



Kinetic studies of marine psychrotolerant microorganisms capable of degrading diesel in the presence of heavy metals

Estudios cinéticos de microorganismos marinos psicrotolerantes capaces de degradar diésel en presencia de metales pesados

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Abstract

The presence of heavy metals in Antarctica is an emerging issue as human influence becomes more discernible over the years. The study of pollution in Antarctica can help people to understand the real influence of human activities on the environmental pollution from polar regions. Bioremediation of petroleum hydrocarbons in the polar environment where toxic metals co-existed involves selecting strictly autochthonous Antarctic strains with dual catabolic competence and tolerance to toxic metals. In this study, diesel degradation was observed in the presence of 1 ppm of eight selected heavy metals; Ag, Al, Cd, Co, Cr, Hg, Ni and Zn. Bacterial growth was inhibited in increasing order of Zn > Cr > Cd > Al > Ni > Hg > Co > Ag. Bacterial growth was the highest in Zn at OD₆₀₀ 0.556 (P>0.05) and lowest in Ag at OD₆₀₀ 0.151 (P<0.05). Diesel degradation was inhibited in the order of Co > Ni > Cd > Ag > Zn > Al > Cr > Hg, which was analysed using gravimetry analysis. Degradation was the highest in Hg at 52.23% (P>0.05) and lowest in Co at 22.76% (P<0.05). This work serves as a pilot study in gathering data to analyse and gather more data for inhibition concentration of heavy metals for the Antarctic marine bacteria.

Keywords: Antarctica, bacteria, diesel, heavy metal, marine.

Resumen

La presencia de metales pesados en la Antártida es un problema que va surgiendo a medida que la influencia humana se vuelve más perceptible a través de los años. El estudio de la contaminación en la Antártida puede ayudar a las personas a comprender la influencia real de las actividades humanas sobre la contaminación ambiental de las regiones polares. La biorremediación de hidrocarburos de petróleo en el ambiente polar donde metales tóxicos coexistieron, implica seleccionar cepas Antárticas estrictamente autóctonas con tolerancia a metales tóxicos y doble competencia catabólica. En este estudio, la degradación de diésel fue observada en presencia de 1 ppm de ocho metales pesados seleccionados; Ag, Al, Cd, Co, Cr, Hg, Ni y Zn. El crecimiento bacteriano se inhibió en el orden creciente de Zn > Cr > Cd > Al > Ni > Hg > Co > Ag. El mayor crecimiento bacteriano fue en Zn a una OD₆₀₀ de 0.556 (P>0.05) y el menor en Ag a una OD₆₀₀ de 0.151 (P<0.05). La degradación de diésel se inhibió en el orden de Co > Ni > Cd > Ag > Zn > Al > Cr > Hg, el cual se determinó usando un análisis de gravimetría. La degradación en Hg a 52.23% (P>0.05) fue la más alta, y la más baja en Co a 22.76% (P<0.05). Este estudio sirve para recopilar información, como un estudio piloto para analizar y reunir más datos para la concentración de inhibición de metales pesados de las bacterias marinas Antárticas.

Palabras clave: Antártida, bacteria, diésel, metales pesados, marino.

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1 Introduction

Heavy metal elements exist in nature within the Earth's crust. However, high industrial consumption of these metals has made it difficult to distinguish the source of heavy metal occurrence (Alcázar-Medina *et al.*, 2020; Villabona-Ortíz *et al.*, 2020). The extent of anthropogenic impacts on the far reaches of the Southern Hemisphere has been well documented over the years with careful studies being done over a large area of studies. Various studies have documented metals such as arsenic (As), cadmium (Cd), copper (Cu), mercury (Hg) and lead (Pb), and zinc (Zn), contributing to local contamination in Antarctica (Tin *et al.*, 2009). Data taken over a time period between 1940-1980 established the presence of Cd, Pb, Cu, and Zn in snow samples in Adelie Land in East Antarctica (Gorlach and Boutron, 1992), suggesting a significant anthropogenic increase of Pb even before the 1940's. Soil samples around Scott Base on Ross Island found contamination of silver (Ag) among Cd, Cu, Pb, Zn and As due to improper waste disposal and chemical and fuel spillage (Sheppard *et al.*, 2000). In sediment profiles taken from Admiralty Bay, Antarctica, evidence of As (2-12 ppm), Cd (0.4-0.9 ppm), chromium (Cr) (2-12 ppm), nickel (Ni) (3-10 ppm) and Pb (3-11 ppm) as well as high concentrations of Cu (47-84 ppm) and Zn (44-89) were found to be sourced from activities associated within the Bay that houses three scientific stations all of which utilise fossil fuel as an energy source (Ribeiro *et al.*, 2011). A study in Roosevelt Island, Antarctica reported findings of heavy metals Pb, manganese (Mn) and Fe in the soil, water and snow (Tuohy *et al.*, 2015).

The Protocol on Environmental Protection to the Antarctic Treaty provides stringent guidelines to protect the Antarctic's natural environment and established obligations for all human activities in Antarctica and the surrounding Southern Ocean. However, events like global warming, increasing demand and aggressive, industrial advancement in countries of the Southern Hemisphere are likely to increase the impact of anthropogenic pollution to the Antarctic ecosystems. In San Jorge Bay in Northern Chile, reports on the distribution of Cu and Pb along the bay's coast have been made evidenced from notorious industrial activity (Valdés *et al.*, 2011). Meanwhile, along the South Australian coastline, varying degrees of metal contamination (As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn) were found

in the seawater with highest levels of Cd, Pb, and Zn being verified (Chakraborty and Owens, 2014). Many countries implement clear and unambiguous guidelines for heavy metal concentrations in surface water (US EPA, 2002; EU, 2000). Unfortunately, there are no accepted international or local standards of metal levels in marine sediments. Only few countries such as the Netherlands and Canada have a long-standing, legislative institution in developing criterias and regulations for sediment quality (Milenkovic *et al.*, 2005).

Cu and Zn are essential to life forms while others like Pb, Cd and Hg have no known biological significance (Allen, 1997). Many recent studies have highlighted the adverse impacts of metal contaminants on invertebrates and cryptogams. Heavy metals can accumulate and biomagnify through the food chain, affecting the higher trophic levels (Chu *et al.*, 2019). Elevated traces of Hg were found in bird feather and mammalian hair, which further indicated biomagnification of metals (Santos *et al.*, 2006). Benthic macroalgae have the capacity to accumulate various concentrations of heavy metals from the environment (Chakraborty and Owens, 2014). Penguins are regarded as the inhabitants the Southern Hemisphere exclusively and studies of trace elements in penguins have been proved to be almost always valuable. Penguins make up 90% of the bird biomass of the Southern Ocean and play a role in transporting pollutants from coastal to terrestrial systems (Celis *et al.*, 2018). A study by Chu *et al.* (2018) compared the concentrations of heavy metals between Fildes Peninsula, Antarctica, which is densely populated with scientific stations and the remote Ardley Island. High amount of contaminants was found in Ardley Island, indicating a powerful impact of penguins in transporting anthropogenic pollutants across Antarctica outweighing even human activities near some research stations (Chu *et al.*, 2018).

Microbiological approaches have attempted to broaden the understanding of heavy metal resistant microorganisms to accompany the effort of a successful bioremediation (Balagurusamy, 2005; Verasoundarapandian *et al.*, 2019). In cold climatic regions, cold adapted microorganism can develop adaptation strategies to compensate the negative effects of low surrounding temperatures on the biochemical reactions (Margesin *et al.*, 2005; Ahmad *et al.*, 2013; Lee *et al.*, 2018; Zakaria *et al.*, 2019). The microbial community that thrives in natural marine environments is known to likely degrade major

fractions of spilled petroleum, consequently reducing the impacts of oil spills (Atlas and Hazen, 2011). The spotlight now falls onto heavy metal-resistant bacteria to obtain better chances of bioremediation success. In a phenol degradation study, *Arthrobacter bambusae* has been seen able to degrade phenol and tolerate most 1 ppm of heavy metals with the exception of Ag and Cd (Ahmad *et al.*, 2018). Mn-resistant *Rhodococcus erythropolis* have been reported from deep-sea environment (Gillard *et al.*, 2019). A similar study has revealed the tolerance level of *Rhodococcus baikonurensis* to 1 ppm of heavy metals while still being able to degrade phenol (Zakaria *et al.*, 2018).

This current work proposes the kinetic studies 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 (Karamba *et al.*, 2016). However, this study only works with a constant concentration across all heavy metals. Hence, in the presence of heavy metals, the extent of the inhibition of microbial growth and the correlation to the degradation of diesel become the question to be answered by this work.

2 Materials and methods

2.1 Screening of bacteria growth on diesel in the presence of 1 ppm heavy metals

The isolate strain, AQ5-AO1 used for this study was isolated from sea water sample collected from Base General Bernado O'Higgins Riqueulme, Cape Legoupil on the Trinity Peninsula, Antarctica. The bacterial isolate was screened for diesel degrading bacteria from seawater abilities in a standardised Bushnell-Haas (BH) salt medium (Bushnell and Haas, 1941) enriched with 1% (v/v) diesel. BH media has a chemical composition of 0.2 g/L MgSO₄, 1.0 g/L NH₄NO₃, 1.0 g/L KH₂PO₄, 1.0 g/L K₂HPO₄, 0.05 g/L FeCl₃ and 0.02 g/L CaCl₂ in dH₂O, adjusted to pH 8.0 ± and further supplemented with 30 ppt (w/v) NaCl to simulate salinity of Antarctic seawater when the samples were taken. The media was aseptically inoculated with 2% (v/v) standardised inoculum and 1 ppm of heavy metals namely Ag, Al, Cd, Cr, Co, Hg, Ni and Zn. The samples were kept on a

shaking incubator at 150 rpm for 7 d under 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.

2.2 Determination of diesel degradation in the presence of heavy metals

Modified gravimetry method was applied to obtain the weight reduction of diesel that defines the degradation of diesel used for this study. Briefly, the residual hydrocarbon in diesel oil from each culture was extracted using a solvent extraction method by destructive sampling of triplicate flask containing culture. n-Hexane (1:1 media to n-hexane) was used to separate the cellular material. After evaporation, the remaining residual hydrocarbon in diesel was quantified gravimetrically (Patowary *et al.* 2017). Diesel degradation percentage was calculated as thus: (Equation 1).

$$\text{Hydrocarbon degradation(\%)} = \frac{X - Y}{X} * 100 \quad (1)$$

where X = Original mass of diesel

Y = mass of residual diesel oil in test sample

After 7 d culture, the amount of degraded hydrocarbon was analysed in the extracted diesel oil samples. Similarly, bacterial growth was determined by measuring the optical density of the media (OD₆₀₀), which was taken in 24 h periodically. 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. A post hoc Tukey's test was then done to determine the significantly different heavy metal.

2.3 Kinetic study of bacterial growth

The microbial growth profile of was used to obtain specific growth rate termed as specific growth rate (μ_{max}). The μ values of the specific growth rate every 24 h was obtained by plotting $\ln x$ (bacterial dry weight) vs time. These values was plotted against time to plot a nonlinear curve. Taking the log of the growth curve thus focusing on the exponential phase of the curve, a linear regression was performed at the highest peak also termed the maximum growth. An exponential growth model was also fitted to the growth curve using the software Graphpad Prism 5.0. To assess the significance of the difference in bacterial growth and diesel degradation in the presence of

heavy metals, an analysis of variance (ANOVA) was performed using graph pad prism 5.0.

3 Results and discussion

3.1 Heavy metal inhibition towards bacterial diesel degradation

According to Figure 1, heavy metal toxicity towards bacterial growth decreased in the order of Zn > Cr > Cd > Al > Ni > Hg > Co > and Ag. It is important to note that in the presence of 1 ppm Zn, bacterial growth was the highest followed by Cr, Cd and Al, then Ni, Co, Hg and lastly but unsurprisingly, Ag. Ag is a known heavy metal with antibacterial properties (Maynard, 2007). The cytotoxicity of Ag towards bacterial cells stemmed from the Ag particles adhering to bacterial cell walls, thus changing membrane structure. The cell membrane became permeable, allowing Ag to accumulate in the cell and cause DNA damage (Morones *et al.*, 2005). This result is consistent with the studies of Ahmad *et al.* (2018) and Zakaria *et al.* (2018), who reported that 1 ppm of Ag significantly inhibited the growth and phenol degradation activities of *Arthrobacter bambusae* strain A5-003 and *Rhodococcus baikomurensis* strain AQ5-001, respectively. The presence of heavy metals can alter the activities of microbial communities, affecting the enzymes involved in different metabolic activities. The presence of various heavy metals is known to impaired microbial degradation of diesel oil.

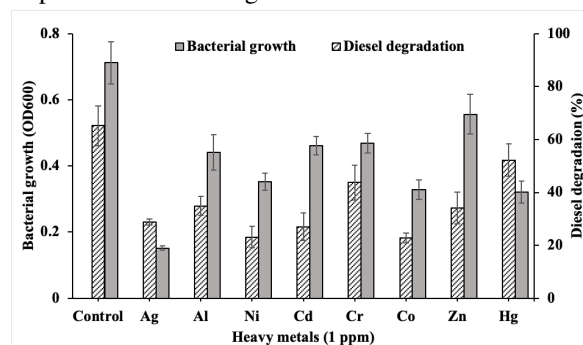


Fig. 1. The effect of 1 ppm heavy metals on the bacterial growth and diesel degradation of Antarctic seawater sample. Growth was carried out at 10 °C using BH media pH 8.0 supplemented with 30 ppt salinity and 1% (v/v) diesel for 7 d. Data represent mean \pm SEM. All data are available in triplicates,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

In the current study, strains AQ5-AO1 continued effective diesel degradation in the presence of 1 ppm concentrations of all the other ions tested (Zn, Cd, Al, Ni, Hg and Cr). This result agrees with Gran-Scheuch *et al.* (2017) who also reported the ability of *Sphingobium xenophagum* D43FB to degrade phenanthrene in the presence of heavy metals present in diesel.

Many microorganisms have developed ways to lessen the impact of heavy metal toxicity, and this is where resistant mechanisms are found (Buendía-González *et al.*, 2019). To date, there are three main types of mechanisms; 1) the flowing out (efflux) of toxic metal ions from bacterial cells, 2) enzymatic transformation of metals, 3) metal-binding proteins incorporating heavy metals into complexes which turns it into something less toxic to the cells (Dziewit and Drewniak, 2016).

Heavy metals can be readily found after oil spills and are bioaccumulated in marine biota. The most frequently found heavy metals post oil spills are Pb > Ni > V > Zn > Cd (Mustafa *et al.*, 2015), which is partly the reason why Ni, Cd and Zn were among the heavy metals of interest for this study. Fossil fuel burning also contributes to the presence of heavy metals in the natural environment (Adriano, 2001). It is worthy to note that the general growth response and degrading abilities may vary in a combined effect of cold temperature, high saline and an alkaline pH with respect to the type of organism and the tested heavy metal (Brown *et al.*, 2017).

Zn is an essential micro-element in the physiology of bacteria. It is used as a catalytic or structural cofactor of not only crucial enzymes but also proteins involved in processes such as protein synthesis and DNA replication (Hantke, 2005). Too much of Zn can however be toxic to bacteria; thus, intracellular levels of Zn are regulated by the bacteria (Hantke, 2005). Bacteria cells are capable of bioaccumulating Zn in the intracellular space. Wei *et al.* (2009) suggested a possibility of an extrusion mechanism that reinforced heavy metal resistance.

Looking at the diesel degradation, inhibition increased in the order of Hg > Cr > Al > Zn > Ag > Cd > Ni > Co. Diesel degraded as much as 34.12% in Zn, 28.70% in Ag, 34.91% in Al, 23.12% in Ni, 27.06% in Cd, 43.71% in Cr and lowest at 22.76% in Co. The highest diesel was degraded in the presence of Hg at 52.23% following Ganesh and Lin (2009) gravimetry method of analysis. Many marine bacteria have been said to be Hg-resistant (De *et al.*, 2008; Deng and Wang, 2012). Hg like Cd plays no known

biological roles in bacteria. These heavy metals are known to accumulate in the cells and impede cell growth (Al Defiery and Reddy, 2014). Regardless of organic or inorganic forms of Hg, cytotoxicity occurs due to the solubility of metal in lipids causing binding of Hg to proteins that contain sulfhydryl groups in cell membranes and enzymes (Robinson and Tuovinen, 1984).

Since bacterial growth seemed inhibited (Figure 1), it can be suggested that there was Hg-resistant taxa inside the sample, while other taxa have been completely inhibited inside the sample. The microbial mechanisms of Hg detoxification were put through volatilisation and known entrapment in the extracellular polymeric substance (De *et al.*, 2008). Independent-culture techniques of hydrocarbon-degrading bacteria have come in agreement that differences in the composition of the microbiome can be inferred when growing on specific hydrocarbons, ultimately suggesting different populations can degrade different hydrocarbons (Garrido-Sanz *et al.*, 2019, Vergenyst *et al.*, 2018).

In Antarctica, high levels of heavy metals such as Cu, Cd and Zn can be found in penguin colonies, which are in favour of the hypothesis of bioaccumulation of heavy metals in bodies and penguins faeces (and other polar animals) when ingesting the heavy metals while feeding in the sea (Espejo *et al.*, 2014). Marine bacteria that can resist high levels of Hg and detoxify Cd have been also reported in a metagenomic analysis by Amer *et al.* (2015). The toxicity of Cd works in the inactivation of dehydratase enzyme where the enzyme activity is arrested upon binding and leads to a stop in key metabolic functions, finally leading to bacterial growth inhibition (Xu and Imlay, 2012). The possibility of intracellular bioaccumulation of Cd by the bacteria is possible, as reported by several authors (Durve *et al.*, 2013; Liu *et al.*, 2019) who related it to an increased in biomass and cell growth (Chiboub *et al.*, 2016).

Cr has been established to be biologically significant to living organisms at all levels. However, at high concentrations of heavy metals, Cr inhibits biodegradation of organic pollutants. According to Ross *et al.* (1981), at 1 ppm, Cr²⁺ is more toxic to Gram-negative bacteria than Gram-positive bacteria. Many genera of microbes like *Bacillus*, *Escherichia* and *Pseudomonas* can bioabsorb and bioaccumulate Cr in bioremediation of metal-contaminated soil and water (Kotas and Stasicka, 2000). Cr removal by the bacteria was attributed to the cellular growth of these organisms (Ray and Ray, 2009).

Ni is known to inhibit bacterial utilisation of petroleum hydrocarbons in contaminated soils (Al-Saleh and Obuekwe, 2009). However, Ni and Co are also the cofactors of bacterial growth and metabolism. Valentine *et al.* (1996) reported on growth associated with uptake of Ni and Co at low concentrations where rapid, pH-binding of Ni and Co occurred before the bioaccumulation of heavy metals intracellularly in the cytoplasm.

Large concentrations of heavy metals block the growth of cells, thus reducing their respiration rate (Dai *et al.*, 2004). However, Asgeir *et al.* (2004) determined that after the initial inhibition of cells by heavy metals, the structural content and function of the cells change. The appearance of the species was also in relation to the tolerance of metals. These metal-tolerant species may replace the more sensitive cells, thus changing the microbial's dynamic and community composition. However, some authors analysed that some metals at low concentrations of heavy metals were able to increase the respiration rate of cells (Giller *et al.*, 1998). There are two key elements for an effective utilisation of hydrocarbons by bacteria; the responsible degradation enzymes and the regulatory elements that control the expression of the specific operons to ensure a more productive yield (Oyetibo *et al.*, 2013). Nies (1999) suggested that the bacteria being studied must adopt one or more mechanisms of heavy metal resistance to sequester while harnessing some homologous enzymes to metabolise the petroleum hydrocarbons. Thus, this study highly recommended the conventional bioremediation strategy towards an environment with the co-existence of both heavy metals and hydrocarbons where a bacterial consortia of metal resistant bacteria and hydrocarbon degraders can be useful to tackle both issues simultaneously. In addition, metal-resistant bacteria were introduced to sequester the toxic metals to non-toxic level for the degraders to better breakdown the hydrocarbons.

3.2 Statistical analysis of diesel degradation in the presence of heavy metals

In this study, a one-way analysis of variance (ANOVA) was performed because only one factor remained independent throughout the entire study; heavy metal. Table 1 shows the results of the one-way ANOVA for bacterial growth in all eight heavy metals and control sample.

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

ANOVA Table	SS	df	MS	P value	F value	R ² value
Treatment (between columns)	0.331	8	0.04148			
Residual (within columns)	1.131	63	0.01795	0.0306	2.310	0.2268
Total	1.463	71				

Table 2. One-way ANOVA table of diesel degradation.

ANOVA Table	SS	df	MS	P value	F value	R ² value
Treatment (between columns)	4148	8	518.5			
Residual (within columns)	1577	18	87.61	0.0009	5.919	0.7246
Total	5725	26				

Statistically, the most significant difference ($P < 0.05$) between the control and the heavy metal samples lied in the Ag sample. There was no statistically significant difference between Al, Cd, Cr, Co, Ni, Hg and Zn.

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 2.10 in the chi-square table when the degrees of freedom of numerator and denominator were 8 and 63, respectively, at the α error level of 0.05. As the observed F test statistic value was 2.310 larger than the critical value, the result may be interpreted as statistically having significant difference among the means of the groups at the α error level of 0.05. The ANOVA results suggested 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 another group means.

Table 2 shows the one-way ANOVA of diesel degradation. The test statistic (F value) given here was 5.919 with P value of 0.0009 where 95% significance in difference was tested. The F critical value for Table 2 was 2.51, which was smaller than the generated F test statistic. The null hypothesis was rejected, thus supporting that there is a significance in at least one of the group means. A post hoc Tukey's test revealed that diesel degradation between the control sample and samples in Ag, Al, Ni, Co, Cd and Zn was statistically significant ($P < 0.05$).

3.3 Growth and degradation kinetics

Table 3 describes the results of linear and nonlinear regression of the bacterial growth in eight heavy metals and a biotic control sample. The exponential

growth equation describes the growth with a constant doubling time. The exponential growth equation was written as in Equation 2.

$$X = X_0 e^{\mu t} \quad (2)$$

X_0 is the value of Y(OD₆₀₀) at time zero

μ is the rate constant, unit (d^{-1})

Tau is the time constant.

Doubling time (T_d): $\ln 2 / \mu$

In the linear scale of the graph, the exponential model equation can also be written as in Equation 3.

$$\ln X = \ln X_0 + \mu t \quad (3)$$

X_0 = Value of Y(OD₆₀₀) at time zero

μ = specific growth rate

t = time with reference to X

Fitness in microbiology is broadly speaking, the ability of microbes to thrive in a viable, competitive environment. It is described as the comparison of growth rates of different microbial strains or species of a mixed culture. Determining the microbial fitness has extensive usage in microbial genetics, biotechnology and evolution. Estimations from growth curves can be done are the simplest ways that allows high throughput. A nonlinear regression with the Exponential growth equation was used as fitting of the data. This step was to minimise the sum of squares (SS) of differences between the predicted and measured values. In predictive microbiology, the data collected from the shape of a growth curve is a fundamental aspect. Bacterial growth curves can be found in many areas of disciplines where lag phase, asymptotic phases, specific growth rates are important. All microbial growth follow the first order of kinetics.

Table 3. Parameters from exponential growth equation.

	Control	Ag	Al	Cd	Cr	Co	Hg	Ni	Zn
Nonlinear Regression									
X_0	0.1477	0.1755	0.1302	0.1513	0.1404	0.1574	0.1984	0.1684	0.2069
μ (h^{-1})	0.2553	0.004896	0.1695	0.1505	0.1722	0.09459	0.04113	0.1191	0.1399
Tau	3.917	204.2	5.899	6.644	5.809	10.57	24.31	8.393	7.147
Doubling time (T_d)	2.715	141.6	4.089	4.605	4.026	7.328	16.85	5.818	4.954
Degrees of Freedom	22	22	22	22	22	22	22	22	22
R^2	0.8852	0.001957	0.6545	0.7496	0.8488	0.5085	0.1607	0.447	0.5402
Absolute Sum of Squares	0.1973	0.04947	0.1228	0.06944	0.05251	0.05258	0.05814	0.1573	0.2809
Goodness of Fit of Linear regression									
F	127.3	0.08429	17	37.45	58.3	19.51	1.281	10.99	8.443
DFn, DFd	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
P value	< 0.0001	0.7753	0.0008	< 0.0001	< 0.0001	0.0004	0.2744	0.0044	0.0103
R^2	0.8884	0.005241	0.5151	0.7007	0.7847	0.5494	0.07412	0.4071	0.3454

Kinetic parameters that are of interest to many authors include 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 (Feher *et al.*, 2012) and inhibition of biofilm production (de la Fuente-Nunez *et al.*, 2012). The purpose of measuring the growth rate is to determine the rate of change in cell number per unit time (min, h and d).

In Figure 2, it is observed that the microbial growth was taken every 24 h for 7 d. The growth curve of the bacteria that revealed bioactivity began 2 d after inoculation and peaked from 5 to 6 d. After 7 d of incubations, the highest growth was reached in the presence of Zn. Statistically however, the most significant difference ($P < 0.05$) between the control and the heavy metal samples lied in the Ag sample. There was no statistically significant difference between Al, Cd, Cr, Co, Ni, Hg and Zn. In all eight heavy metals studied, a lag phase was observed at the beginning from day 0 to day 2 before the microbial growth started increasing all the way to day 6. The presence of a lag phase indicated the adjustment period of the cells to the toxicity of the heavy metals.

Ideally, bacteria growth consists of lag phase, exponential, stationary, and decline and/or death phase. The first phase observed is the lag phase when the growth rate is essentially zero, which is defined as a transition to the exponential phase after the initial population has doubled (Yates and Smotzer, 2007). In Figure 2, the lag phase can be seen from the graph which indicates that between days 0 and 2, there is no increase in bacterial growth through turbidity (OD_{600})

The lag phase is thought to be caused by the cells physiologically adapting to the new culture conditions and low initial density of inoculum. According to Sengor *et al.* (2009), heavy metals create the microbial onto the metabolic activities of growing cells.

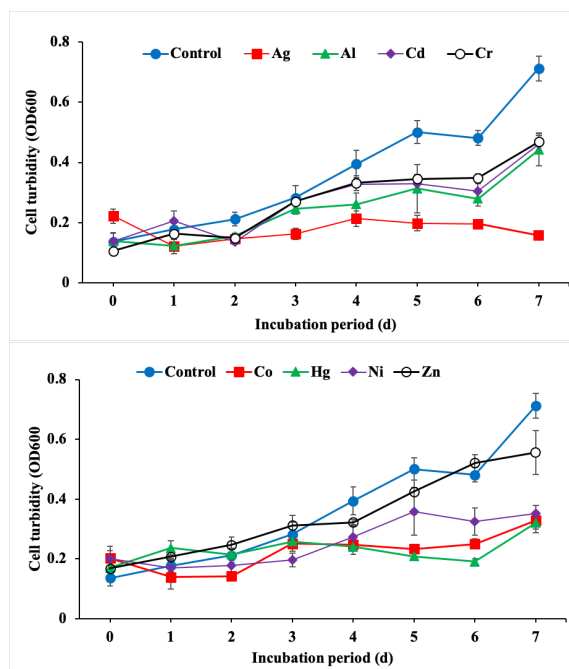


Fig. 2. The microbial growth curve of sample in optimised media supplemented with 1 ppm a) Al, Ag, Cd, Cr and positive control b) Co, Hg, Ni, Zn and positive control. Growth was carried out at 10 °C using BH media pH 8.0 supplemented with 30 ppt salinity and 1% (v/v) diesel for 7 d. Data represent mean \pm SEM. All data are available in triplicates, $n = 3$.

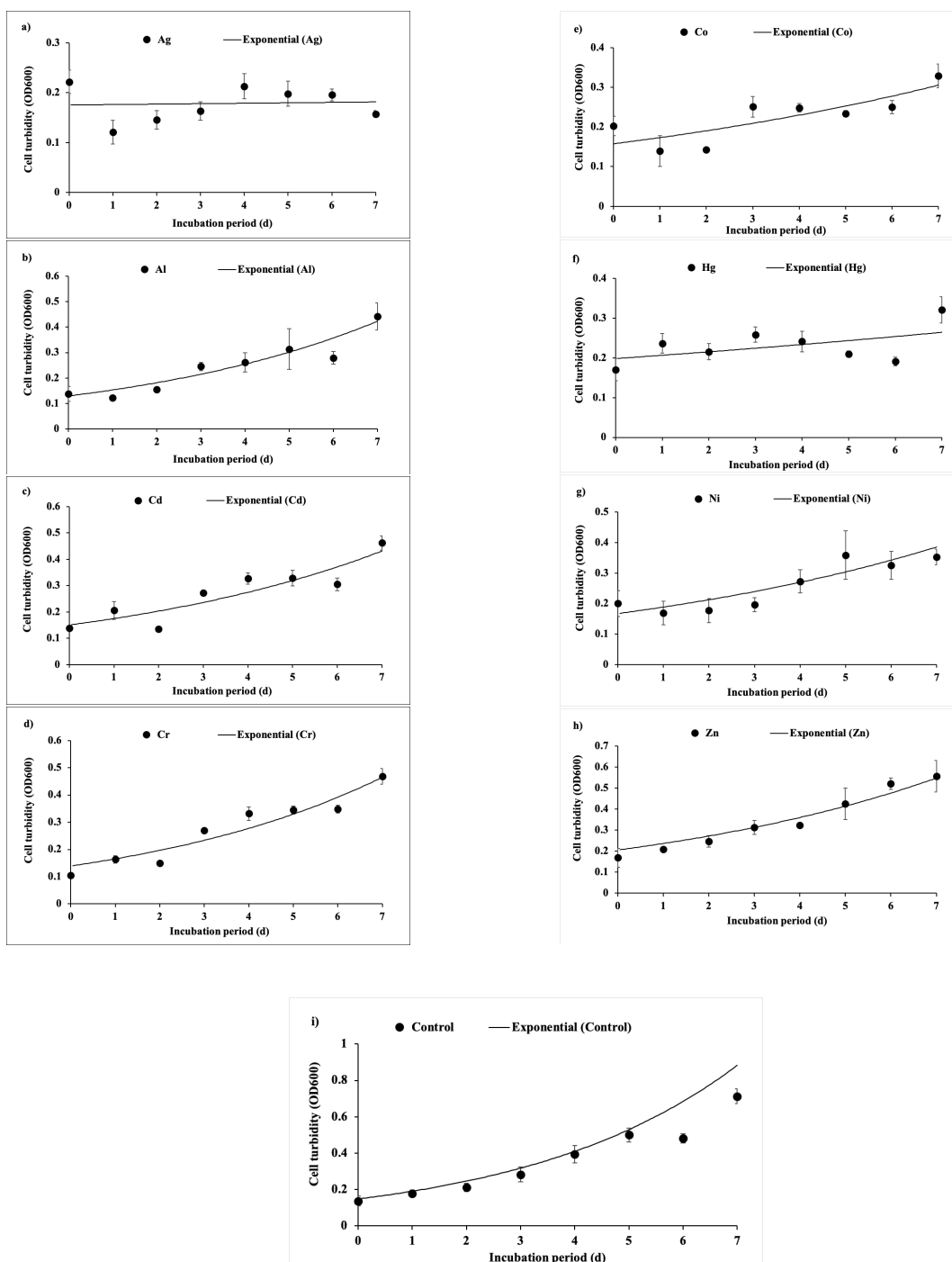


Fig. 3. The nonlinear regression model using exponential growth equation of (a) 1 ppm Ag with specific growth rate, $\mu = 0.004896 \text{ d}^{-1}$, (b) in 1 ppm Al with specific growth rate, $\mu = 0.1695 \text{ d}^{-1}$. (c) Sample in 1 ppm Cd with specific growth rate, $\mu = 0.1505 \text{ d}^{-1}$, (d) Sample in 1 ppm Cr with specific growth rate, $\mu = 0.1722 \text{ d}^{-1}$, (e) Sample in 1 ppm Co with specific growth rate, $\mu = 0.09459 \text{ d}^{-1}$ (f) Sample in 1 ppm Hg with specific growth rate, $\mu = 0.04113 \text{ d}^{-1}$ (g) Sample in 1 ppm Ni with specific growth rate, $\mu = 0.1191 \text{ d}^{-1}$ (h) Sample in 1 ppm Zn with specific growth rate, $\mu = 0.1399 \text{ d}^{-1}$ (i) Sample in biotic control with specific growth rate, $\mu = 0.2553 \text{ d}^{-1}$ All data are available in triplicates, $n = 3$. The straight line represents the exponential model $X_0 e^{\mu t}$ fitted to the data points. All data points represent mean \pm SEM.

Coming to the point of interest of many scientific works, growth curve studies lie in the exponential phase also known as a log phase. Exponential growth produces a J-shaped curve, while logistic growth produces a famously termed sigmoid curve or simply an S-shaped curve. Exponential growth may happen for a while if there are few cells in a substrate-rich environment. But when the cell numbers get large enough, resources will start to get used up, thus slowing the growth rate. Eventually, the growth rate will plateau or level off, making an S-shaped curve.

A valuable kinetic parameter of the growth curve is the maximum specific growth rate (μ_m). Zwietering *et al.* (1990) describes this parameter as the slope of the line when the bacteria grow exponentially. This parameter is estimated by subjectively deciding which part of the curve is decidedly linear (logging the y axis) and then determining the slope by linear regression. 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 growth of the bacteria in the presence of 1 ppm heavy metals is related to their antecedent resistance (Oyetibo *et al.*, 2013; Oyetibo *et al.*, 2010).

Figure 3 a, b, c, d, e, f, g, h and i reveal the nonlinear regression using exponential growth model on same sample conditions of 1 ppm Ag, Al, Cd, Co, Cr, Hg, Ni, Zn and a biotic control. The control flask showed a specific growth rate, $\mu = 0.2553 \text{ d}^{-1}$. Out of all eight heavy metal conditions, Figure 3d, in the presence of 1 ppm Cr, generated the highest specific growth rate $\mu = 0.1722 \text{ d}^{-1}$ followed by Figure 3b, Al with $\mu = 0.1695 \text{ d}^{-1}$. Low specific growth rates were found in 1 ppm Hg, $\mu = 0.04113 \text{ d}^{-1}$ with the lowest coming from Ag with a specific growth rate, $\mu = 0.004896 \text{ d}^{-1}$. These low values thus presenting the inhibition effect of Ag and Hg towards the growth of the bacteria. The growth rates decreased in the order of Cr>Al>Cd>Zn>Ni>Co despite the values of bacterial growth in decreasing order of heavy metals Zn>Cr>Cd>Al>Ni>Hg>Co>Ag (Figure 1).

Growth curves describe the turbidity of cell populations in a liquid culture over a certain period of time. Cell absorbance and density is interchangeable in many work but this study opted for the theoretically correct term for cell growth; cell turbidity. The simplest way to deduce the microbial fitness from growth curves is by assessing the growth rate during the exponential growth phase. This can be observed at the slope of the log of the growth curve. However,

exponential growth rates do not completely attain the dynamics of typical growth curves because the data is only evaluated at the exponential phase. Many literatures across the decade have used other mathematical models like the Logistics model (Nagel *et al.*, 1999), Baranyi-Roberts model (Ram *et al.*, 2019) and modified Gompertz model (Giotta *et al.*, 2006) for pure cultures. Many of those also performed growth studies on monocultures rather than mixed bacterial consortium (Gikas, 2008; Sengor *et al.*, 2009). Growth estimation in mixed culture or consortium has to incorporate competition parameters (Ram *et al.*, 2019). However, fitting an exponential model to the exponential growth phase can always be used as a benchmark for further studies. Markwiese and Colberg (2000) proposed a nonlinear regression model incorporating a lag time before microbial activity increased. The microbial lag is crucial because disregarding it may result in significant overestimation of the substrate (diesel) being degraded (Wood *et al.*, 1995). This is especially valuable for predicting bioremediation in metal-contaminated environmental systems. A microbial lag time created by heavy metals towards the bacterial growth is in due response to substrate availability which has not been incorporated into microbial kinetic models (Sengor *et al.*, 2009). More data can be collected over a series of time, where the time taken is more frequent to acquire maximum growth rate. Further studies can also be done when knowing the time of inflection and can incorporate the differences of heavy metal concentrations to evaluate more inhibition kinetics in accordance to bacterial growth relying solely on diesel.

Conclusions

As a summary of this work, it is worthy to note that the marine bacteria can resist heavy metals in the marine ecosystem of Antarctica. These bacteria may be able to cope and/or adapt to the presence of pollutants. The present study confirmed strain AQ5-AO1, a cold-adapted Antarctic diesel-degrading marine bacterium was capable of tolerating exposure to the heavy metals Al, Cd, Co, Cr, Hg, Ni, and Zn, which are common co-pollutants in diesel pollution events. This finding supports that the bacterial strain have potential to contribute to the bioremediation of diesel oil polluted soils and wastewaters in Antarctica and other cold environments even in the presence of some heavy metals. The metals Ag strongly inhibited diesel

degradation by strain AQ5-AO1, at concentrations of 1 ppm.

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