted to the growth curve using the software Graphpad Prism 5.0.

## RESULTS AND DISCUSSION

Data was taken every day to monitor the bacterial growth in the presence of 1 ppm As, Cu and Pb with reference to a biotic control and diesel degradation was evaluated on the final day of incubation period (7d). The conditions of the bacterial growth with 30 ppt salinity and pH 8.0 served to assimilate the natural conditions of seawater. Marine environment has a pH that sits dynamically in the range of 7.5 to 8.5. Surface ocean water around

the globe is alkaline, ranging from pH 7.9 to 8.2 in the year 2005 [29]. Rivaro et al. [30] concluded the same pH for a study done in the Ross Sea in 2008. The bacterial consortium has a stronger efficacy at pH 8.0 using phosphate buffer as the presence of phosphorus element in the buffer might have serve either a functional or nutritional role towards the microbial cells. In the oceanic environment, natural concentration of P is readily available for microbial uptake; however, oil spills can cause an imbalance of natural concentration and thus becomes a limiting factor. Heavy metals can be readily found after oil spills and are bioaccumulated in marine biota. The most frequently found heavy metals are Pb>Ni>V>Zn>Cd [31].

From **Figure 1**, the weight reduction of diesel was inhibited the most by Pb, followed by As and lastly Cu. In the control flask, diesel was degraded up to 65.19% according to gravimetry method of obtaining weight reduction. In the presence of Cu, diesel degraded up to 48.91%, followed by As, 34.35%. This was followed by a 31.75% diesel degradation which was inhibited in the presence of Pb. On the other hand, bacterial growth in the different types of heavy metals showed contrasting results. Growth was the highest in Pb followed by As ($P<0.05$) and lastly Cu ($P<0.05$). High levels of Cu have been found in marine sediments in a study by Ribeiro [32], in agreement with the result obtained by Trevizani et al. [33]. Cu is an essential element for animal and plant metabolism. However, they are also very toxic to aquatic life. While Cu is importnat to life forms like animals and plant metabolism, Pb like As and Hg have no known biological significance. However, many authors have discussed that high levels of Cu in marine samples may not entirely put to be blamed on anthropogenic issues. Machado et al. [34] suggests that the mineralisation of chalcopyrite plays a role in the presence of Cu. Older works by Fourcade [35] believes that Cu along with other metals like Zn is incorporated by glacial erosion of volcanic rocks.

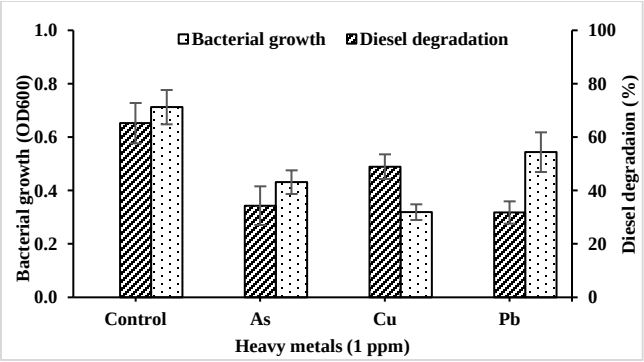


**Figure 1.** The effect of 1 ppm heavy metals on the bacterial growth and diesel degradation of Antarctic seawater sample. Growth was carried out at 10℃ using BH media pH 8.0 supplemented with 30 ppt salinity and 1% (v/v) diesel for 7 d. Data represent mean ± SEM. All data are available in triplicates, $n = 3$.

Cu-resistant bacterial community is found when, in a chronic exposure to Cu, develops mobile Cu genetic determinants in polluted environments over time [36]. Bacterial communities from long-term Cu-polluted soils can well adapt to high Cu content. Short-term Cu pollution in soil generates significant alterations in the structure of bacterial community, but these changes are resilient after a few weeks or months [37]. Previous study also showed that Cu, Pb and Zn do not significantly shift the bacterial diversity after long-term contamination [38]. Heavy metal Cu proves to be toxic to most microorganisms because it binds to the bacteria, giving it a more immediate effect on cell division rather than on general metabolism [39]. However, Cu resistance is acquired by the bacteria when cell division recommences following increasing concentrations of Cu. This is then followed by physical and chemical adaptations by the bacteria such as loss of motility and changes in shape, increased osmotic sensitivity, reduced cytochrome content and loss of cellular magnesium.

As was chosen as a heavy metal of interest as many works have agreed upon the bioaccumulation of As in many marine organisms typically algae [33, 40]. However, Farías et al. [41] argues that bioaccumulation of As can also be attributed to natural processes. Algae have metabolic adaptations to assimilate arsenic as a phosphorous (P) analog during the metabolic processes. As-dependent bacterial growth has been also reported [42], suggesting that some bacteria have the ability to assimilate As into biomolecules. This would explain mediocre bacterial growth in the presence of As. In some organisms, As induces specific resistance genes to cope with the toxicity of As [43], whereas some As-utilising microbes are able to conserve energy for growth from the oxidation of reduced As species as a terminal electron acceptor [44].

Many literatures have studied the presence of Pb bioaccumulation that has become a more commonly found heavy metal in Antarctic marine ecosystems. Ribeiro et al. [32] reported higher levels of Pb than those observed by Grotti et al. [45]. However, **Table 1** shows the results obtained for the bacterial growth in Pb, which has little significance ($P>0.05$) in comparison to As, Cr and Cu. Pb is an element detrimental to living organisms. To lessen the effects, some microbes have resistance mechanisms from adsorption by extracellular polysaccharides, cell exclusion and sequestration as insoluble phosphates and ion efflux to the cell exterior [46].

**Table 1.** One-way ANOVA table of bacterial growth

| | Sum of Squares | Df | Mean Squares | F | P value | $R^2$ |
|---|---|---|---|---|---|---|
| Treatment (between columns) | 0.2533 | 3 | 0.08444 | 10.68 | 0.0036 | 0.8002 |
| Residual (within columns) | 0.06327 | 8 | 0.007909 | | | |
| Total | 0.3166 | 11 | | | | |

The ANOVA statistical analysis assessed the relative size of variance among the group means compared to the average variance within groups. A multiple comparison *post-hoc* test was applied using Tukey's test. The critical F value was 4.07 in the F table when the degrees of freedom of numerator and denominator were 3 and 8, respectively at the α error level of 0.05. As the observed F value was 10.68 larger than the critical value, the result may be interpreted as statistically has significant difference among the means of the groups at the α error level of 0.05. The ANOVA results suggest rejecting the null hypothesis that all the group mean values are the same. Additionally, the results supported that at least one group mean differs from other group means.

**Growth kinetic studies in the presence of 1 ppm heavy metals**

A fundamental aspect of predictive microbiology is the data collected from the shape generated by the microbial curve. Microbial growth has been described by a variety of mathematical models where the population size can be described. Each model comes with its own properties to describe how well they fit and predict experimental growth data. All microbial growth follows the first order of kinetics. Kinetic parameters that are of interest to many works include the specific growth rate, maximum specific growth rate and lag time. Growth rates have long been used in microbiology for many purposes including in antibacterial studies [47] and inhibition of biofilm production 48]. The purpose of measuring the growth rate is to determine the rate of change in cell numbers per unit time (min, h and d).

Ideally, the bacteria growth consists of lag phase, log phase or exponential phase, stationary phase and decline phase or death phase. **Figure 2** illustrates the microbial growth of sample in the presence of 1 ppm As, Cu and Pb with 1% (v/v) diesel as sole carbon source. The first phase observed is the lag phase when the growth rate is essentially zero. The lag phase is defined as transition to the exponential phase after the initial population has doubled [49] bearing in mind that not all cells divide at the same time. The lag phase is thought to be due to the cells physiologically adapting to the culture conditions. The lag phase is also thought to be due to the low initial density of inoculum. As a result, the exoenzymes that leak from growing cells is diluted and not easily taken up. Thus, transition to exponential phase is slowed.

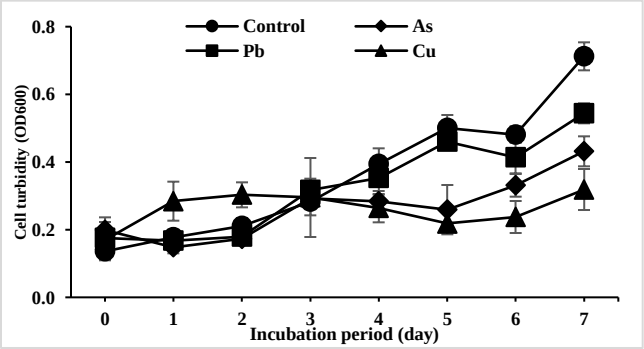


**Figure 2.** The microbial growth curve of sample in optimised media supplemented with 1 ppm As, Cu, Pb and positive control. Growth was carried out at 10℃ using BH media pH 8.0 supplemented with 30 ppt salinity and 1% (v/v) diesel for 7 d. Data represent mean ± SEM. All data are available in triplicates, $n = 3$.

The general point of interest in growth curve studies is the exponential phase or log phase. In exponential growth, a population's per capita (per individual) growth rate stays the same regardless of population size, making the population to grow faster as it gets larger. Exponential growth produces a J-shaped curve, while logistic growth produces an S-shaped curve or generally termed as sigmoidal. Exponential growth is not a very sustainable state of affairs since it depends on infinite amounts of resources (which are likely not to exist when applied in the real world).

Exponential growth may happen for a while if there are few individuals and many resources. But when the number of individuals gets large enough, resources start to get used up, slowing the growth rate. Eventually, the growth rate will plateau, or level off, making an S-shaped curve. This study only collected data for 7 d following a preliminary study relating to diesel biodegradation of the sample. **Figure 2** shows exponential phases for As and Pb on day 2 but stops plateaus slightly at day 6 before continuing to climb on day 7.

The exponential growth equation describes the growth with a constant doubling time. Doubling time or some literatures also refers it to 'specific growth rate' is denoted as $\mu$. The exponential growth equation is written as in Equation 1.

$X = X_0 * \exp^{\mu t}$ (Equation 1)
$X_0$ = Value of Y ($OD_{600}$) at time zero
K is the rate constant, unit ($d^{-1}$)
Tau is the time constant.
Doubling time: ln2/K

In the linear scale of the graph, the exponential model equation can also be written as in Equation 2.
$\ln X = \ln X_0 + \mu t$ (Equation 2)
$X_0$ = Value of Y($OD_{600}$) at time zero
$\mu$ = specific growth rate
t = time with reference to X

**Table 2** tabulates the results of nonlinear regression and linear regression of the slope of control, As, Cu and Pb. When comparing slopes, a P value of <0.05 signifies significance of the slopes. **Figure 3** records that only slope of Cu has a P value >0.05, thus automatically being insignificantly different. P values of control, As and Pb all have the P values <0.05.

**Table 2.** Parameters from Exponential growth equation

| Exponential growth equation | | | | |
|---|---|---|---|---|
| Best-fit values | **Control** | **As** | **Pb** | **Cu** |
| Y0 | 0.1477 | 0.1731 | 0.1733 | 0.2347 |
| K | 0.2553 | 0.1189 | 0.1636 | 0.04480 |
| Tau | 3.917 | 8.409 | 6.112 | 22.32 |
| Doubling Time | 2.715 | 5.829 | 4.237 | 15.47 |
| Goodness of Fit | | | | |
| Degrees of Freedom | 22 | 22 | 22 | 22 |
| R square | 0.8852 | 0.4671 | 0.5881 | 0.07374 |
| Absolute Sum of Squares | 0.1973 | 0.1512 | 0.2621 | 0.2446 |
| Linear regression of slopes | | | | |
| F | 218.8 | 19.49 | 33.28 | 1.778 |
| P value | < 0.0001 | 0.0002 | < 0.0001 | 0.1960 |

Figures 3 a, b, c and d show the usage of exponential growth equation on four different sample conditions of heavy metals. Out of all three heavy metal conditions, Fig 3d generated the highest specific growth rate $\mu = 0.1636\ d^{-1}$ followed by Fig 3b, As with $\mu = 0.1189\ d^{-1}$ and lowest by Cu with a specific growth rate, $\mu = 0.04480\ d^{-1}$. Comparison of growth rates or lag times is commonly used to estimate the fitness of individual microbial isolates despite clear, observational evidence that it provides an inadequate alternative.

Growth curves describe the density of cell populations in a liquid culture over a certain period of time. The simplest way to infer fitness from growth curves is estimating the growth rate during the exponential growth phase, using the slope of the log of the growth curve. However, exponential growth rates do not

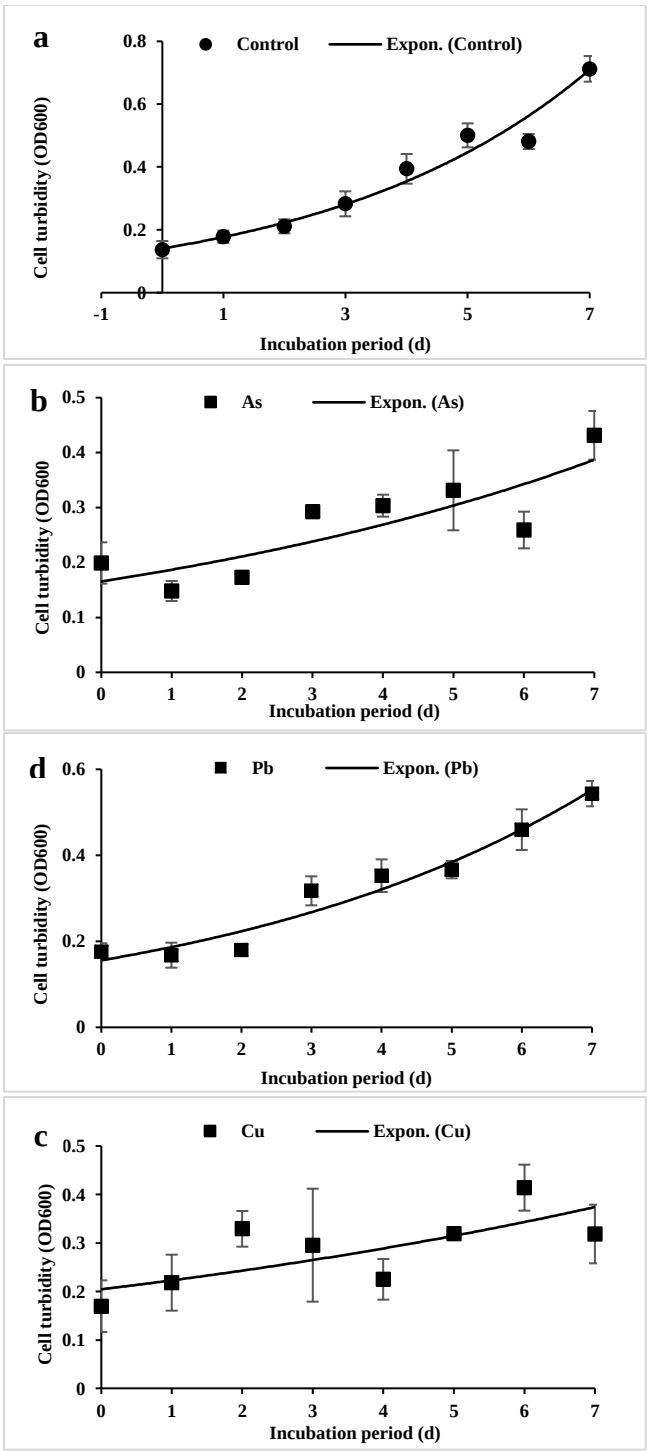


**Figure 3.** The nonlinear regression model using exponential growth equation of (a) control with specific growth rate, $\mu = 0.2553\ d^{-1}$, (b) in 1 ppm As with specific growth rate, $\mu = 0.1189\ d^{-1}$. (c) Sample in 1 ppm Cu with specific growth rate, $\mu = 0.04480\ d^{-1}$, (d) Sample in 1 ppm Pb with specific growth rate, $\mu = 0.1636\ d^{-1}$. All data represent mean ± SEM. All data are available in triplicates, $n = 3$.

capture the complete dynamics of typical growth curves because data is only evaluated at the exponential phase. Many literatures across the decade have used other mathematical models like the Logistics model, the Baranyi-Roberts model, the dual-Monod model and so forth with many incongruities in the data being performed across literature. Many literatures also perform growth studies on monocultures rather than mixed bacterial consortium [50, 51]. However, fitting an exponential model to the exponential growth phase can always be used as a benchmark for further studies. More data can be collected over a series of closer time is required in order to acquire maximum growth rate at the time of inflection, and further studies can incorporate the differences of heavy metal concentrations in order to evaluate more inhibition kinetics in accordance to bacterial growth relying solely on diesel.

## CONCLUSION

It is well documented that heavy metals influence microbial growth whether it be the type of metal and the type of microorganisms. The current approach of obtaining specific growth rates from growth curves are easily obtained despite discrepancies across literature. However, the objective of the study was to evaluate the bacterial growth of marine bacteria isolated form Antarctic seawater to degrade diesel in the presence of heavy metals. This study shows that Antarctic marine bacteria isolated from the sea was able to degrade diesel in the presence of 1 ppm Cu and As with higher inhibition in the presence of Pb. Since diesel pollution occurs with consequent increase in heavy metals to the surroundings, simply put there is a possibility of bioremediation to occur. However, there is still a paucity of data in the findings of heavy metals related to the marine ecosystem of Antarctica. There is still room for improvement in the techniques and the careful collection of data. The results of this work only serve to add to the library of knowledge on the biodegradation of natural marine bacteria for diesel hydrocarbon and heavy metals. The results could prove useful for future monitoring and understanding how heavy metal serve to act towards microbes in the interest of bioremediating hydrocarbons at low temperatures in seawater.

## ACKNOWLEDGEMENTS

This work was supported by Universiti Putra Malaysia (Matching Grant PUTRA (9300436) and PUTRA Berimpak (9678900)), Centro de Investigacion y Monitoreo Ambiental Antàrctico (CIMAA) and Yayasan Penyelidikan Antartika Sultan Mizan (YPASM). The authors also would like to thank Chilean Army and the Antarctic General Bernardo O'Higgins Station staff especially Sargento Segundo Augusto Antonio Barra Morale, Sargento Segundo Flavio Marcelo Nahuelcoy Perez and Sargento Segundo Claudio Durand Ibacache, National Antarctic Research Centre (NARC) and Instituto Antártico Chileno (INACH). We also thank the Public Service Department of Malaysia (JPA) for granting a master programme scholarship to Ahmad Fareez Ahmad Roslee.

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