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Statistical Assessment of Phenol Biodegradation by a Metal-Tolerant Binary Consortium of Indigenous Antarctic Bacteria

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Abstract: Since the heroic age of Antarctic exploration, the continent has been pressurized by multiple anthropogenic activities, today including research and tourism, which have led to the emergence of phenol pollution. Natural attenuation rates are very slow in this region due to the harsh environmental conditions; hence, biodegradation of phenol using native bacterial strains is recognized as a sustainable remediation approach. The aim of this study was to analyze the effectiveness of phenol degradation by a binary consortium of Antarctic soil bacteria, *Arthrobacter* sp. strain AQ5-06, and *Arthrobacter* sp. strain AQ5-15. Phenol degradation by this co-culture was statistically optimized using response surface methodology (RSM) and tolerance of exposure to different heavy metals was investigated under optimized conditions. Analysis of variance of central composite design (CCD) identified temperature as the most significant factor that affects phenol degradation by this consortium, with the optimum temperature ranging from 12.50 to 13.75 °C. This co-culture was able to degrade up to 1.7 g/L of phenol within seven days and tolerated phenol concentration as high as 1.9 g/L. Investigation of heavy metal tolerance revealed phenol biodegradation by this co-culture was completed in the presence of arsenic (As), aluminum (Al), copper (Cu), zinc (Zn), lead (Pb), cobalt (Co), chromium (Cr), and nickel (Ni) at concentrations of 1.0 ppm, but was inhibited by cadmium (Cd), silver (Ag), and mercury (Hg).

Keywords: cold climate; pollution; statistical optimization; mixed culture; metal ion

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1. Introduction

Antarctica and its surrounding Southern Ocean are widely considered to be among the Earth's last pristine wildernesses, compared with the rest of the world, and has only recently been discovered and subsequently impacted by humans. The negotiation of the Antarctic Treaty in 1959, which came into force in 1961, declared that this 'white canvas' was to be a land of science, research, and peaceful cooperation between nations. Since then, the continent has seen inexorable growth in scientific exploration, numbers, and extent of research stations, research activities, marine transportation and, more recently,

tourism [1,2]. Human presence and activity present a distinct set of threats to the Antarctic environment, one of which is pollution [3,4]. Despite the strict guidelines provided in the Protocol on Environmental Protection to the Antarctic Treaty to protect the Antarctic environment, there remains potential for significant marine and terrestrial impacts at various scales due to accidents such as shipwrecks and pollution associated with research activities and research stations [2]. Historical and, in some cases, ongoing application of inappropriate waste management practices have led to the release of contaminants both to the surrounding terrestrial and near-shore aquatic environments [5–7].

The primary routes by which phenol has entered the Antarctic environment are through oil spills and inappropriate waste management practices. The most recent major oil spill in the marine environment took place in November 2007 when the cruise vessel, MS Explorer, carrying 1200 L of petrol, 24,000 L of lubricant oil, and 190,000 L of marine gas oil was holed by ice and sank soon after in the Bransfield Strait between the Antarctic Peninsula and South Shetland Islands [8]. Further diesel spills have occurred at research stations, such as at the Argentinian Carlini Station, King George Island, in 2009 [9]. Following such spills, POPs, including phenol, can be transported more widely through physical and chemical processes, resulting from the addition of dispersants, subsequent shoreline erosion, water washing, or volatilization, while retaining their toxicity [10]. In addition to catastrophic marine accidents, refueling operations for stations on land, pipeline faults within the stations' transport, and smaller-scale operations such as refueling of vehicles are the sources of the majority of terrestrial fuel spills [11]. Phenol is one of the major components used in the production of diesel fuel [12] and Singh et al. [13] reported that petroleum wastewater containing 140–480 mg/L of oil included phenol at concentrations ranging from 1.2 to 3.71 mg/L. Moreover, discharging lightly treated grey water into the sea from coastal research stations and, historically, also onto ice and other parts of the local environment from inland stations, can lead to phenol pollution [14], as phenol is one of the main components in many household and pharmaceutical products [15,16]. Phenol and phenol-derived pollutants such as vanillic acid, homovanilic acid, syringic acid, p-coumaric acid, and polychlorinated biphenyls are persistent in the Antarctic environment due to the continent's frigid and often dry climate, limiting the rates of both biological processes and abiotic degradation [17–19].

The hazardous effects of phenol, even at low concentrations in the environment, are more than sufficient to justify mitigation. In recent decades, research into the treatment of phenol pollution has gained momentum [20,21]. Remediation in polar regions has traditionally relied upon excavation and removal of polluted soil, which may still be appropriate in instances of severe contamination. However, this type of remediation has a considerable direct impact on the Antarctic environment and can result in soil shrinkage, land slumping, permafrost melt, or alteration of groundwater flow. Such approaches are also expensive and logistically challenging [22]. Therefore, bioremediation using native microorganisms has attractive potential as a sustainable approach for restoring contaminated sites. Polluted wastewater is often compounded by the presence of various heavy metals as co-contaminants, which can have further adverse effects on the function and availability of microorganisms [23–26]. Concordantly, traces of heavy metals were detected in the wastewater discharged from various Antarctic research stations [27,28]. Thus, heavy metal toxicity may compromise the biodegradation of phenol. Studies of the relationship between bacterial tolerance to heavy metals and degradation of phenolic compounds are, therefore, an important element of the development of bioremediation applications.

In recent years, studies using mixed microbial cultures have attracted increasing research attention, especially recognizing the potential role of cooperative or synergistic effects result from the possession of different but complementary biochemical degradation pathways in the different strains present [29]. A range of studies are now available using mixed culture methods, encouraging investigation of the application of this approach in Antarctica using native microorganisms. Nevertheless, several limitations exist, such as the limited capability of microbial species in the field and the absence of benchmark values

against which to test the technique's effectiveness for extensive field application [30]. In addition, the effectiveness of biodegradation depends on several external factors such as temperature, pH, nutrients availability, salinity, and site characteristics [21,31]; hence, investigation of effect of these factors is crucial to indicate the optimal conditions that play a role in bioremediation. A statistical approach using design of experiment (DoE) tools is efficient and versatile for the optimization of processes comprising multiple variables as it reduces the number of experimental trials and identifies the influence of individual factors, in addition to the interaction effects on the desired response [32,33]. Central composite design (CCD) and response surface methodology (RSM) comprising factorial designs and regression analyses are some of the DoE tools used to design such experiments, evaluate the significant factors, and build models to reveal optimum conditions for the biodegradation process [34].

The present study focuses on the statistical evaluation of phenol degradation by a binary consortium of the Antarctic soil bacteria, *Arthrobacter* sp. strain AQ5-06, and *Arthrobacter* sp. strain AQ5-15 in combination with investigation of the consortium's tolerance of exposure to different heavy metals.

2. Materials and Methods

2.1. Bacterial Culture, Maintenance and Media Preparation

Arthrobacter sp. strains AQ5-06 (KX946127) and AQ5-15 (MK744046) from King George Island, which were previously isolated and identified as diesel and phenol-degrading bacteria [35–37], and maintained in glycerol stock under −80 °C at the Eco-Remediation Technology Laboratory, Universiti Putra Malaysia, were used in this study. These two strains were inoculated individually in nutrient broth (NB) and incubated at 10 °C at 150 rpm for 96 h. Then 1 mL culture broth was transferred to degradation culture medium containing 0.5 g/L phenol and incubated for 96 h. Repeated transfers were performed several times under the same conditions as earlier to acclimatize the cultures. Minimal salt medium (MSM) (pH 7.5) was prepared by adding (g/L) K_2HPO_4 (0.4), KH_2PO_4 (0.2), NaCl (0.1), Mg_2SO_4, (0.1), $(NH_4)_2SO_4$ (0.4), $MnSO_4 \cdot H_2O$ (0.01), $Fe_2(SO_4) \cdot H_2O$ (0.01), and $NaMoO_4 \cdot H_2O$ (0.01). Phenol was added after autoclaving the medium at 121 °C for 20 min. The working quantity of phenol medium (PM) was 50 mL for all experiments conducted in 250 mL Erlenmeyer flasks.

2.2. Phenol Biodegradation

A binary consortium of strains AQ5-06 AQ5-15 with initial inoculum size of OD_{600} nm $\approx 1.00 \pm 0.10$ was prepared by blending them to a final concentration of 10.0% (v/v) and inoculating them into the PM prior to incubation. Phenol medium without bacterial inoculation served as a control sample. Phenol degradation was assessed using the 4-aminoantipyrine assay following the American Public Health Association method and analyzed by UV-*vis* spectrophotometry at 510 nm [38]. Optical density at 600 nm was used as a proxy measure of bacterial growth [39]. In brief, 1 mL of the culture medium was centrifuged at 12,000× *g* for 15 min. The supernatant was separated from the pellet and further used in the 4-AAP assay, while the pellet was diluted with 1 mL sterile distilled water and used to measure bacterial growth. All experiments were carried out in triplicate in a batch shake flask. The percentage of phenol degradation was calculated using Equation (1):

$$\text{Phenol degradation } (\%) = \frac{x_2 - x_1}{x_2} \times 100\% \quad (1)$$

where x_2 is the initial phenol concentration and x_1 is the residual phenol concentration.

2.3. Design of Experiment (DoE) by Response Surface Methodology (RSM)

The central composite design (CCD)-based RSM is a statistical tool widely used for investigational design, modeling, and optimization. In this study, the experimental design

and statistical analyses were generated using Design-Expert® version 6.0.8 (Stat-Ease Inc. Minneapolis, MN, USA) to optimize the culture conditions. In total, 30 runs with four significant variables were considered at five different levels (−2, −1, 0, +1, +2) containing 16 factorial points, eight axial points, and six replications at the center point (Table 1). The experimental values' range of each factor was selected based on the optimum range of pure culture. Phenol degradation was used as the response and the significance of the model and each coefficient in the equation were examined using Fischer's F-test and ANOVA. Upon completion of DoE, biodegradation of phenol was carried out in the batch culture to obtain an appropriate model. The model was validated by comparing the predicted response with experimental data.

Table 1. Experimental ranges of four different variables tested in CCD.

Variables	Symbol	Experimental Values				
		−2	−1	0	+1	+2
(NH4)2SO4 concentration (g/L)	A	0.2	0.3	0.4	0.5	0.6
NaCl concentration (g/L)	B	0.08	0.10	0.13	0.15	0.17
pH	C	6.75	7.00	7.25	7.50	7.75
Temperature (°C)	D	7.0	10.0	12.5	15.0	17.50

2.4. Heavy Metal Tolerance

Tolerance of exposure to the heavy metals arsenic (As), aluminum (Al), silver (Ag), copper (Cu), cobalt (Co), chromium (Cr), cadmium (Cd), lead (Pb), nickel (Ni), zinc (Zn), and mercury (Hg) was evaluated separately at a concentration of 1.0 ppm in MSM containing 0.5 g/L phenol. Pure cultures of strain AQ5-06, strain AQ5-15, and the binary consortium were inoculated individually and incubated under optimized conditions at 150 rpm. A quantity of 10.0% (v/v) of bacterial strain was inoculated into MSM in the absence of heavy metals to act as control [40]. Data analysis for dose-response inhibition was performed using GraphPad Prism version 9.0.2 and relative inhibition was plotted vs. the heavy metal concentration and fitted to a standard inhibition dose-response curve to generate a half maximal inhibitory concentration (IC_{50}) value [41].

3. Results and Discussion

3.1. Statistical Optimization of Phenol Degradation Using RSM

Prior to statistical analysis, the effect of inoculum ratio of the two strains was determined by adding, to 50 mL MSM containing 0.5 g/L of phenol, the acclimatized cell cultures at varying amounts in such a way as to obtain AQ5-06:AQ5-15 ratios of 1:1, 1:3, and 3:1. In each of the three cases, there was no apparent lag, but a better growth was observed when the inoculum ratio was 1:1 (Table 2). Consortia with inoculum ratios of 1:3 and 3:1 have both slower growth and degradation rates. In agreement, Tecon and Or [42] also reported that strains mixed in equal fractions are expected to have optimal mutualistic interaction. The active performance of the selected binary consortium was confirmed by comparing the outcome with the control sample. Pure culture of strain AQ5-06 was reported to completely degrade 0.5 g/L of phenol within 120 h [35], and strain AQ5-15 to completely degrade 0.5 g/L of phenol within 108 h [36]. As shown in Table 2, these two strains performed better as the co-culture favored convergence of 1:1 partner ratio.

Table 2. Time taken for binary consortium with different inoculum ratios to completely degrade 0.5 g/L phenol at 10 °C.

DMC	Inoculum Ratio	Growth Rate (h^{-1})	Incubation Time (h)
AQ5-06 + AQ5-15	1:1	0.0191 (±0.0263)	48
	1:3	0.0106 (±0.0017)	84
	3:1	0.0121 (±0.0227)	84

The investigational results of phenol biodegradation by the binary consortium were examined through RSM to generate an empirical model. Based on these outcomes, the relationship between the response and independent variables was identified and is described by the second-order polynomial equation (Equation (2)):

$$Y = +94.78 - 0.022x_1 - 1.47x_2 + 1.26x_3 + 8.12x_4 - 4.45{x_1}^2 - 4.67{x_2}^2 - 4.59{x_3}^2 - 13.12{x_4}^2 \quad (2)$$

where Y is the phenol degradation percentage, x_1, x_2, x_3, and x_4 are the corresponding independent variables $(NH_4)_2SO_4$ concentration (g/L), NaCl concentration (g/L), pH, and temperature (°C), respectively. These four factors were employed based on the RSM analysis undertaken in previous studies on pure cultures of strains AQ5-06 and AQ5-15 [43,44]. Clarke [45] noted that a well-balanced medium comprising carbon and nitrogen is essential for cell growth and maintenance, and Varjani and Upasani [46] found that nutrient availability, especially nitrogen demand, is crucial in hydrocarbons' biodegradation. A tolerable level of NaCl has a positive effect on the biological performance of microorganisms and exceeding that level can negatively affect the cell numbers and distribution, thus reducing the rate of microbial metabolism [47]. Most microbes have lower tolerance to high or low pH due to the significant effect on the biochemical reactions required for biodegradation [48]. Finally, temperature has a direct influence on the microbial growth rates and metabolic processes, and can affect the physical state of pollutants [46].

Table 3 shows the ANOVA results of CCD on phenol degradation by the binary consortium. The overall model was well supported and there is significant correlation between the factors and response. One linear term D, and all four quadratic terms A^2, B^2, C^2, and D^2, were significant for phenol degradation. This indicated that the temperature has the most significant effect on phenol degradation by this binary consortium. Although the linear terms A, B, and C were not significant, significant second orders of these factors indicated that they affect the response. The insignificant "lack of fit" value and high coefficient of determination (R^2) and adjusted R^2 values confirm the model provides an appropriate fit to the experimental data.

Table 3. Analysis of variance (ANOVA) of phenol degradation by the binary consortium.

Source	Sum of Squares	DF	Mean Square	*F*-Value	Prob > *F*
Model	6879.31	8	859.91	37.51	<0.0001 ***
A	0.01	1	0.012	5.3×10^{-3}	0.9819
B	51.92	1	51.92	2.26	0.1472
C	38.35	1	38.35	1.67	0.2099
D	1581.13	1	1581.13	68.97	<0.0001 ***
A^2	544.17	1	544.17	23.74	<0.0001 ***
B^2	598.61	1	598.61	26.11	<0.0001 ***
C^2	577.34	1	577.34	25.18	<0.0001 ***
D^2	4718.10	1	4718.10	205.80	<0.0001 ***
Residual	481.43	21	22.93		
Lack of Fit	378.55	16	23.66	1.15	0.4773
Pure Error	102.88	5	20.58		
Cor Total	7360.74	29			
Standard deviation		4.79	**R^2**		0.9346
Mean		73.31	**Adjusted R^2**		0.9097
Coefficient variance		6.53	**Predicted R^2**		0.7573
PRESS		1786.11	**Adequate Precision**		26.1944

A: Ammonium sulphate concentration (g/L); B: Sodium chloride concentration (g/L); C: pH; D: Temperature (°C). *** $p < 0.001$.

Figure 1a indicates the close similarity between the predicted and experimentally achieved value for phenol degradation by the binary consortium, and Figure 1b reveals no indication of non-normality, confirming the quadratic model is satisfactory for the purpose of this analysis.

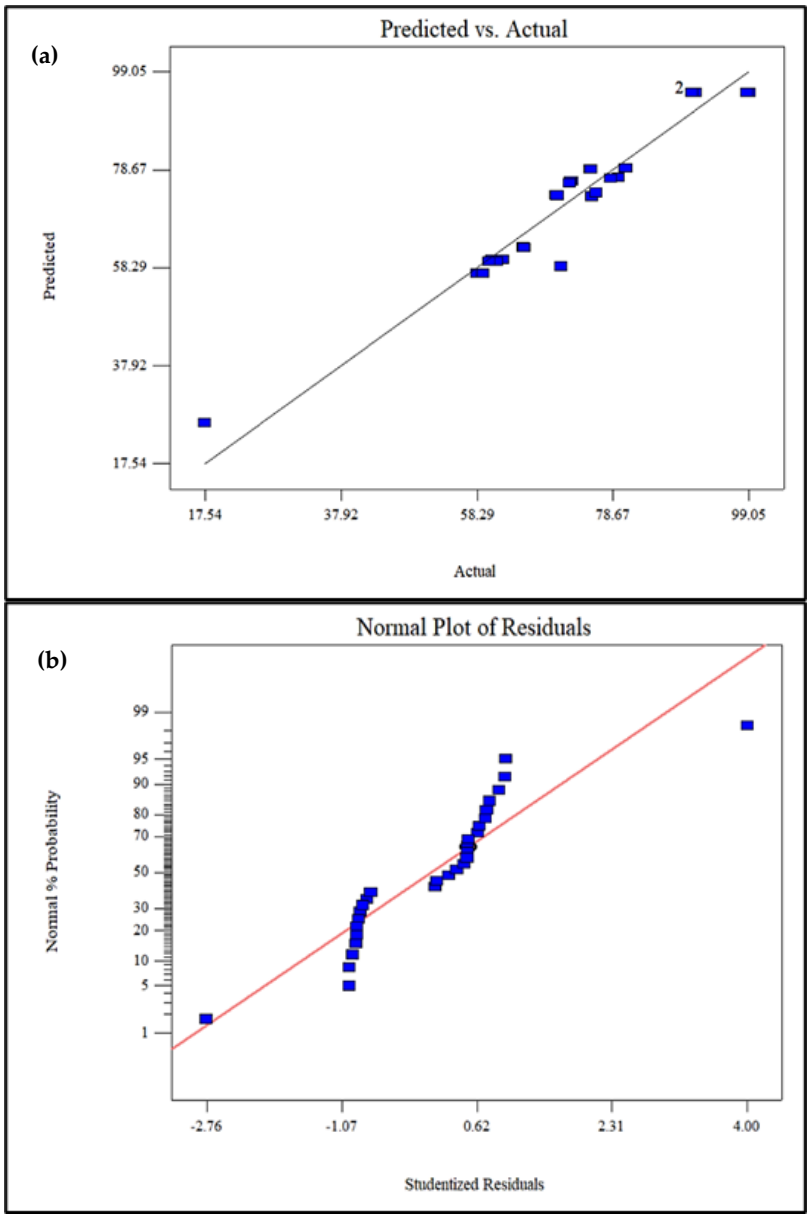

Figure 1. Diagnostic plots of the central composite design model of phenol degradation by the binary consortium. (**a**) Similarity plot between predicted and actual values for phenol degradation; (**b**) Normal probability plot against residuals for phenol degradation.

The highly significant influence of the temperature on phenol degradation is shown in Figure 2. This indicated that even a small deviation in temperature can have a disproportionate effect on phenol degradation achieved by the binary consortium. Temperature is a crucial variable, especially in the polar regions. The optimum temperature for phenol degradation by this consortium was 12.50–13.75 °C (Figure 2), supporting the potential use of this consortium in phenol degradation in Antarctica during summer periods where the soil surface temperature may reach up to 19.8 °C [49]. Although not crucial, the presence of $(NH_4)_2SO_4$ and NaCl, and near-neutral pH had little impact on phenol degradation. None of the interaction effects between variables were significant. CCD analysis on pure culture of strain AQ5-06 revealed that the optimum conditions for phenol degradation by this strain were between 10.0 and 12.5 °C with NaCl concentration range of 0.10–0.13 g/L and pH 6.5–7.0, with these three, and not $(NH_4)_2SO_4$ concentration, being significant factors [43]. Optimum conditions for strain AQ5-15 are at a pH range of 7.13–7.50, and 15.0–17.5 °C with interaction between these two factors being significant [44]. In a previous

study, ANOVA of RSM data for phenol degradation by a microbial consortium revealed that pH and temperature were significant factors but that the interaction between them was not significant [31].

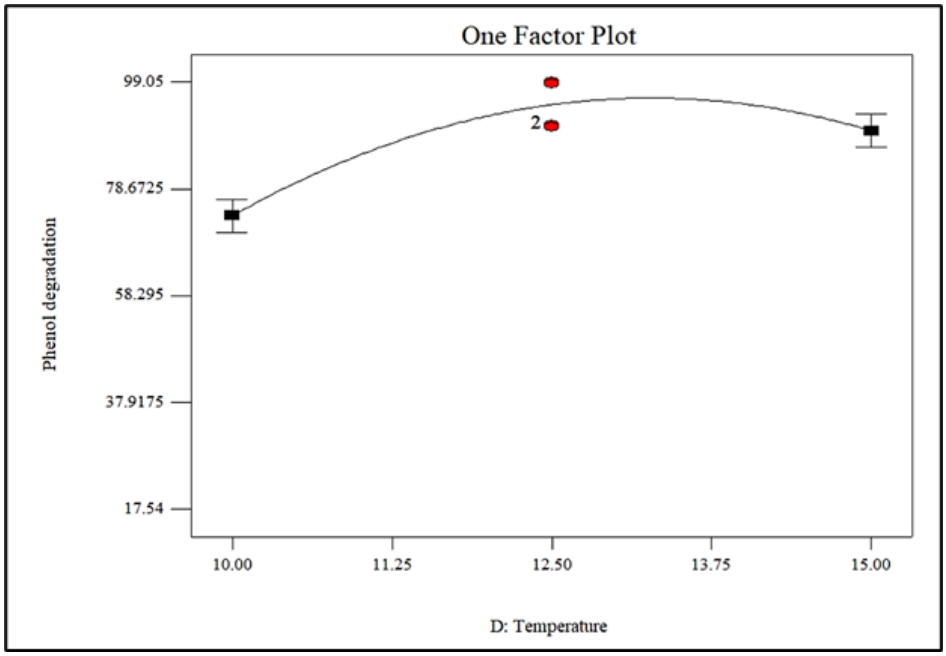

Figure 2. The one-factor plot showing the significant effect of temperature on phenol degradation by the binary consortium.

The RSM model was validated using the values predicted for each parameter. This identified no significant difference (*t*-test, $p = 0.0671$) between the percentage of phenol degradation predicted by the model (91.14%) and measured experimentally approaches (93.84%), supporting the validity of the model.

3.2. Effect of Phenol Initial Concentration on Consortium Degradation Activity

Figure 3 shows the bacterial growth and degradation of different initial concentrations of phenol by the binary consortium under the optimized conditions obtained from CCD. The strains, when in consortium, had the ability to degrade phenol up to an initial concentration of 1.7 g/L within 168 h. When the initial concentration was increased to 1.9 g/L, the strains started to lose their degradation ability despite growth being visible. High concentration of phenol hinders the microbial growth and its metabolic capacity. The bactericidal property of phenol allows it to partition into the cellular membrane, thereby disrupting membrane function and resulting in cell mortality [50,51]. Strain AQ5-06 in monoculture did not exhibit significant growth at a phenol concentration of 1.25 g/L [43], whereas strain AQ5-15 was inhibited at a phenol concentration of 1.7 g/L [44]. Similar to these pure cultures, Wen et al. [52] also reported that strain *Rhodococcus* sp. SKC showed no significant degradation or growth over 15 days at a phenol concentration of 1.5 g/L due to the inhibitory nature of the target pollutant. The ability of the binary mixture of the strains studied here to tolerate up to 1.9 g/L initial phenol concentration and to metabolize phenol at a concentration as high as 1.7 g/L supports the advantage gained from a defined mixed culture in phenol biodegradation. Similarly, Senthilvelan et al. [53] reported that a mixed culture of *Pseudomonas putida* strain Tan-1 and *Staphylococcus aureus* strain Tan-2 can degrade a high concentration of phenol in a short period of time compared to both strains individually. It is possible that the metabolic capacity of monocultures may be limited to certain concentration ranges of substrates (in this case, phenol), but that the use of consortia broadens their enzymatic capabilities and provides greater capacity to degrade higher concentrations of phenol [54,55].

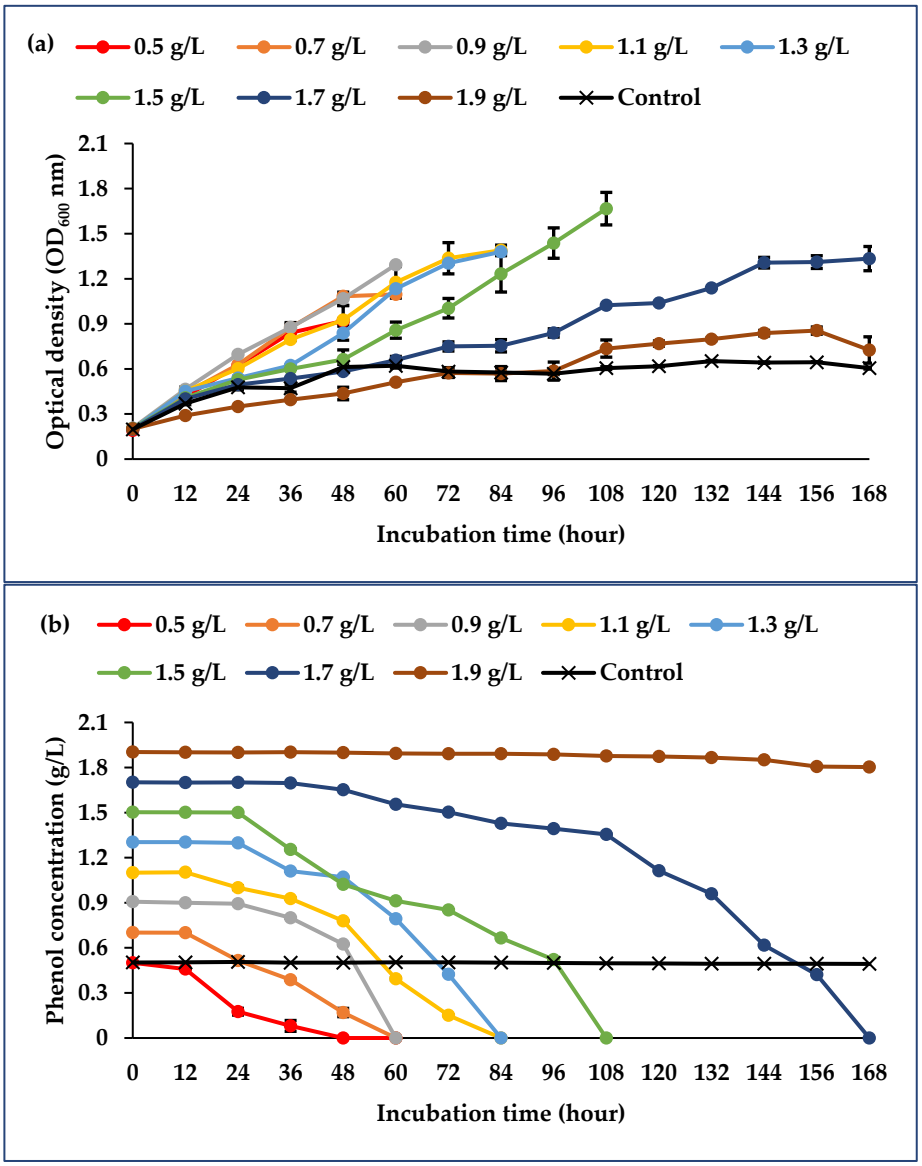

Figure 3. (**a**) Bacterial growth and (**b**) degradation of different initial concentrations of phenol by the binary consortium under optimized conditions (0.40 g/L $(NH_4)_2SO_4$, 0.13 g/L NaCl, pH 7.25, 12.50°C). The control (cross mark) shows the initial phenol concentration in the absence of the bacterial strain. Error bars represent the mean ± standard deviation of three replicates.

3.3. Impact of Heavy Metals on Phenol Biodegradation

Metal ions have proven to have significant effects on phenol biodegradation as they inhibit the activity of catechol dioxygenase which serves as a key enzyme in phenol metabolism [56,57]. Studies on baseline values for heavy metals in many Antarctic regions have reported significant levels. A study on King George Island showed the presence of Hg, Cd, Pb, Zn, Ni, Cu, As, and Cr in benthic samples [58]. Similarly, assessment of soil from Fildes Peninsula and Ardley Island revealed the presence of significant concentrations of Pb, Cu, Zn, Hg, and Cd [59]. Lischka et al. [60] reported the presence of Ag, Cd, Hg, Cr, Pb, Ni, Co, As, Zn, and Cu in different species of Antarctic octopod sampled near Elephant Island, South Shetland Islands. For the purposes of the current study, Cu, Cr, Zn, Pb, As, Ni, Al, Co, Ag, Hg, and Cd were chosen as heavy metals of interest. Our previous studies on Antarctic isolates revealed that these metals negatively affected the biodegradation processes [61–64].

3.3.1. *Arthrobacter* sp. Strain AQ5-06

The heavy metal tolerance of strain AQ5-06 was assessed by evaluating residual phenol concentration in culture media amended with 1.0 ppm of various heavy metal ions (Figure 4). ANOVA confirmed that significant differences in both bacterial growth [$F_{11,12}$ = 200.7, $p < 0.0001$] and phenol degradation [$F_{11,12}$ = 141158, $p < 0.0001$] for strain AQ5-06 were apparent with exposure to the different metals. Degradation by this strain was not inhibited by 1.0 ppm of Cu, Zn, Pb, As, Ni, Al, Co, or Cd, with 100% degradation of 0.5 g/L phenol achieved within 120 h. Exposure to Cr led to 83.52% (±0.0042) degradation and to Hg led to 11.82% (±0.0021) degradation within 120 h. Exposure to Ag completely inhibited phenol degradation. Strain AQ5-06 exhibited different growth patterns in response to exposure to the different heavy metals, with the highest growth observed in the presence of Cu. Tukey's multiple comparison test confirmed that exposure Cu led to significantly greater growth (1.1890 ± 0.0050) than both the control conditions and all other metals tested. Growth was inhibited in the presence of Ag and was lowest in the presence of Hg (0.2555 ± 0.0149), and in both cases was significantly lower than in control conditions and relative to all other metals tested.

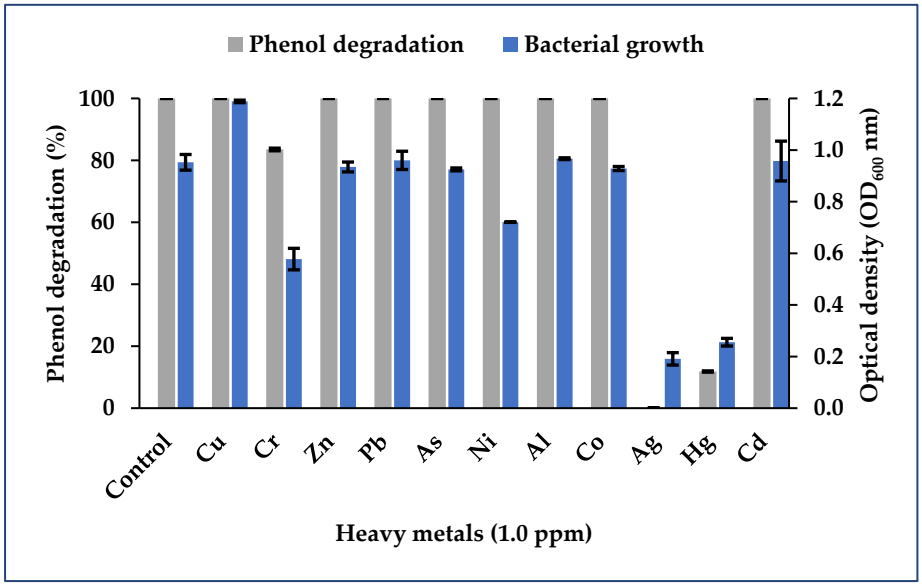

Figure 4. Phenol degradation and growth of *Arthrobacter* sp. strain AQ5-06 in the presence of 1.0 ppm concentrations of different heavy metals. Error bars represent the mean ± standard deviation of three replicates.

Following the initial screening, phenol degradation by strain AQ5-06 was tested in the presence of different concentrations of inhibiting metals (Figure 5). For Ag, ANOVA confirmed that both bacterial growth [$F_{6,7}$ = 325.80, $p < 0.0001$] and phenol degradation [$F_{6,7}$ = 501.10, $p < 0.0001$] were significantly influenced by the metal concentration applied. Inhibition of phenol degradation commenced at an Ag concentration of 0.7 ppm (38.30% inhibition), whereas at 0.8 ppm inhibition increased to 66.57%. A similar pattern was apparent in bacterial growth, with strong inhibition becoming apparent at 0.8 ppm. For Hg, ANOVA again confirmed that both bacterial growth [$F_{5,6}$ = 767.80, $p < 0.0001$] and phenol degradation [$F_{5,6}$ = 1518, $p < 0.0001$] were significantly influenced by the metal concentration applied. Phenol degradation started to show inhibition by Hg at 0.6 ppm (26.30% inhibition). At higher concentrations, degradation and growth were progressively reduced, reaching 88.18% inhibition at 1.0 ppm.

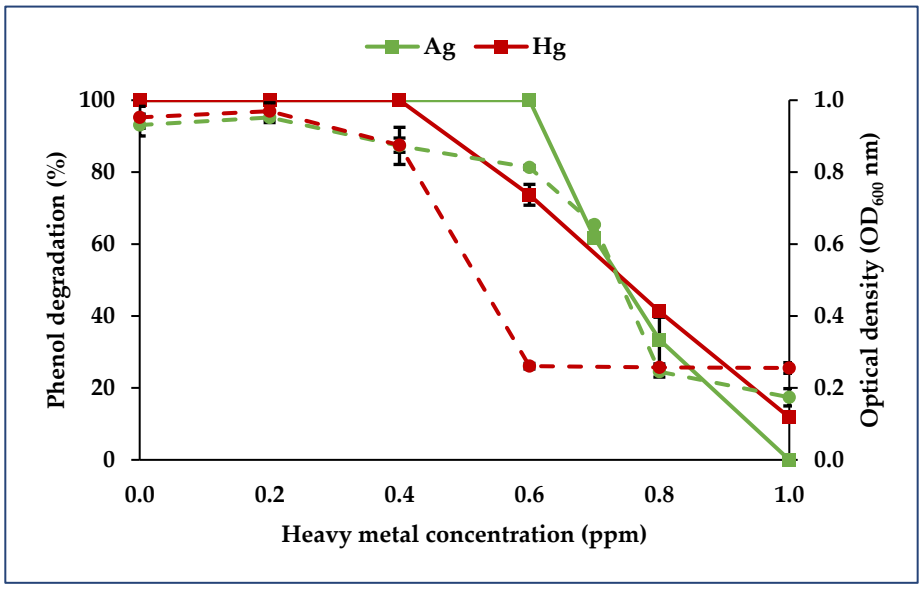

Figure 5. Effects of exposure to different concentrations of Ag and Hg on phenol degradation (solid line) and growth (dotted line) of *Arthrobacter* sp. strain AQ5-06. Error bars represent the mean ± standard deviation of three replicates.

The IC_{50} value is the concentration of a substance that reduces an enzyme's activity by 50%. The IC_{50} of these two heavy metals was determined using dose-response curves (Figure 6). Based on the modelled curve, both Ag and Hg exhibited similar levels of inhibition of phenol degradation with IC_{50} values of 0.75 and 0.74 ppm, respectively. Cu is known as an essential trace element, especially in aerobic organisms where it acts as a cofactor for enzymes that are responsible for diverse redox reactions [65]. Similarly, other heavy metals are essential for growth of microorganisms at trace concentrations [66]. Conversely, heavy metals such as Cd, Hg, and Pb have no known biological roles and their bioaccumulation in the cell over time can lead to negative impacts [66,67]. However, despite their toxicity, Pb and Cd did not inhibit phenol degradation by this strain, similar to the report of Yoo et al. [68]. The significant inhibitory effects on phenol degradation detected in this study are similar to those reported by Al-Defiery and Reddy [57] who confirmed that phenol degradation by *Rhodococcus pyridinivorans* strain GM3 was inhibited by Ag and Hg at low concentration. The inhibitory effects of Ag and Hg are caused by their ability to bind to sulfhydryl (-SH) groups of enzymes responsible for microbial metabolism, thereby impeding enzymatic actions [69]. These metals cause disturbance in cellular functions and trigger oxidative stress in microorganisms [70]. Ag and Hg are generally recognized as strong inhibitors of phenol degradation with bacteria unable to resist their impacts [57].

3.3.2. *Arthrobacter* sp. Strain AQ5-15

The heavy metal tolerance of strain AQ5-15 was assessed by evaluating residual phenol concentration in culture media amended with 1.0 ppm of various heavy metal ions (Figure 7). ANOVA showed that bacterial growth [$F_{11.12} = 727.50$, $p < 0.0001$] was significantly influenced by exposure to different metals. As phenol degradation was either 0% or 100% ANOVA could not be applied. Tukey's multiple comparison test showed that growth under exposure to Cu was greater (1.2785 ± 0.0135) but not significantly different from the control, and otherwise significantly greater (all $p < 0.05$) in comparison with all other heavy metals tested. The growth of this strain was least in the presence of Ag (0.2675 ± 0.0025) and Hg (0.2065 ± 0.0045) and was moderate in the presence of Cd (0.503 ± 0.001), although still significantly lower than the control ($p < 0.05$).

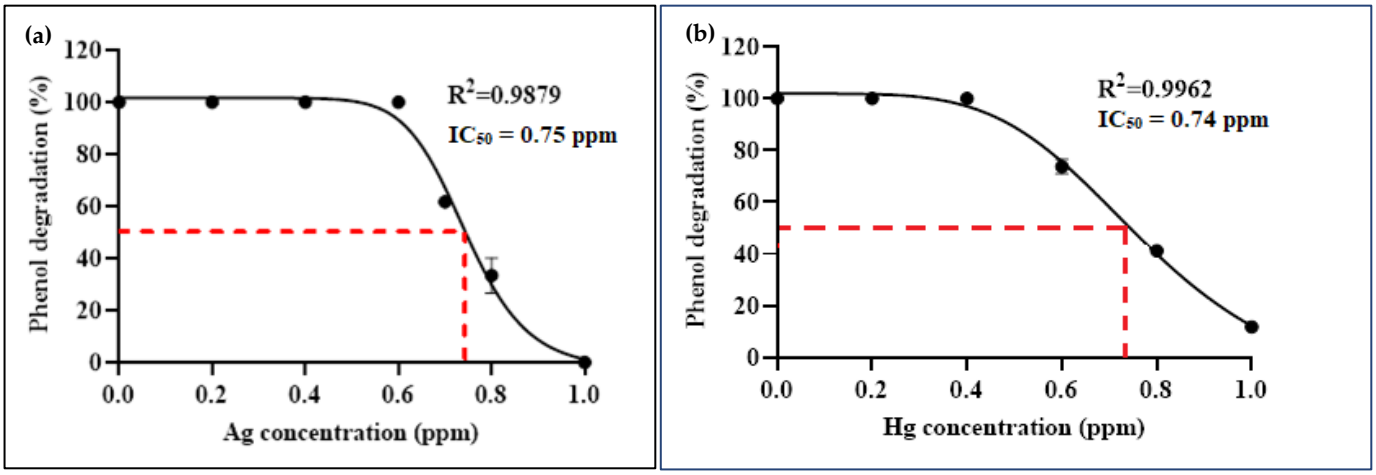

Figure 6. Dose-response curves identifying IC_{50} values for *Arthrobacter* sp. strain AQ5-06 phenol degradation after 120-h exposure to different concentrations of (**a**) Ag and (**b**) Hg.

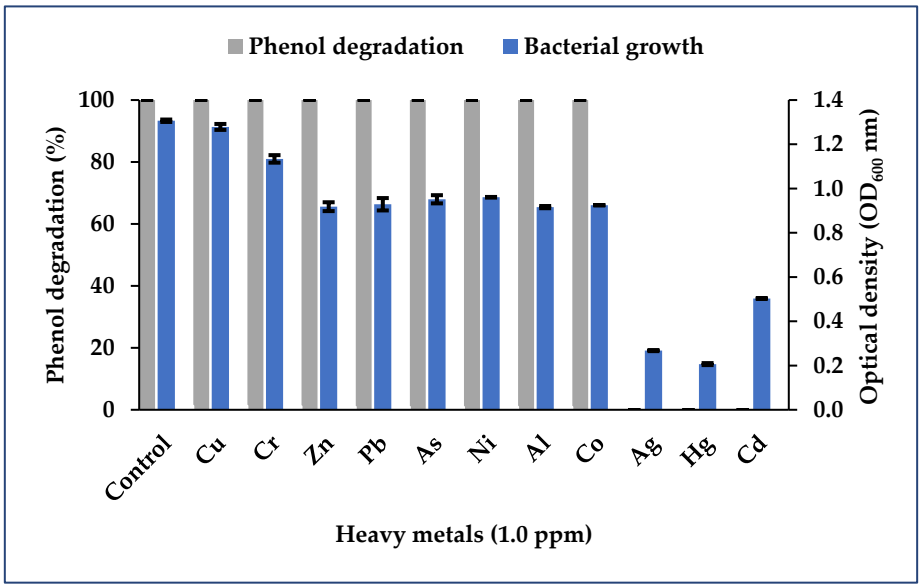

Figure 7. Phenol degradation and growth of *Arthrobacter* sp. strain AQ5-15 in the presence of 1.0 ppm concentrations of different heavy metals. Error bars represent the mean ± standard deviation of three replicates.

Different concentrations of inhibiting metals were added to media with 0.5 g/L phenol to determine the minimum concentration required for inhibition within a 96 h incubation (Figure 8). For Ag, ANOVA identified a significant influence on both bacterial growth [$F_{5,6}$ = 1504, $p < 0.0001$] and phenol degradation [$F_{5,6}$ = 264.7, $p < 0.0001$]. Significant inhibition of phenol degradation started to become apparent at Ag concentration of 0.6 ppm (44.06% inhibition), increasing to 61.20% inhibition at 0.8 ppm. Bacterial growth similarly started to be inhibited at 0.6 ppm. For Hg, ANOVA confirmed a significant influence on both bacterial growth [$F_{5,6}$ = 3807, $p < 0.0001$] and phenol degradation [$F_{5,6}$ = 12230, $p < 0.0001$]. Significant inhibition of phenol-degradation became apparent at Hg concentration of 0.2 ppm (57.03% inhibition). At a concentration of 0.4 ppm both degradation and growth were completely inhibited. For Cd, ANOVA again confirmed a significant influence on both bacterial growth [$F_{5,6}$ = 250.8, $p < 0.0001$] and phenol degradation [$F_{5,6}$ = 1959000, $p < 0.0001$]. Phenol degradation was significantly inhibited at a Cd concentration of 0.2 ppm (79.88% inhibition). At a concentration of 0.4 ppm, degradation was completely inhibited although growth continued, decreasing with higher Cd concentration.

This shows that this metal only hinders the metabolic process and does not kill the bacteria. The growth without the availability of phenol may indicate that this strain utilized nutrients recycled from dead cells [71].

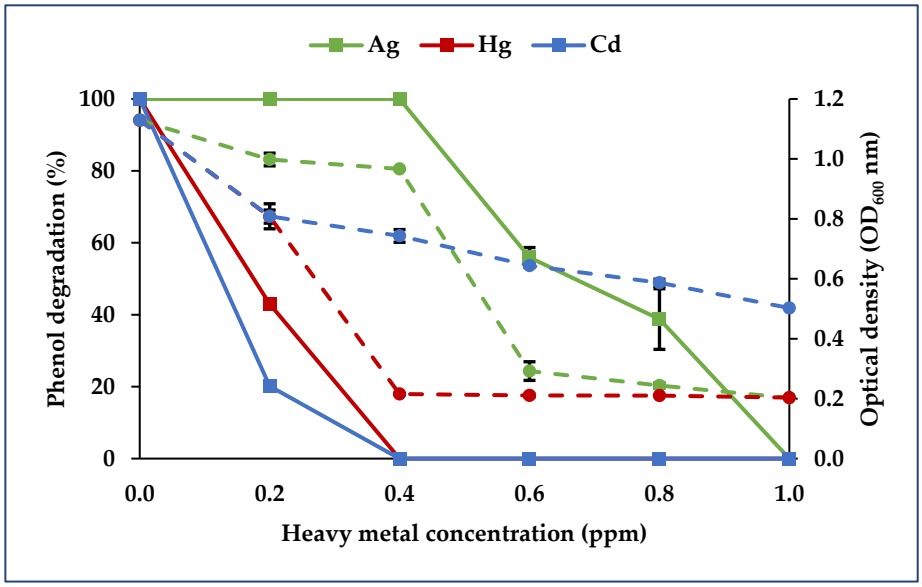

Figure 8. Effects of different concentrations of Ag, Hg, and Cd on phenol degradation (solid line) and growth (dotted line) of *Arthrobacter* sp. strain AQ5-15. Error bars represent the mean ± standard deviation of three replicates.

IC_{50} of these heavy metals was determined using dose-response curves (Figure 9). Based on the modelled curve, the metal with the greatest inhibitory effect on phenol degradation by strain AQ5-15 was Cd which had the lowest IC_{50} value of 0.06 ppm, followed by Hg with IC_{50} of 0.14 ppm and Ag with IC_{50} of 0.70 ppm. Reduction in phenol degradation rate with increasing metal concentration is likely to be due to toxic effects on metabolic pathways, although not necessarily to population decline [72], as illustrated by the continued growth of strain AQ5-15 at increasing Cd concentration. Similarly, Ahmad et al. [40] reported that *Arthrobacter bambusae* strain AQ5-003 exhibited intolerance to 0.6 ppm of Ag with <50% phenol degradation and to a Cd concentration of 0.2 ppm with 20% phenol degradation. These heavy metals have cytotoxic effects on both structural and permeability properties of cell membranes and organelles, thereby inhibiting cellular enzymatic activities, in this case the enzyme system responsible for phenol degradation.

3.3.3. Binary Consortium

The tolerance of heavy metals by this binary consortium comprising strains AQ5-06 and AQ5-15 was identified by evaluating residual phenol concentration in culture media with 1.0 ppm of heavy metal (Figure 10). ANOVA confirmed that bacterial growth [$F_{11,12} = 756.40$, $p < 0.0001$] was significantly influenced by exposure to different metals. ANOVA could not be applied for phenol degradation because degradation by this consortium was not inhibited by 1.0 ppm Cu, Cr, Zn, Pb, As, Ni, Al, or Co, with 100% degradation of 0.5 g/L phenol within 48 h, whereas Ag, Hg and Cd completely inhibited degradation. The growth patterns differed with exposure to the different heavy metals, with the highest growth observed in the absence of heavy metals. Tukey's multiple comparison tests confirmed that growth under control conditions (1.350 ± 0.0090) was significantly greater than with any of the metals tested. Growth was completely inhibited by Hg and was significantly lower than either control or other metals in the presence of Ag (0.240 ± 0.0200) and Cd (0.4140 ± 0.0055). This consortium's inhibition pattern was similar to that described for strain AQ5-15, with Ag, Hg, and Cd being strong inhibitors of phenol degradation. Cd did not show an inhibitory effect on pure culture of strain AQ5-06; however, when

combined with strain AQ5-15, Cd completely inhibited phenol degradation but not growth. Lu et al. [73] reported similar results, where the effect of metal ions on polychlorinated biphenyl (PCB) dechlorination by a binary mixture of *Dehalococcoides mccartyi* strain CG1 and *Geobacter lovleyi* strain LYY exhibited similar trends with respect to a pure culture of *D. mccartyi* strain CG1. These data suggest use of a mixed culture does not necessarily have a positive influence on tolerance of exposure to toxic heavy metals at high concentration.

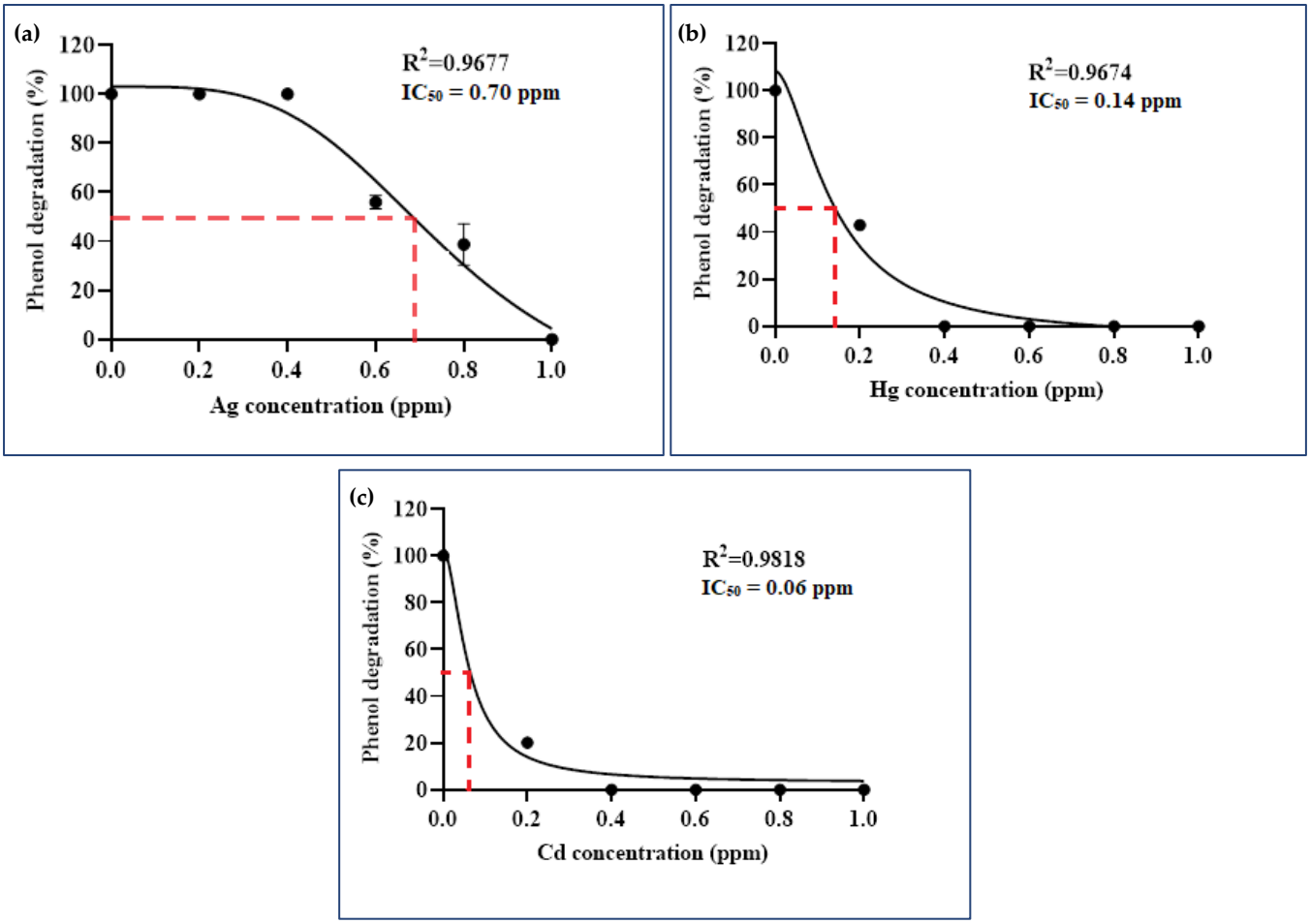

Figure 9. Dose-response curves indicating IC_{50} values for *Arthrobacter* sp. strain AQ5-15 phenol degradation after 96-h exposure to different concentrations of (**a**) Ag, (**b**) Hg, and (**c**) Cd.

Different concentrations of inhibiting heavy metals were added to media with 0.5 g/L phenol to determine the starting concentration of inhibition over a 48 h incubation (Figure 11). For Ag, ANOVA confirmed that both bacterial growth [$F_{5,6} = 2296$, $p < 0.0001$] and phenol degradation [$F_{5,6} = 14477$, $p < 0.0001$] were significantly influenced by metal concentration. Inhibition of phenol degradation by the mixed culture started at an Ag concentration of 0.2 ppm (48.64% inhibition) and further increase in concentration resulted in complete inhibition. Bacterial growth showed a similar response to increasing concentration. For Hg, ANOVA again confirmed that both bacterial growth [$F_{6,7} = 17518$, $p < 0.0001$] and phenol degradation [$F_{6,7} = 45824$, $p < 0.0001$] were significantly influenced by metal concentration. Inhibition of degradation started at a concentration of 0.5 ppm (58.25% inhibition). At a concentration of 0.6 ppm, inhibition reached 89.76% and further increase in concentration resulted in complete inhibition. For Cd, ANOVA also confirmed that both bacterial growth [$F_{6,7} = 17086$, $p < 0.0001$] and phenol degradation [$F_{6,7} = 16423$, $p < 0.0001$] were significantly influenced by metal concentration. Inhibition of degradation started at Cd concentration of 0.8 ppm (48.81% inhibition). At 0.9 ppm inhibition reached 89.76%. In this case, how-

ever, growth was strongly inhibited, having initially increased slightly when exposed to concentrations of up 0.4 ppm before decreasing with further increase in concentration.

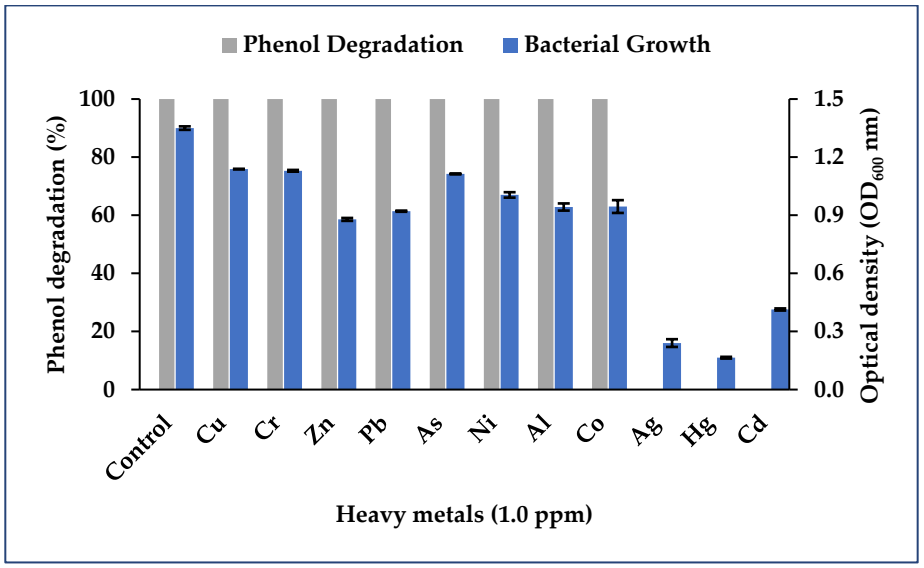

Figure 10. Effects of 1.0 ppm concentration of different heavy metals on phenol degradation and growth on the binary consortium. Error bars represent the mean ± standard deviation of three replicates.

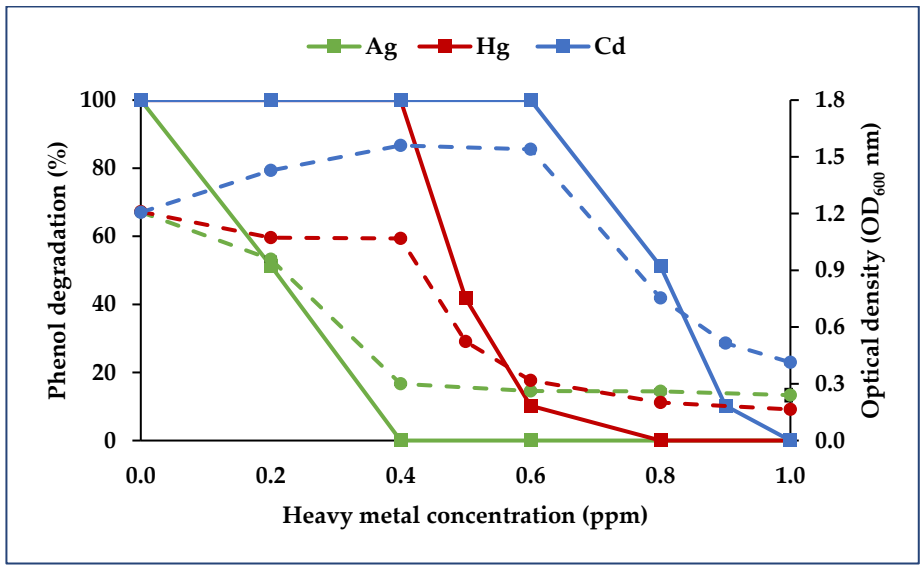

Figure 11. The influence of exposure to different concentrations of Ag, Hg and Cd on phenol degradation (solid line) and the growth (dotted line) of the binary consortium.

The pure cultures of strains AQ5-15 and AQ5-06 could tolerate Ag concentrations up to 0.4 and 0.6 ppm, respectively, whereas the binary mixture could only tolerate up to 0.2 ppm, at which concentration phenol degradation was already reduced to half of the control level. This result contrasts with a number of studies reporting greater resistance of mixed microbial cultures compared with pure cultures towards toxic heavy metals [74,75]. However, this mixture showed increased tolerance to higher concentrations of Hg and Cd than did pure culture of strain AQ5-15, possibly suggesting that strain AQ5-06 may have a positive synergistic impact on its co-culture's tolerance. IC_{50} of these heavy metals was determined from dose-response curves (Figure 12). Based on the modelled curve, Ag showed the strongest inhibition of phenol degradation with the lowest IC_{50} value of 0.15 ppm, followed by Hg with an IC_{50} of 0.48 ppm and Cd with an IC_{50} of 0.80 ppm.

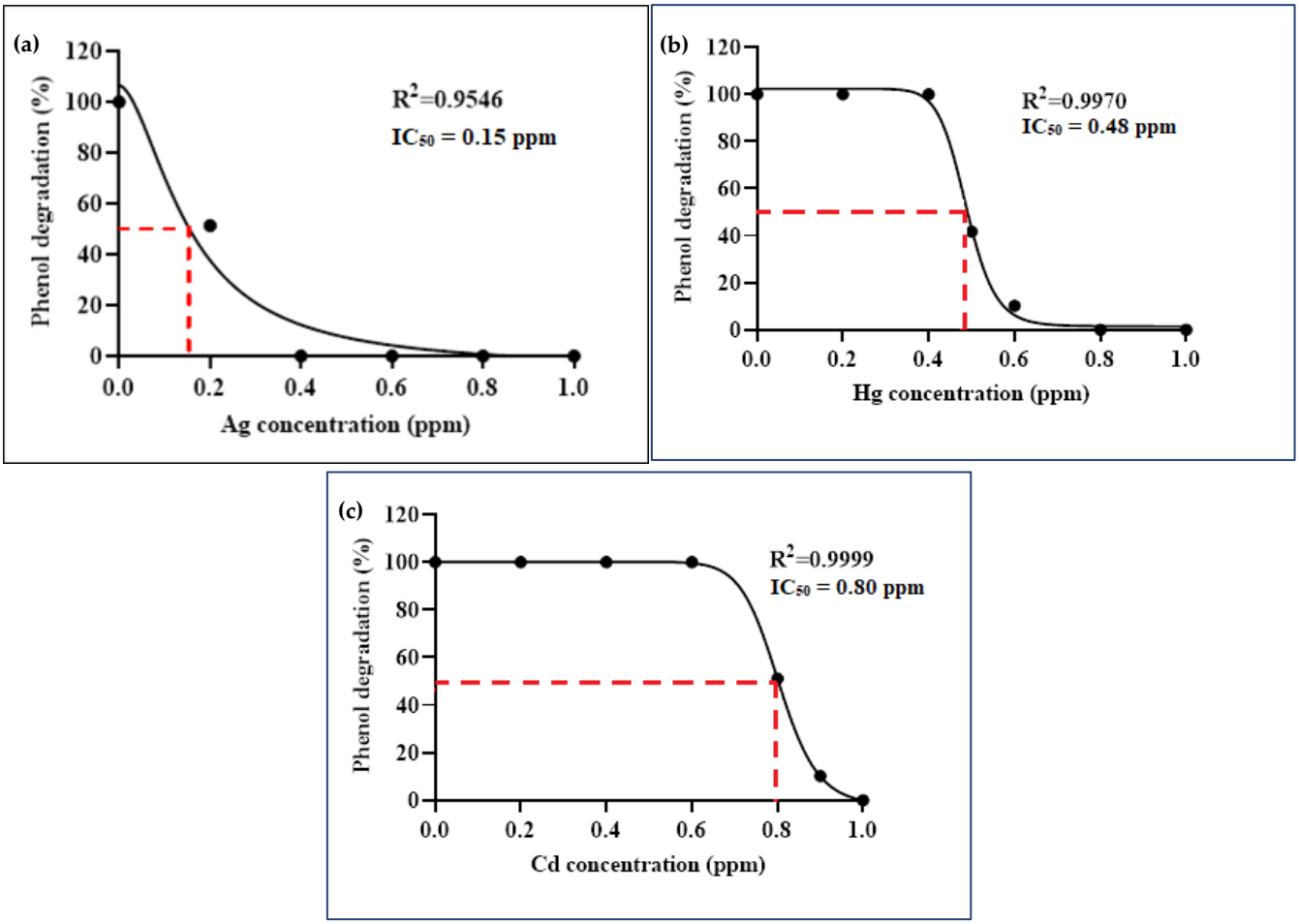

Figure 12. Dose-response curves indicating IC_{50} values for the binary consortium phenol degradation after 48-h exposure to different concentrations of (**a**) Ag, (**b**) Hg, and (**c**) Cd.

4. Conclusions

This study utilized the advantage of statistical experimental design to optimize phenol biodegradation by a binary consortium of native Antarctic bacteria. The outcome sheds light on the potential application of RSM in the modeling and optimization of environmental factors in order to improve phenol degradation. Under optimized conditions, the mixed culture had better tolerance to a high concentration of phenol compared to pure culture. In terms of heavy metal tolerance, the binary consortium did not present any advantage over pure culture at high concentrations. Hence, further studies are needed to ensure the effectiveness of this binary consortium to bioremediate phenol pollution in the presence of different co-contaminants. The data from this study provides a solid foundation that will support the development of practical field bioremediation strategies, particularly in wastewater treatment systems in the Antarctic environment.

Author Contributions: Conceptualization, S.A.A., P.C., A.Z. and N.A.S.; methodology, A.Z., S.A.A., K.S. and K.A.K.; software, K.S. and K.A.K.; validation, K.S., A.Z., S.A.A., P.C., N.A.S. and K.A.K.; formal analysis, K.S., A.Z., S.A.A. and K.A.K.; investigation, K.S.; resources, S.A.A. and C.G.-F.; original draft preparation, K.S.; Writing—review and editing S.A.A., P.C., N.A.S., K.A.K., T.A.T.-M., C.G.-F. and A.Z.; supervision, S.A.A., P.C., N.A.S., K.A.K. and A.Z.; project administration, S.A.A., P.C., A.Z. and C.G.-F.; funding acquisition, A.Z., S.A.A. and C.G.-F. All authors have read and agreed to the published version of the manuscript.

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